Signal Regulation of Protease-activated Receptor-2 and Structural Determinants of Gα$_q$-dependent Activation and Deactivation of Phospholipase C-β

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ABSTRACT

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Signal Regulation of Protease-activated Receptor-2 and Structural Determinants of $G\alpha_q$-dependent Activation and Deactivation of Phospholipase C-β
(Under the direction of Dr. T. Kendall Harden and Dr. JoAnn Trejo)

Cells respond to changes in their environment by relaying information from extracellular cues to intracellular compartments, and receptors play an important role in the transmission of these signals. We examined signal transduction of G protein-coupled protease-activated receptor-2 (PAR2), a cell surface receptor for serine proteases. Unlike most GPCRs, PAR2 is irreversibly activated by proteolytic cleavage, and the mechanisms regulating desensitization and trafficking are essential for the fidelity of PAR2 signaling. Most activated GPCRs are rapidly desensitized and internalized following phosphorylation and β-arrestin binding. However, the role of phosphorylation in signaling and trafficking of PAR2 is unknown. We found that PAR2 phosphorylation is required for receptor desensitization and β-arrestin binding. Phosphorylated PAR2 internalized through a canonical dynamin-, clathrin-, and β-arrestin-dependent pathway. In contrast, phosphorylation-deficient PAR2 constitutively internalized through a dynamin-dependent but clathrin- and β-arrestin-independent pathway. Collectively, we show that phosphorylation of PAR2 is critical for β-arrestin binding and desensitization but not endocytic trafficking.

Activated PAR2 and GPCRs catalyze GTP exchange on heterotrimeric G proteins. GTP-bound Gα subunits activate protein effectors including phospholipases C-β (PLC-β) isozymes. PLC-β isozymes are stimulated by Gα_q and also accelerate GTP hydrolysis of on their activating G protein. The mechanisms that regulate Gα_q-dependent activation and deactivation of PLC-β are not clearly understood. Inspection of a three-dimensional crystal structure of the PLC-β3·Gα_q complex revealed three novel contacts within the binding interface. A small region following the C2 domain of PLC-β3 inserts into the effector binding pocket of Gα_q. Gα_q also makes electrostatic interactions with a region preceding the C2 domain of PLC-β3. In addition, a loop between the third and fourth EF hands of PLC-β3 contacts the nucleotide binding pocket of Gα_q. Mutation of this loop abrogated PLC-β3 GAP activity, and a GAP-deficient PLC-β3 mutant displayed a much slower rate of deactivation. Consequently, PLC activity was largely unchanged compared to rapid termination of wildtype PLC-β3. Our studies define the important domains within the PLC-β3/Gα_q binding interface that are required for activation and deactivation of PLC-β isozymes. The studies presented herein describe the mechanisms regulating PAR2 and GPCRs at the cell surface and controlling PLC-β isozymes within intracellular compartments.

To my parents, Cecil and Deborah Ricks, who showed me through their hard work and

dedication that there are no limits to what I can achieve.

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ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid
Ala or A	Alanine
AlF_4	Aluminum tetrafluoride
AMSH	Associated molecule with the Src homology 3 domain of STAM
APC	Activated protein C
AP-2	Adaptor protein complex-2
Arg or R	Arginine
Arr	Arrestin
Asn or N	Asparagine
Asp or D	Aspartic acid
ATP	Adenosine 5′-trisphophate
β_2AR	β2-adrenergic receptor
βarr	β-arrestin
BSA	Bovine serum albumin
cAMP	Adenosine 3′,5′-cyclic monophosphate
cDNA	Complementary deoxyribonucleic acid
CHC	Clathrin heavy chain
C-tail	Cytoplasmic tail
Cys or C	Cysteine
DH	Dbl homology
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid

Dyn	Dynamin
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosomal antigen-1
EF	Elongation Factor
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
ERK1/2	Extracellular regulated kinase 1/2
FBS	Fetal bovine serum
GAP	GTPase activating protein
GDP	Guanosine 5′-diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
Gln or Q	Glutamine
Glu or E	Glutamic acid
Gly or G	Glycine
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	Guanosine 5′-triphosphate
GTPγS	Guanosine 5′-O-(3-thiotriphosphate)
His or H	Histidine
Hrs	Hepatocyte growth factor regulated substrate
$Ins(1,4,5)P_3$	Inositol (1,4,5)trisphosphate
Iso or I	Isoleucine

kDa	Kilodalton
LAMP1	Lysosomal-associated membrane protein-1
Leu or L	Leucine
Lys or K	Lysine
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated kinase
MEF	Mouse embryonic fibroblast
Met or M	Methionine
mGluR	Metabotropic glutamate receptor
MMP	Matrix metalloproteinase
MVB	Multivesicular body
NHERF	Na^+/H^+ exchanger regulator factor
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDEγ	Phosphodiesterase γ
PDZ	PSD-95/Dlg/ZO-1
PH	Pleckstrin homology
Phe or F	Phenylalanine
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C

Pro or P	Proline
PtdIns(4,5)P$_2$	Phosphatidylinositol (4,5)-bisphosphate
P2YR	Purinergic P2Y receptor
RGS	Regulator of G protein signaling
RNAi	Ribonucleic acid interference
SDS-PAGE	Sodium dodecyl sulfate-polyacrylimide gel electrophoresis
Ser or S	Serine
SH2	Src homology 2
SH3	Src homology 3
shRNA	Short interfering ribonucleic acid
siRNA	Small interfering ribonucleic acid
SNX	Sorting nexin
STAM	Signal-transducing adaptor molecule
TF	Tissue factor
Thr or T	Threonine
TIM	Triose phosphate isomerase
Trp or W	Tryptophan
Tsg101	Tumor suppressor gene 101
Tyr or Y	Tyrosine
UBPY	Ubiquitin-associated protease Y
UTP	Uridine 5′-triphosphate
Val or V	Valine
WT	Wildtype

CHAPTER 1

INTRODUCTION – PROTEASE-ACTIVATED RECEPTORS

Protease-activated receptors (PARs) are members of the G protein-coupled receptor (GPCR) family and transmit signals of extracellular proteases in response to tissue injury and inflammation mainly within the vasculture (Coughlin, 2005). There are four members of the PAR family encoded in the mammalian genome. PAR1, PAR3, and PAR4 are expressed primarily on platelets, endothelial cells, and leukocytes and elicit cellular responses to thrombin, the primary effector protease of the coagulation cascade. PAR1 is also activated by coagulant protease factor Xa (FXa), plasmin, and activated protein C (APC). PAR2 is expressed in intestinal and airway epithelial cells, fibroblasts, as well as cell types of the vasculature and is activated by trypsin, mast cell tryptase, coagulant proteases FVIIa and FXa but not by thrombin (Nystedt et al., 1994; Bohm et al., 1996b; Molino et al., 1997a; D'Andrea et al., 1998; Camerer et al., 2000). Unlike other GPCRs, PARs are uniquely activated through a proteolytic cleavage event (Coughlin, 2000). Serine proteases cleave the extracellular N-terminus of PARs, exposing a new N-terminus that intramolecularly binds and activates the receptors. PARs are also fully activated in the absence of proteolytic cleavage by small synthetic peptides that mimic the new N-terminus of the receptors.

1.1 PAR1

Coughlin and colleagues first discovered PAR1 as the receptor that mediates thrombin signaling in the vasculature (Vu et al., 1991a). Vu et al. injected *Xenopus* oocytes with mRNA from megakaryocyte cell lines that were responsive to thrombin and demonstrated conferrance of a thrombin-dependent increase in intracellular calcium and inward Cl$^-$ currents (Vu et al., 1991a). They then enriched for thrombin receptor mRNA by size fractionation on sucrose gradients, generated a cDNA library from the enriched mRNA pool, and identified a single cDNA that retained thrombin sensitivity in *Xenopus* oocytes. Characterization of the predicted protein sequence revealed that the thrombin receptor contains a seven transmembrane spanning region similar to known GPCRs and a relatively long N-terminal extension (Vu et al., 1991a). The PAR1 N-terminus contains the sequence LDPR^{41}/S^{42}FL and Arg^{41} at the P1 site that are essential for protease recognition and cleavage by thrombin and other serine proteases (Fig. 1.1A). A second location of thrombin binding is an acidic region (WEDEEKNES) C-terminal to the thrombin cleavage site of PAR1 (Fig. 1.1A). Thrombin interacts with this acidic region to confer high affinity binding to PAR1. A similar acidic region (DFEEIPEEY) is also found in the leech anticoagulant hirudin, which also binds thrombin. Removing the hirudin-like domain causes a 100-fold decrease in the potency of thrombin at PAR1, and replacing the region with the leech hirudin domain recovers receptor function (Vu et al., 1991b).

Thrombin recognizes both the PAR1 hirudin-like domain and LDPR^{41}/S^{42}FL cleavage site in the receptor N-terminus and cleaves the peptide bond between Arg^{41}/Ser^{42} (Fig. 1.1A), exposing a tethered ligand domain that intramolecularly binds and activates the receptor (Vu et al., 1991a; Vu et al., 1991b). Substituting the essential

Arg[41] residue with alanine prevents activation by thrombin (Vu et al., 1991b). In addition, replacing the entire LDPR[41] protease cleavage sequence with the enterokinase or trypsin cleavage sequences also inhibits activation by thrombin and allows activation by enterokinase or trypsin, respectively (Vu et al., 1991b; Hammes and Coughlin, 1999). These results show that cleavage and formation of the tethered ligand domain is sufficient for receptor activation. PAR1 is activated by picomolar concentrations of thrombin and is also fully activated independent of proteolytic cleavage by a small synthetic peptide agonist S[42]FLLRN (Vu et al., 1991a). S[42]FLLRN mimics the newly formed N-terminus of PAR1 but is less potent (~5 μM) than thrombin.

PAR1 also acts as a cell surface sensor for other serine proteases including those that function upstream of thrombin in the coagulation cascade (Fig. 1.1B and 1.2). The coagulant protease FXa cleaves and activates PAR1 as a soluble protease or in complex with tissue factor (TF)-FVIIa (Camerer et al., 2000; Riewald and Ruf, 2001). Tissue factor, the initiator of the coagulation cascade, is constitutively expressed on cells extrinsic to the vasculature or induced on monocytes and endothelial cells in the presence of inflammatory mediators. When the vasculture is disrupted, TF can associate with circulating zymogen FVII in plasma to convert it to active FVIIa. The TF-FVIIa complex then generates FXa, which can elicit responses on its own through PARs or can generate thrombin from prothrombin with the aid of an additional cofactor, FVa (Camerer et al., 2000; Riewald and Ruf, 2001). Thus, PAR1 transmits cellular responses of thrombin and upstream coagulant proteases under conditions of vascular injury and inflammation.

Thrombin and coagulant proteases activate PAR1 to elicit pro-inflammatory responses and promote endothelial barrier permeability within the vasculature (Coughlin, 2005). Alternatively, activated protein C (APC) stimulates PAR1 to induce opposing effects including anti-inflammatory responses and endothelial barrier protection (Riewald et al., 2002; Feistritzer and Riewald, 2005). Protein C is localized to the cell surface by its cofactor endothelial protein C receptor (EPCR) and is converted to anti-coagulant protease APC by the thrombin-thrombomodulin complex (Stearns-Kurosawa et al., 1996). While bound to EPCR, APC cleaves and inactivates FVa and FVIIa to prevent thrombin generation and thus, inhibits the coagulation cascade and thrombin signaling. EPCR also localizes APC for activation of PAR1 on endothelial cells. Recent studies show that compartmentalization of APC, EPCR, and PAR1 is required for PAR1 to induce its anti-inflammatory effects and promote endothelial barrier protection (Bae et al., 2007a; Bae et al., 2007b; Russo et al., 2009a). APC, EPCR, and PAR1 are localized to lipid rafts in endothelial cells and are present in membrane fractions containing caveolin-1, a structural scaffolding protein that is needed for invagination of caveolae lipid microdomains (Bae et al., 2007b). Unlike the soluble ligand thrombin, APC activation of PAR1 requires caveolin-1 in membrane microdomains and compartmentalization for protease-selective cytoprotective signaling in endothelial cells (Russo et al., 2009a).

Initial characterization of the PAR1 exodomain suggested that receptor activation is strictly mediated by extracellular serine proteases. Serine proteases recognize and cleave a specific recognition sequence, $LDPR^{41}/S^{42}FL$ in the PAR1 N-terminal exodomain (Vu et al., 1991a), which requires a basic residue at the P1 site (Fig. 1.1A).

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Interestingly, Kuliopulos and colleagues showed that matrix metalloproteinase-1 (MMP-1) also activates PAR1 on breast cancer cells and platelets but not PAR2, PAR3, or PAR4 (Boire et al., 2005; Trivedi et al., 2009). The MMP-1 cleavage site is distinct from the site required for thrombin and other serine proteases. MMPs usually prefer a hydrophobic residue at the P1′ site, a basic residue at the P2′ site, and a small residue at P3′ (Fig. 1.1A). As such, MMP-1 cleaves PAR1 at $LD^{39}/P^{40}RSFL$ within the exodomain, two residues N-terminal to the thrombin cleavage site (Trivedi et al., 2009). Mutation of Pro^{40} to an Asn prevented activation of PAR1 by MMP-1 but not by thrombin and inhibited signaling to RhoA, p38 mitogen-activated protein kinase (MAPK), and cell migration in MCF-7 breast cancer cells (Trivedi et al., 2009). Furthermore, in the absence of PAR1 proteolytic cleavage, a synthetic peptide PR-SFLLRN that mimics the MMP-1-generated tethered ligand also stimulated platelet activation and signaling (Trivedi et al., 2009).

Activated PAR1 couples to multiple heterotrimeric G protein subtypes to transmit intracellular signaling (Fig. 1.1B). Activated PAR1 couples to $G\alpha_i$ and inhibits adenylyl cyclase activity and accumulation of cAMP (Hung et al., 1992b). PAR1 also interacts with $G\alpha_q$ and stimulates PLC-β isozymes, which hydrolyze $PtdIns(4,5)P_2$ to second messengers, diacylglycerol and $Ins(1,4,5)P_3$ (Hung et al., 1992b; Baffy et al., 1994). These second messengers mobilize intracellular calcium and activate protein kinase C to elicit various cellular responses. The initial, rapid phase of PAR1-stimulated phosphoinositide hydrolysis is mediated by PLC-β isozymes whereas sustained phospholipid signaling occurs through activation of $G\alpha_{12/13}$ and PLC-ϵ (Kelley et al., 2006). Additional studies have found that PAR1 coupling to $G\alpha_{12/13}$ subtypes also

promotes RhoGEF activity and actin remodeling (Offermanns et al., 1994; Gohla et al., 1999). Thus, PAR1 activates several heterotrimeric G proteins to transmit signals from extracellular proteases, and coupling to a specific pathway appears to depend on expression of different effectors in a particular cell type, protease selectivity, and compartmentalization of PAR1 and its signaling partners into membrane microdomains.

1.2 PAR2

PAR2 was discovered through a mouse genomic library screen of GPCRs using transmembrane probes of the substance K receptor and was identified as a second protease-activated receptor (Nystedt et al., 1994; Nystedt et al., 1995; Bohm et al., 1996b). The PAR2 protein sequence has an overall 30% identity to the PAR1 sequence and 44% sequence identity within the transmembrane domains. PAR2 lacks the acidic hirudin-like domain of PAR1 and is not activated by thrombin. PAR2 is cleaved and activated at picomolar concentrations by trypsin and other serine proteases (Nystedt et al., 1994; Nystedt et al., 1995; Bohm et al., 1996b). Similar to PAR1 activation, serine proteases cleave the Arg^{34}/Ser^{35} peptide bond, and the newly formed N-terminus activates the receptor (Fig. 1.1A). The PAR2-specific agonist peptide $S^{35}LIGKV$ also activates PAR2 at low micromolar concentrations in the absence of proteolytic cleavage (Nystedt et al., 1995; Bohm et al., 1996b).

Upstream coagulant proteases FVIIa and FXa directly cleave and activate PAR2 expressed in the vasculature (Fig. 1.2). The TF-FVIIa complex cleaves and activates PAR2 at nanomolar concentrations in both fibroblasts and endothelial cells induced to express TF in the presence of cytokines (Camerer et al., 2000; Riewald and Ruf, 2001). A recent study showed that a fraction of PAR2 and TF localize to lipid raft microdomains

and co-localize with caveolin-1 by immunofluorescence microscopy (Awasthi et al., 2007). Additionally, disruption of caveolae by cholesterol depletion and siRNA-silencing of caveolin-1 impaired TF-FVIIa signaling whereas signaling induced by free PAR2 agonist peptide was not affected. These results suggest that TF-FVIIa signaling may require compartmentalization of TF and PAR2 in lipid raft microdomains for protease-selective signaling.

The TF-FVIIa complex generates FXa (Fig. 1.2), which cleaves and activates PAR2 as a free monomer or in a tertiary complex with TF-FVIIa (Camerer et al., 2000; Riewald and Ruf, 2001). Endothelial cells derived from PAR2 knockout mice were markedly impaired in FXa signaling compared to wild-type endothelial cells (Camerer et al., 2002). PAR1$^{-/-}$ endothelial cells also displayed defects in FXa signaling but not to the same extent as endothelial cells lacking PAR2. These findings indicate that FXa signaling is mostly attributed to activation of PAR2. Thus, similar to PAR1, PAR2 is a cell surface receptor for upstream coagulant proteases and may require formation of membrane signaling platforms for activation within the vasculature.

PAR2 is also implicated as a cell surface receptor for other serine proteases. For example, PAR2 is directly cleaved and activated by mast cell tryptase. Mast cell tryptase is a protease expressed in the airways and infiltrating immune cells where it induces inflammatory responses and tissue remodeling (Molino et al., 1997a). Tryptase was found to activate PAR2 in heterologous systems as well as cell types that endogenously express PAR2, and tryptase signaling was selectively inhibited by PAR2-specific blocking antibodies (Molino et al., 1997a; Akers et al., 2000; Berger et al., 2003; Ramachandran and Hollenberg, 2008). PAR2 is also expressed on immune cells and

elicits cellular responses of proteinase-3, a protease secreted from neutrophils that promotes inflammation (Uehara et al., 2002). In a recent study, PAR2 was found to induce the cellular functions of kallikreins, a large family of serine proteases that are widely expressed and exhibit trypsin and chymotrypsin activity (Oikonomopoulou et al., 2006). Although a few proteases have been shown to activate PAR2 mainly in the vasculature and at sites of inflammation, these proteases probably represent only a handful of the potential physiological activators. To fully understand PAR2 function in physiological and pathological processes, identification of other activating proteases of PAR2 will be an important future pursuit.

There is no direct evidence demonstrating PAR2 coupling to specific heterotrimeric G proteins. However, numerous studies show that PAR2-selective agonists stimulate phosphoinositide hydrolysis and calcium mobilization, suggesting that PAR2 couples to $G\alpha_q$, $G\alpha_i$, and possibly $G\alpha_{12/13}$ subtypes (Arora et al., 2007). Some PAR2 responses are also sensitive to pertussis toxin treatment, indicating a role for $G\alpha_i$ proteins in some cellular contexts (Schultheiss et al., 1997; Yu et al., 1997). In addition, activated PAR2 appears to signal independent of heterotrimeric G proteins through a stable complex with β-arrestins in endocytic compartments (DeFea et al., 2000; Stalheim et al., 2005). β-arrestins function as signaling scaffolds and promote sustained MAPK activation, actin remodeling, cell migration, and cell permeability (DeFea et al., 2000; Ge et al., 2003; Jacob et al., 2005b; Zoudilova et al., 2007).

1.3 PAR3

Characterization of PAR1$^{-/-}$ knockout mice revealed the existence of an additional member of the PAR family (Connolly et al., 1996). In humans, PAR1 is required for

thrombin-mediated activation of platelets. Surprisingly, PAR1-deficient mouse platelets respond normally to thrombin and exhibit platelet activation similar to those derived from wildtype mice (Connolly et al., 1996). These results implicated an additional PAR in mediating thrombin signaling in mouse platelets and suggested species-specific expression and function of different PARs. Consequently, Coughlin and colleagues identified PAR3 using PCR and degenerate primers derived from conserved regions of PAR1, PAR2, and glycoprotein hormone GPCRs (Connolly et al., 1996; Ishihara et al., 1997). Human PAR3 has 27% amino-acid sequence similarity to PAR1 and 28% similarity to PAR2.

PAR3 has a similar mechanism of activation as other PAR family members (Ishihara et al., 1997). The N-terminus of PAR3 is proteolytically cleaved at residues Lys^{38}/Thr^{39} to unmask a tethered ligand domain that binds and activates the receptor (Fig. 1.1A). PAR3 also contains a hirudin-like domain in its N-terminus that confers protease specificity for thrombin. Thrombin activates PAR3 at picomolar concentrations in COS-7 cells overexpressing the receptor. Unlike PAR1 and PAR2, addition of synthetic agonist peptides that mimic the new N-terminus of PAR1, PAR2, or PAR3 do not activate the receptor or induce transmembrane signaling even at high concentrations of peptide (Ishihara et al., 1997).

Studies of PAR3 signaling show that the receptor is less efficacious in transmitting signals through heterotrimeric G proteins (Coughlin, 2000; Coughlin, 2005). PAR3 is essential for mouse platelet activation at low thrombin concentrations, but does not promote inositol phosphate accumulation or calcium mobilization despite cleavage of its exodomain by thrombin (Kahn et al., 1998; Nakanishi-Matsui et al., 2000). Instead,

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PAR3 acts as a cell surface cofactor for PAR4, the second thrombin receptor in platelets. Thrombin binds to the PAR3 cleavage site and hirudin-like domain for localization and activation of PAR4, a PAR with lower affinity for thrombin (Nakanishi-Matsui et al., 2000). In the presence of PAR3, thrombin cleaves PAR4 more efficiently and shows an increase in potency at PAR4. In a recent study, PAR3 was found to elicit cellular responses without the aid of an additional PAR family member. Seminario-Vidal et al. showed that human PAR3 was the only PAR expressed in A549 lung epithelial cells and was sufficient to induce thrombin-promoted ATP release through a RhoA- and calcium-dependent pathway (Seminario-Vidal et al., 2009). Future work is needed to better characterize the signaling pathways downstream of PAR3 depending on its expression in different cell types and the presence of other PAR family members.

1.4 PAR4

PAR1 is required for human platelet activation, but PAR1-deficient mouse platelets remain responsive to thrombin (Kahn et al., 1998). Consequently, PAR3 was identified as the thrombin receptor expressed in mouse platelets. At low thrombin concentrations, PAR3-deficient mouse platelets exhibit little to no activation compared to wildtype platelets but at higher concentrations exhibit a delayed response, suggesting the existence of another thrombin receptor (Kahn et al., 1998). PAR4 was discovered by searching for expressed tag sequences (ESTs) related to PAR1, PAR2, and PAR3 (Kahn et al., 1998; Xu et al., 1998). The PAR4-specific agonist peptide stimulated wildtype and PAR3-deficient mouse platelets as well as PAR1-desensitized human platelets (Kahn et al., 1998; Xu et al., 1998; Kahn et al., 1999). These results suggested that PAR4 is the second thrombin receptor in both mouse and human platelets and required for platelet

responses at high concentrations of thrombin (Kahn et al., 1998; Xu et al., 1998; Kahn et al., 1999).

Serine proteases cleave the PAR4 N-terminus (Kahn et al., 1998; Xu et al., 1998), and the exposed tethered ligand domain binds and activates the receptor (Fig. 1.1). The PAR4-specific agonist peptide GYPGKF fully activates PAR4 although at a lower potency than the PAR1 agonist peptide at PAR1. Thrombin is less potent at PAR4 than PAR1 but induces a similar maximal response, explaining the increased thrombin-sensitivity in PAR3-deficient mouse platelets with higher concentrations of thrombin. PAR4 is also activated by other serine proteases including trypsin, TF-FVIIa-FXa, plasmin, and cathepsin G (Kahn et al., 1998; Xu et al., 1998). In fibroblasts, activated PAR4 stimulates phosphoinositide hydrolysis but not inhibition of adenylyl cyclase, suggesting that PAR4 couples to $G\alpha_q$ and possibly $G\alpha_{12/13}$ but not $G\alpha_i$ (Faruqi et al., 2000).

1.5 Physiology of PARs

PARs primarily function in the vasculature and transmit signals from extracellular proteases in response to tissue injury and inflammation (Coughlin, 2000; Coughlin, 2005). PAR1, PAR3, and PAR4 have overlapping and distinct tissue distribution and are expressed on cell types of the vasculature including platelets, endothelial cells, leukocytes, smooth muscle cells, fibroblasts, and neurons (Coughlin, 2000; Coughlin, 2005). PAR2, unlike the other PAR family members, is not found on platelets or activated by thrombin but is present on endothelial cells, fibroblasts, smooth muscle cells, neurons, and immune cells within the vasculature (Nystedt et al., 1994; Nystedt et al., 1995; D'Andrea et al., 1998). PAR2 is also expressed on epithelial cells in the lung,

kidney, skin, and, eye and found at high levels in the gastrointestinal tract where it is cleaved by one of its physiological activators, trypsin (Nystedt et al., 1994; Nystedt et al., 1995).

1.5.1 PARs in Vascular Biology

Thrombin is the primary effector protease of the coagulation cascade and the most potent activator of platelets. Thrombin elicits its cellular responses through cleavage and activation of cell surface receptors PAR1, PAR3, and PAR4 (Coughlin, 2000; Coughlin, 2005). Thrombin is generated through a series of zymogen conversions initiated by the transmembrane protein, tissue factor. Tissue factor is basally expressed on the surface of epithelial cells, macrophages, and other cell types that normally are not in contact with blood. Under conditions of vascular injury, tissue factor associates with circulating coagulant proteases and initiates thrombin formation (Fig. 1.2). Consequently, thrombin elicits cellular responses through activation of PARs on cell types of the vasculature. Thrombin also generates fibrin from circulating fibrinogen, which polymerizes into a meshwork over sites of vascular injury to form blood clots.

Studies of PAR knockout mice and PAR-specific agonist peptides and inhibitors have uncovered particular roles for PAR subtypes in vascular biology. In humans, PAR1 and PAR4 are expressed on platelets (Vu et al., 1991a; Ishihara et al., 1997; Kahn et al., 1998; Kahn et al., 1999; Nakanishi-Matsui et al., 2000). Addition of PAR1-specific agonist peptide, SFLLRN, activates human platelets, resulting in platelet shape change and aggregation, ATP release, and activation of α_{IIb}/β_3 integrin, a cell adhesion receptor that binds fibrinogen and von Willebrand Factor to promote platelet aggregation (Vu et al., 1991a; Vassallo et al., 1992; Coughlin, 2005). Thrombin also promotes synthesis and

secretion of thromboxane A_2 and expression of pro-inflammatory mediators, P-selectin and CD40 ligand on the surface of platelets (Jennings, 2009). In human platelets, thrombin responses were inhibited with PAR1 blocking antibodies but only at low thrombin concentrations (Brass et al., 1992; Hung et al., 1992a). Incubation with both PAR1 and PAR4 blocking antibodies completely ablated platelet activation at high and low concentrations of thrombin, indicating that both receptors are required for cellular responses to thrombin in human platelets (Kahn et al., 1999). Thus, PAR1 is the main cell surface receptor for thrombin in human platelets and stimulates platelet aggregation and secretion in the presence of low levels of thrombin whereas PAR4 can elicit these same responses in the absence of PAR1 activity at high levels of thrombin.

In contrast to human platelets, mouse platelets use PAR3 and PAR4 as cell surface receptors for thrombin signaling. PAR1 agonist peptides were shown to fully activate human platelets as well as PAR1 expressed in various cell types. In mouse platelets, however, PAR1 agonist peptides had no effect (Connolly et al., 1994; Derian et al., 1995; Connolly et al., 1996; Kahn et al., 1998). In addition, platelets from PAR1^{-/-} knockout mice are still responsive to thrombin stimulation, indicating that PAR1 is dispensable in mouse platelet activation and that another thrombin receptor mediates this response (Connolly et al., 1996).

Coughlin and colleagues cloned PAR3 and showed that PAR3-deficient mouse platelets are unresponsive to low concentrations of thrombin (Ishihara et al., 1997). PAR4 was identified as the second thrombin receptor in mouse platelets and responsible for cellular functions of thrombin at high concentrations (Kahn et al., 1998; Xu et al., 1998). Studies examining activation and signaling of recombinant PAR3 in COS-7 cells

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revealed that PAR3 does not promote signaling on its own but requires expression of PAR4 to mediate cellular responses to thrombin (Nakanishi-Matsui et al., 2000). Expression of PAR3 increases thrombin cleavage of PAR4 and promotes signaling at low thrombin concentrations (Nakanishi-Matsui et al., 2000). In addition, expression of just the N-terminal domain of PAR3 on the cell surface also increased thrombin cleavage and activation of PAR4 in the presence of low levels of thrombin (Nakanishi-Matsui et al., 2000). Collectively, these results suggested that PAR3 is a cofactor for PAR4 and localizes thrombin to the cell surface for PAR4 activation. In PAR4-deficient mice, loss of PAR4 completely ablates platelet activation at high and low concentrations of thrombin, indicating that PAR4 is essential for thrombin signaling in mouse platelets and the presence of PAR3 alone cannot rescue this effect (Sambrano et al., 2001).

PARs are also found on other cell types of the vasculature either surrounding or in blood vessels. In the event of vascular injury, generation and release of thrombin, upstream coagulant proteases FVIIa and Xa, or other extracellular proteases may result in activation of PARs expressed on endothelial cells, smooth muscle, immune cells, neurons, or other cell types (Coughlin, 2000; Coughlin, 2005). Activation of PAR1 on endothelial cells induces release of von Willebrand factor and expression of P-selectin on the cell surface for rolling and adhesion of leukocytes and platelets. Thrombin activation also induces expression of platelet-activating factor, chemokines, prostaglandins, and COX2 (Weksler et al., 1978; Houliston et al., 2002). In the vasculature, PAR2 is expressed on endothelial and smooth muscle cells and is upregulated by inflammatory mediators and tissue damage (Nystedt et al., 1996; Damiano et al., 1999). Activation of both PAR1 and PAR2 regulates vascular tone, primarily by inducing relaxation of blood

vessels through release of nitric oxide and prostaglandins (al-Ani et al., 1995; Hamilton et al., 1998; Saifeddine et al., 1998; Hamilton et al., 2001). In addition, thrombin activation of PAR1 promotes endothelial cell permeability whereas proteolytic cleavage by APC induces endothelial barrier protection, suggesting that PARs may mediate different responses depending on the activating protease and downstream signaling pathways (Feistritzer and Riewald, 2005; Finigan et al., 2005). PAR1 and PAR2 expressed on vascular smooth muscle and the endothelium promote cell proliferation and production of growth factors and matrix proteases for tissue remodeling (Mirza et al., 1996). On sensory neurons, activation of PAR1 and PAR2 induces neurogenic inflammation, edema, and hyperalgesia (de Garavilla et al., 2001; Vergnolle et al., 2001). Thus, PARs elicit responses to extracellular proteases within the vasculature and function in various events related to vascular injury and inflammation.

1.5.2 PARs in Hemostasis and Thrombosis

Hemostasis is essential to prevent uncontrolled bleeding at sites of vascular injury (Jennings, 2009). Under pathological conditions, however, this process can lead to thrombi formation in blood vessels, which obstruct blood flow and can lead to various vascular diseases including stroke, angina, and myocardial infarction (Jennings, 2009). Hemostasis and thrombosis requires the presence of platelets and thrombin as well as many other agonists that activate potentially redundant signaling pathways of these events. Since PAR4-deficient mouse platelets are completely unresponsive to thrombin activation, studies of PAR4-deficient mice have provided an *in vivo* model to specifically examine the function of thrombin-dependent platelet activation and PARs on hemostasis and thrombosis (Coughlin, 2005).

PAR4-deficient mice exhibited prolonged bleeding times and displayed significant resistance to thrombolytic challenge in experimental hemostasis and thrombosis models (Sambrano et al., 2001; Hamilton et al., 2004; Vandendries et al., 2007). This phenotype was reversed when PAR4$^{+/+}$ bone marrow was reconstituted into lethally irradiated PAR4-deficient mice (Hamilton et al., 2004). These observations suggest that the protective effect is completely due to loss of PAR4 signaling in platelets and not other cell types within the vasculature that could contribute to hemostasis and thrombosis. Since PAR4-deficient mice do not exhibit spontaneous bleeding or excessive bleeding after giving birth, PARs may serve as potential therapeutic targets for the treatment of vascular diseases associated with thrombosis without affecting the normal physiological process of hemostasis (Sambrano et al., 2001). In accordance with these genetic experiments, studies of non-human primates have provided promising results for the use of PAR antagonists as antiplatelet drugs. Similar to humans, PAR1 signaling is considered to be more important than PAR4 signaling in monkeys. Inhibiting PAR1 with either a blocking antibody or the small molecule antagonist, RWJ-58259, protected monkeys from experimental arterial thrombosis without affecting PAR4 signaling (Cook et al., 1995; Derian et al., 2003). These results suggest that inhibiting PAR1 alone is sufficient to block thrombosis in humans.

1.5.3 PARs in Cancer

The tumor microenvironment contains a multitude of extracellular proteases, including PAR activators, thrombin, coagulant proteases FVIIa and FXa, trypsin, and matrix metalloproteases. PAR1 is overexpressed in breast, colon, and prostate cancer as well as melanoma (Even-Ram et al., 1998; Chay et al., 2002; Tellez and Bar-Eli, 2003;

Darmoul et al., 2004). PAR1 and PAR2 expression is also upregulated in stromal fibroblasts surrounding malignant tumors whereas neither receptor is present in normal or benign tumor samples (D'Andrea et al., 2001). Ectopic expression of PAR1 transforms NIH-3T3 cells, suggesting an oncogenic role for PAR1 in cancer progression (Whitehead et al., 1995). In addition, thrombin activation of PAR1 promotes tumor cell invasion of highly invasive breast cancer cells *in vitro* (Even-Ram et al., 1998; Booden et al., 2004). Boire et al. found that overexpression of PAR1 in non-invasive breast carcinoma cells increased tumor cell migration and invasion, and injection of these PAR1-expressing cells into the mammary fat pads of nude mice markedly increased tumor cell growth compared to wildtype cells (Boire et al., 2005). In accordance with these studies, shRNA depletion of PAR1 from highly invasive breast carcinoma cells markedly impaired tumor cell growth in a mouse xenograft tumor model compared to control cells (Arora et al., 2008).

PAR2 is also expressed in breast carcinoma and highly invasive cancer cell lines, and its physiological activator trypsin is upregulated in colon, ovarian, lung, and gastric cancers (Ducroc et al., 2002; Arora et al., 2007). The PAR2 activators, TF-FVIIa and Xa were shown to induce breast cancer cell migration and invasion through a PAR2-dependent pathway (Ge et al., 2004; Morris et al., 2006). In addition, siRNA depletion of PAR2 prevented invasion and migration of breast carcinoma cells toward NIH-3T3 conditioned medium (Morris et al., 2006). In a recent study, Versteeg et al. validated these *in vitro* results using the mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) mouse model (Versteeg et al., 2008). This breast cancer model closely resembles breast tumor progression in humans, and mice exhibit widespread transformation of

mammary epithelium and metastasis to the lymph nodes and lungs (Fantozzi and Christofori, 2006). Genetic crossing of PAR2$^{-/-}$ and MMTV-PyMT mice resulted in a significant delay in breast cancer progression whereas mice lacking PAR1 were identical to wildtype MMTV-PyMT mice (Versteeg et al., 2008). These results imply a role for PAR2 and not PAR1 in breast cancer progression, suggesting that PAR2 may be a therapeutic target for the treatment of breast cancer.

1.6 Canonical paradigm of GPCR signal regulation

G protein-coupled receptors (GPCRs) are the largest and most diverse family of cell surface receptors encoded in the mammalian genome. In humans, there are ~1000 GPCRs that transduce signals of a variety of extracellular stimuli such as hormones, peptides, neurotransmitters, proteases, ions, photons, and many others. Signaling downstream of these receptors regulates an array of physiological responds including cardiac function, vision, olfaction, and neurotransmission. Consequently, approximately half of the drugs currently available target GPCRs either directly or indirectly to modulate intracellular signaling and cellular physiology, indicating the huge success of targeting GPCR signaling for therapeutic treatments of many diseases.

GPCRs elicit their cellular response through association with heterotrimeric G proteins. Upon binding of agonist, GPCRs undergo conformational changes and expose intracellular domains for heterotrimeric G protein binding. GPCRs act as guanine nucleotide exchange (GEF) factors and catalyze exchange of GDP for GTP on Gα subunits. GTP-bound Gα subunits dissociate from G$\beta\gamma$ dimers, and both signaling molecules directly bind and activate various effector proteins including adenylyl cyclases, ion channels, phospholipases, and GEF proteins for small monomeric GTPases.

In addition, GPCRs also signal independent of heterotrimeric G proteins through stable association with the adaptor proteins, arrestins. Arrestins can function as scaffolds and form stable complexes with some GPCRs and a variety of signaling proteins.

GPCR signaling is tightly regulated by mechanisms of receptor desensitization and trafficking. The mechanisms controlling receptor desensitization are best characterized for the photoreceptor rhodopsin and β_2-adrenergic receptor (β_2AR). In the classic model, agonist-occupied GPCRs undergo rapid phosphorylation by G protein-coupled receptor kinases (GRKs) or second messenger kinases (Krupnick and Benovic, 1998; Pitcher et al., 1998). GRKs recognize activated receptors and phosphorylate serine and threonine residues within the third intracellular loop and/or cytoplasmic tail (C-tail) of GPCRs. The cytosolic adaptor proteins, β-arrestin 1 and β-arrestin 2 (also known as arrestin 2 and 3) translocate to the plasma membrane and bind to the activated and phosphorylated receptor. β-arrestins competitively bind and sterically hinder heterotrimeric G protein binding to receptors.   Thus, β-arrestins uncouple activated GPCRs from G proteins and attenuate transmembrane signaling.

GPCR signal regulation is also tightly controlled by receptor trafficking and is best characterized for the β_2AR (Fig. 1.3). β_2AR and other GPCRs internalize through a β-arrestin-dependent pathway (Krupnick and Benovic, 1998; Pitcher et al., 1998). β-arrestins directly associate with clathrin and adaptor protein complex-2 (AP-2), components of the endocytic machinery, and facilitate receptor internalization through clathrin-coated pits (Goodman et al., 1996; Laporte et al., 1999). β_2AR internalization requires dephosphorylation of β-arrestin 1 and 2 to enhance clathrin binding and targeting of the β_2AR/β-arrestin complex to clathrin-coated pits (Lin et al., 1997; Lin et al., 2002).

In addition, β_2AR internalization also requires ubiquitination of β-arrestin 2 by the E3 ubiquitin ligase mouse double minute-2 (MDM2) (Shenoy et al., 2001). Once internalized, β_2AR is sorted to recycling endosomes where it is dephosphorylated and targeted to the plasma membrane for additional signaling (Fig. 1.3). After prolonged agonist exposure, the receptor is targeted to lysosomes for degradation through a ubiquitin-dependent pathway.

1.7 Signal regulation of PARs

In contrast to β_2AR, PARs are irreversibly activated by proteolytic cleavage and contain a tethered ligand domain that does not diffuse away. Studies of PAR signal regulation indicate that the regulatory mechanisms are relatively unique from reversibly activated GPCRs. Once activated, PARs are rapidly desensitized, endocytosed, and quickly sorted to lysosome for degradation without recycling to the cell surface for subsequent signaling (Arora et al., 2007). Thus, the mechanisms controlling desensitization and trafficking of PARs are essential for regulating the temporal and spatial aspects of receptor signaling. In fact, a chimeric receptor consisting primarily of PAR1 and the C-tail domain of substance P receptor was fully activated by thrombin cleavage but recycled to the cell surface and displayed prolonged receptor signaling due to the intact tethered ligand domain (Trejo et al., 1998). In highly invasive breast carcinoma cells, proteolytically cleaved PAR1 was not sufficiently sorted to lysosomes and degraded (Booden et al., 2004). Consequently, PAR1 exhibited prolonged phosphoinositide and MAPK signaling after thrombin was removed. These results demonstrate the importance of receptor internalization and lysosomal degradation in terminating signaling downstream of PARs.

Signal regulation of PARs is best characterized for PAR1. Upon proteolytic cleavage, PAR1 is rapidly desensitized despite irreversible activation by extracellular proteases. GRKs phosphorylate PAR1 on intracellular serine and threonine residues, and β-arrestin binding uncouples the receptor from heterotrimeric G proteins (Ishii et al., 1994; Tiruppathi et al., 2000; Paing et al., 2002). While PAR1 phosphorylation is required for receptor endocytosis (Fig. 1.3), PAR1 internalizes independent of β-arrestin 1 and β-arrestin 2 through a clathrin and dynamin-dependent pathway (Trejo et al., 2000; Paing et al., 2002). Once internalized, PAR1 is quickly sorted through the endocytic pathway and targeted to lysosomes for degradation at a half-life of 1 hour (Fig. 1.3) (Trejo et al., 1998). Unlike β_2AR and other GPCRs, degradation of PAR1 is not regulated by ubiquitination. A PAR1 mutant lacking all intracellular lysine residues was shown to degrade normally in HeLa cells (Wolfe et al., 2007). Other studies found that PAR1 degradation is regulated by sorting nexin 1 (SNX1), a protein involved in vesicular trafficking (Wang et al., 2002; Gullapalli et al., 2006). A dominant negative mutant of SNX1 significantly impaired PAR1 degradation, and these results were confirmed by siRNA depletion (Gullapalli et al., 2006).

PARs are considered single use receptors because they are quickly internalized and sorted from endocytic compartments to lysosomes for degradation. Thus, activated PARs are not recycled to the cell surface for additional signaling, and PAR1 resensitization occurs through a distinct mechanism from other GPCRs. Under basal conditions, an internal pool of unactivated PAR1 constitutively cycles between the cell surface and an intracellular compartment and is protected from cleavage by thrombin and other extracellular proteases (Fig.1.3). After protease exposure, PAR1 is cleared from the

cell surface by internalization, and the protected pool of PAR1 cycles to the plasma membrane for subsequent stimulation (Hein et al., 1994). Constitutive internalization of unactivated PAR1 is regulated by a tyrosine-based motif ($Y^{420}XX\Phi$; where X is any amino acid and Φ is a bulky hydrophobic residue) in the PAR1 cytoplasmic tail (Paing et al., 2006). The tyrosine-based motif is recognized by the μ-subunit of AP-2, which binds protein cargo for sorting to clathrin-coated pits. Mutation of the tyrosine-based motif in the PAR1 cytoplasmic tail or depletion of AP-2 abrogated constitutive PAR1 internalization but not agonist-promoted endocytosis (Paing et al., 2006). Surface plasmon resonance studies confirmed that the μ-subunit of AP-2 binds directly to synthetic peptides of the PAR1 cytoplasmic tail but not peptides mutated at the tyrosine-based motif (Paing et al., 2006).

Studies of PAR1 intracellular trafficking revealed a novel role for receptor ubiquitination (Fig. 1.3). PAR1 is basally ubiquitinated and deubiquitinated upon agonist exposure (Wolfe et al., 2007). A PAR1 mutant lacking all intracellular lysine residues exhibited enhanced constitutive internalization, and this effect was blocked by fusing a ubiquitin moiety to the cytoplasmic tail (Wolfe et al., 2007). These results suggested that basal PAR1 ubiquitination negatively regulates constitutive internalization. Similar to wildtype PAR1, the ubiquitin-deficient mutant also required clathrin, dynamin, and the endocytic adaptor protein, AP-2 to facilitate constitutive internalization (Trejo et al., 2000; Paing et al., 2006; Wolfe et al., 2007). Unlike wildtype PAR1, however, siRNA depletion of AP-2 also blocked agonist-promoted internalization of ubiquitin-deficient PAR1 (Wolfe et al., 2007). The authors speculated that PAR1 ubiquitination might regulate the interaction between AP-2 and PAR1. Interestingly, within the AP-2 binding

sequence (Y^{420}KKL) of PAR1 are two lysine residues. Mutation of Lys^{421} and Lys^{422} revealed that these are the primary sites of PAR1 ubiquitination and that these sites negatively regulate PAR1 constitutive internalization (Wolfe et al., 2007). While this evidence is intriguing, studies examining direct binding of AP-2 and ubiquitinated PAR1 are needed to support these observations. Collectively, these studies show that unlike the role of ubiquitination in controlling degradation of β_2AR and other GPCRs, ubiquitination of PAR1 instead functions to negatively regulate receptor internalization.

Receptor desensitization and trafficking are also essential for PAR2 signal regulation, but the regulatory mechanisms are less well defined (Bohm et al., 1996a). Previous studies found that pharmacological inhibitors of protein kinase C increased PAR2-stimulated calcium mobilization in two epithelial cell lines, KNRK and hBRIE 380 cells, suggesting a role for phosphorylation in mediating receptor desensitization (Bohm et al., 1996a). PAR2 contains multiple serine and threonine residues on its C-tail and intracellular loops that could function as potential sites of receptor phosphorylation. However, it remains to be determined if PAR2 is directly phosphorylated and what is the function of this modification on PAR2 regulatory mechanisms. In addition, the role of specific GRKs and/or second messenger kinases in controlling PAR2 signaling requires further investigation.

Previous studies also have demonstrated a role for β-arrestins in regulating PAR2 signaling and trafficking (Fig. 1.3). PAR2 displayed enhanced phosphoinositide signaling in mouse embryonic fibroblast (MEFs) lacking β-arrestin 1 and β-arrestin 2 compared to wildtype MEFs, suggesting that β-arrestins are required for uncoupling PAR2 from heterotrimeric G proteins (Stalheim et al., 2005). In addition, agonist-

induced internalization of PAR2 was severely impaired in β-arrestin1,2$^{-/-}$ MEFs, confirming previous results using dominant negative β-arrestin mutants (DeFea et al., 2000; Stalheim et al., 2005). β-arrestins were also found to co-localize with internalized PAR2 in endosomal compartments and form a stable complex consisting of PAR2, β-arrestins, raf-1, and activated ERK1/2 (DeFea et al., 2000). Formation of this stable complex was required for sustained ERK1/2 signaling and was proposed to occur independent of heterotrimeric G protein activation (DeFea et al., 2000; Stalheim et al., 2005; DeFea, 2007).

Binding of β-arrestins to activated GPCRs involves multiple interactions with intracellular loops and/or cytoplasmic tails of receptors. Sequential deletion of the PAR2 C-tail had no effect on rapid translocation of β-arrestins to the cell surface or β-arrestin-dependent desensitization, suggesting that the C-tail is not required for β-arrestin recruitment or uncoupling from heterotrimeric G proteins (Stalheim et al., 2005). In contrast, internalized PAR2 truncation mutants failed to form stable complexes with β-arrestins in endocytic compartments or stimulate sustained ERK1/2 activation (Stalheim et al., 2005). Collectively, these results show that β-arrestins are critical for signal regulation of PAR2, but the molecular determinants controlling the diverse functions of β-arrestins in PAR2 signaling and trafficking are not clearly understood.

Internalized PAR2 is quickly sorted through the endocytic pathway to lysosomes where the receptor is degraded at a half-life of 3h (Fig. 1.3). Similar to β$_2$AR and CXCR4, lysosomal sorting of PAR2 requires ubiquitination (Marchese and Benovic, 2001; Shenoy et al., 2001; Jacob et al., 2005a). A PAR2 mutant lacking all intracellular lysine residues is not ubiquitinated or degraded after prolonged agonist exposure (Jacob

et al., 2005a). Co-localization studies found that ubiquitin-deficient PAR2 internalizes and sorts to an early endosomal compartment like wildtype PAR2 but fails to sort to lysosomes. Further analysis suggested that the E3 ubiquitin ligase Cbl catalyzes ubiquitination of PAR2 (Jacob et al., 2005a). The molecular determinants for Cbl binding and ubiquitination of PAR2 remain to be determined and are likely unique from other proteins since Cbl normally recognizes tyrosine phosphorylated substrates for binding.

PAR2 lysosomal sorting and degradation are also tightly regulated by two deubiquitinating enzymes, AMSH (associated molecule with the Src homology 3 domain of STAM (signal-transducing adaptor molecule)) and UBPY (ubiquitin-associated protease Y) (Hasdemir et al., 2009). AMSH and UBPY are present on endosomal membranes and associate with endosomal adaptor proteins to regulate lysosomal sorting of ubiquitinated protein cargo (Clague and Urbe, 2006). Catalytically inactive mutants and siRNA knockdown of AMSH and UBPY prevented deubiquitination of PAR2 and blocked sorting of the receptor from endosomal compartments to lysosomes for degradation (Hasdemir et al., 2009). These results suggest that deubiquitination is required for proper post-endocytic trafficking of PAR2 and receptor downregulation.

Research Goals

Protease-activated receptor 2 (PAR2) is a G protein-coupled receptor (GPCR) that acts as a cell surface sensor for extracellular proteases such as trypsin, mast cell tryptase, and coagulant proteases, factor VIIa and Xa (Nystedt et al., 1994; Nystedt et al., 1995; Bohm et al., 1996b; Molino et al., 1997b; Camerer et al., 2000). PAR2, like other members of the PAR family, has a unique mechanism of activation. Extracellular

proteases cleave the N-terminus of the receptor to unmask a tethered ligand domain that intramolecularly binds to the body of PAR2 and activates the receptor. Upon activation, PAR2 couples to $G\alpha_q$, $G\alpha_i$, and possibly $G\alpha_{12/13}$ to induce transmembrane signaling, primarily leading to migratory and inflammatory responses to vascular injury. The mechanisms controlling PAR2 signaling, however, are not clearly defined.

Signal regulation of GPCRs involves receptor desensitization and internalization. Most GPCRs are rapidly phosphorylated on their intracellular loops and/or cytoplasmic tails upon ligand binding and recruit the multifunctional adaptor proteins, β-arrestins, to the cell surface (Krupnick and Benovic, 1998; Pitcher et al., 1998). β-arrestins recognized both the activated and phosphorylated form of GPCRs and sterically hinder binding of heterotrimeric G proteins. In addition, β-arrestins also interact with components of the endocytic machinery and facilitate GPCR internalization, removing activated receptors from the cell surface to prevent further stimulation.

Unlike classic GPCRs, PARs are irreversibly activated by extracellular proteases, and the molecular mechanisms of signal regulation are likely unique from reversible activated GPCRs. We hypothesize that the mechanisms controlling receptor desensitization and trafficking are critical for regulating the magnitude and duration of PAR2 signaling. The goal of this research is to define the role of receptor phosphorylation in regulating PAR2 signaling and trafficking. We will measure PAR2 phosphorylation directly and define the region of phosphorylation using mutational analysis. In addition, we will use phosphorylation-deficient mutants to assess the function of phosphorylation in PAR2 signaling, internalization, and endocytic sorting.

A

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          P3-P2-P1/P1'-P2'-P3'
PAR1  M<34>ATLDPR/SFLLRNPNDKYEPFWEDEEKNESGLTEYRLVSINKSSPLQKQLPARISEDA - TM1

PAR2  M<18>SLSCSGTIQGTNRSSKGR/SLIGKVDGTSHVTGKGVTVETVFSVDEFSASVLTGKLTT - TM1

PAR3  M<30>AKPKPTLPIK/TFRGAPPNSFEEFPFSALEGWTGATITVKIKCPEEESAAGLGVKNAT - TM1

PAR4  M<10>VLGFSLSGGTQTPSVYDESGSTGGGDDSTPSILPAPR/GYPGQVCANDSDTLELPDSS - TM1
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B

Receptor	Tethered ligand	Activating proteases	Signaling partners
PAR1	SFLLRN	Thrombin TF-VIIa-Xa or Xa Trypsin APC-EPCR MMP-1 Plasmin Granzyme A	$G\alpha_q$ $G\alpha_i$ $G\alpha_{12/13}$
PAR2	SLIGKV	Trypsin TF-VIIa, TF-VIIa-Xa Tryptase Proteinase-3 Kallikreins Matriptase (MT-SP1) Der p3 D9 Granzyme A	$G\alpha_q$ $G\alpha_i$ $G\alpha_{12/13}$ β-arrestins
PAR3	TFRGAP	Thrombin	?
PAR4	GYPGQV	Thrombin Trypsin TF-VIIa-Xa Plasmin	$G\alpha_q$ $G\alpha_{12/13}$

Figure 1.1. **Protease-activated receptor family**. (**A**) Amino-terminal sequences of human PARs preceding the first transmembrane domain (TM1). The tethered ligand domain is shown in red, and the hirudin-like domain in PAR1 and PAR3 is underlined and required for high affinity thrombin binding. The protease recognition sites are defined as P3-P2-P1/P1′-P2′-P3′ where (P) and (P′) represent unprimed and primed sites respectively, and / depicts the site of proteolytic cleavage. The critical basic residues recognized by serine proteases are shown in blue. The number of amino acids not listed is shown in brackets. (**B**) Table of PAR family members and their respective tethered ligand domains, activating proteases, and signaling partners. Synthetic peptides that mimic the tethered ligand domain fully activate PARs in the absence of proteolytic cleavage except for PAR3. Figure was modified from Russo et al., 2009.
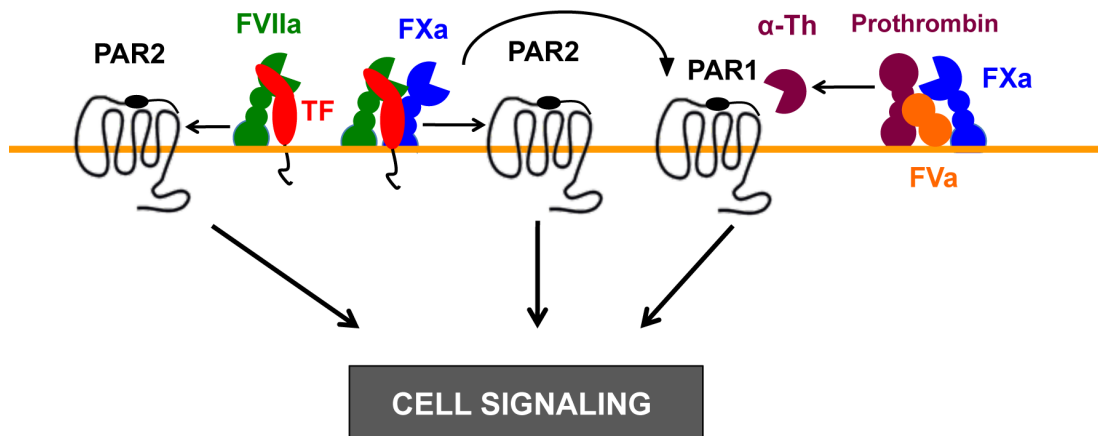
Figure 1.2. **Activation of PARs by coagulant proteases**. Tissue (TF) acts as a transmembrane cofactor for FVIIa, which converts coagulant protease FX to activated FXa. FXa with the aid of an additional cofactor, FVa, generates α-thrombin (α-Th) from prothrombin. α-Th elicits cellular responses through proteolytic cleavage and activation of PAR1, PAR3, and PAR4 but not PAR2. The TF-FVIIa complex directly cleaves and activates PAR2 whereas the tertiary TF-FVIIa-FXa complex activates both PAR1 and PAR2 to induce transmembrane signaling.
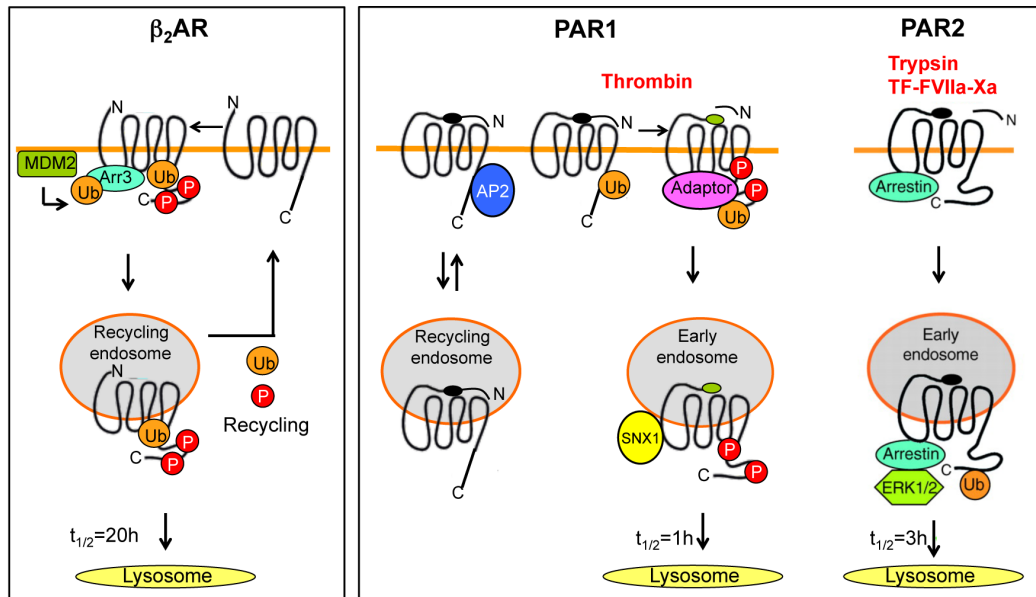
Figure 1.3. **Endocytic trafficking of β₂AR vs. PARs.** Reversibly activated β₂-adrenergic receptor (β₂AR) requires phosphorylation, arrestin binding, and arrestin ubiquitination by the E3 ubiquitin ligase MDM2 for endocytosis. Internalized β₂AR is sorted to recycling endosomes where it is dephosphorylated and recycled to the cell surface for additional signaling. Prolonged agonist exposure leads to degradation of β₂AR through a ubiquitin-dependent pathway. In contrast, PARs are considered single use receptors due to irreversible activation by extracellular proteases and are rapidly internalized and sorted to lysosomes for degradation. PAR1 exhibits two types of internalization. The endocytic adaptor protein, AP2, facilitates constitutive internalization of a pool of PAR1 that tonically cycles between the cell surface and recycling endosomes. Ubiquitination of PAR1 negatively regulates this constitutive internalization. Agonist-induced PAR1 internalization requires receptor phosphorylation but occurs independent of arrestin binding. Internalized PAR1 is rapidly sorted from endosomes to lysosomes through a SNX1-dependent pathway and degraded. Unlike PAR1, PAR2 internalizes through an arrestin-dependent pathway and appears to signal independent of G proteins through its interaction with arrestins within intracellular compartments. Internalized PAR2 is sorted to lysosomes and degraded through a ubiquitin-dependent pathway. Figure was adapted from Arora et al., 2007.

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CHAPTER 2

PHOSPHORYLATION OF PROTEASE-ACTIVATED RECEPTOR-2 DIFFERENTIALLY REGULATES DESENSITIZATION AND INTERNALIZATION

2.1 Abstract

Protease-activated receptor 2 (PAR2) is a G protein-coupled receptor (GPCR) irreversibly activated by extracellular proteases. Activated PAR2 couples to multiple heterotrimeric G protein subtypes including $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$. Most activated GPCRs are rapidly desensitized and internalized following phosphorylation and β-arrestin binding. However, the role of phosphorylation in regulation of PAR2 signaling and trafficking is not known. To investigate the function of phosphorylation, we generated a PAR2 mutant in which all serines and threonines in the C-tail were converted to alanines and designated it PAR2 0P. In mammalian cells, the addition of agonist induced a rapid and robust increase in phosphorylation of wildtype PAR2 but not the 0P mutant, suggesting that the major sites of phosphorylation occur within the C-tail domain. Moreover, desensitization of PAR2 0P signaling was markedly impaired compared to wildtype receptor. Wildtype phosphorylated PAR2 internalized through a canonical dynamin, clathrin- and β-arrestin-dependent pathway. Strikingly, PAR2 0P mutant internalization proceeded through a dynamin-dependent but clathrin- and β-arrestin-independent pathway in both a constitutive and agonist-dependent manner. Collectively, our studies show that PAR2 phosphorylation is essential for β-arrestin binding and

uncoupling from heterotrimeric G protein signaling and that the presence of serine and threonine residues in the PAR2 C-tail hinder constitutive internalization through a non-canonical pathway. Thus, our studies reveal a novel function for phosphorylation that differentially regulates PAR2 desensitization and endocytic trafficking.

2.2 Introduction

Protease-activated receptor 2 (PAR2) is a member of the protease-activated G protein-coupled receptor (GPCR) family that includes PAR1, PAR3 and PAR4 (Arora et al., 2007). PAR2 is expressed in intestinal and airway epithelial cells, fibroblasts and in a variety of cell types of the vasculature and functions in inflammatory processes associated with tissue injury. PAR2 is also expressed in certain types of metastatic cancers and stimulates tumor cell migration and invasion (Ge et al., 2004; Morris et al., 2006). Multiple extracellular proteases cleave and activate PAR2 including trypsin, mast cell tryptase, and the coagulation protease factor VIIa in complex with tissue factor and Xa and others, but not thrombin (Bohm et al., 1996b; Dery et al., 1999; Camerer et al., 2000). Similar to other PARs, proteolytic cleavage of PAR2 results in the formation of a new N-terminus that acts like a tethered ligand by binding intramolecularly to the receptor to trigger transmembrane signaling (Nystedt et al., 1994; Bohm et al., 1996b). Synthetic peptides that mimic the tethered ligand sequence of the newly exposed N-terminus can activate PAR2 independent of proteolytic cleavage. Upon activation, PAR2 couples to multiple heterotrimeric G protein subtypes including $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$ which signal to a variety of effectors and promotes diverse cellular responses (Arora et al., 2007). Unlike other PARs, however, activated PAR2 also signals independently of G proteins through its interaction with β-arrestins, which promotes sustained mitogen-

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activated protein (MAP) kinase signaling, actin remodeling, and cell migration (Ge et al., 2003; Jacob et al., 2005b; Zoudilova et al., 2007). The molecular determinants that specify PAR2 coupling to distinct heterotrimeric G protein subtypes and binding to β-arrestins remain to be determined.

The known regulatory processes that control GPCR signaling are based largely on studies of the β_2-adrenergic receptor (β_2-AR) (Pitcher et al., 1998). In the classic paradigm, ligand-activated GPCRs are rapidly phosphorylated on serine and threonine residues localized within the third intracellular loop or cytoplasmic tail (C-tail) by G protein-coupled receptor kinases (GRKs). β-arrestins are then rapidly recruited and associate with activated and phosphorylated GPCRs at the plasma membrane. The binding of β-arrestins to activated and phosphorylated GPCRs mediates receptor uncoupling from G proteins and facilitates receptor internalization (Ferguson et al., 1996; Goodman et al., 1996; Laporte et al., 1999). Once internalized, some GPCRs signal from intracellular compartments through stable interaction with β-arrestins, which functions as a scaffold and transducer of MAP kinase signaling independent of G proteins (Luttrell, 2003). Internalized GPCRs are then targeted to recycling endosomes, dephosphorylated and returned to the cell surface, or sorted to lysosomes and degraded.

Unlike most classic GPCRs, PAR2 is irreversibly activated by extracellular proteases. Thus, we hypothesize that PAR2 signal regulatory mechanisms are likely unique since most other GPCRs are reversibly activated. We previously reported that β-arrestins are essential for activated PAR2 desensitization and internalization. Indeed, in mouse embryonic fibroblasts (MEFs) deficient in β-arrestin 1 and 2 expression, PAR2 desensitization was significantly impaired compared to wildtype β-arrestin expressing

cells (Stalheim et al., 2005). Internalization of activated PAR2 was also virtually abolished in cells lacking β-arrestin 1 and 2 expression (Stalheim et al., 2005). The binding of β-arrestins to activated GPCRs is known to involve multiple interactions with the receptor intracellular loops and C-tail (Gurevich and Gurevich, 2006). Interestingly, activated PAR2 C-tail truncation mutants displayed normal agonist-induced internalization, caused rapid redistribution of β-arrestins to the plasma membrane, and desensitized in a β-arrestin-dependent manner similar to wildtype PAR2 (Stalheim et al., 2005), suggesting that the C-tail of PAR2 is not critical for rapid β-arrestin recruitment. However, activated PAR2 C-tail mutants lost the capacity to form stable complexes with β-arrestins, and failed to promote sustained MAP kinase signaling. These findings suggest that the PAR2 C-tail regulates the stability of β-arrestin interaction and sustained MAP kinase signaling, but is not essential for rapid β-arrestin recruitment nor β-arrestin-dependent receptor desensitization or internalization. These findings further suggest that the diverse functions of β-arrestins in the regulation of PAR2 signaling and trafficking are likely to be controlled by distinct determinants. However, the PAR2 specific determinants that are critical for the diverse functions of β-arrestins in regulation of receptor signaling and trafficking remain poorly understood.

In the present study, we examine for the first time the role of phosphorylation in regulation of PAR2 signaling and trafficking. We report that phosphorylation of PAR2 distinctly regulates desensitization and endocytic trafficking. Our findings further demonstrate that PAR2 phosphorylation is critical for receptor desensitization and β-arrestin binding and that serine and threonine residues in the PAR2 C-tail domain impede constitutive internalization and lysosomal degradation. These studies reveal a novel

function for phosphorylation in differentially regulating PAR2 desensitization and endocytic trafficking.

2.3 Materials and Methods

Reagents and Antibodies

Agonist peptides TFLLRNPNDK (PAR1-specific) and SLIGKV (PAR2-specific) were synthesized as the carboxyl amide and purified by reverse-phase high-pressure chromatography (UNC Peptide Facility, Chapel Hill, NC). α-Trypsin treated with tosylamide-2-phenylethyl chloromethyl ketone, leupeptin, and cycloheximide were from Sigma-Aldrich (St. Louis MO). [^{32}P]orthophosphate was purchased from Perkin-Elmer (Boston, MA). Monoclonal M1 and M2 anti-FLAG, polyclonal anti-FLAG, and anti-β-actin antibodies were from Sigma-Aldrich (St. Louis, MO). Anti-early endosome antigen-1 (EEA1) monoclonal antibody was purchased from BD Biosciences. Anti-clathrin monoclonal antibody X22 was from GeneTex, Inc (San Antonio, TX). Anti-lysosomal-associated membrane protein 1 (LAMP1) H4A3 monoclonal antibody was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Anti-β-arrestin polyclonal antibody A1CT was a gift from R. J. Lefkowitz (Duke University). Horseradish peroxidase (HRP) conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Bio-Rad (Hercules, CA). Alexa Fluor-488 and Alexa Fluor-594 conjugated goat anti-mouse and goat anti-rabbit antibodies were from Invitrogen (Eugene, OR).

cDNAs, siRNAs, and Cell Lines

A cDNA encoding the wildtype PAR2 containing an N-terminal FLAG epitope was previously described (Stalheim et al., 2005). PAR2 mutants were generated by site-

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directed mutagenesis using QuikChange (Stratagene, La Jolla, CA), and mutations were confirmed by dideoxy sequencing. The PAR1 chimera containing the substance P receptor C-tail (P/S) was previously described (Trejo and Coughlin, 1999). The green fluorescent protein (GFP) tagged β-arrestin 1 and 2 constructs were a gift from M. Caron (Duke University). The GFP-tagged dynamin K44A mutant was a gift from M. McNiven (Mayo Clinic and Foundation).

HeLa cells and Rat1 fibroblasts were cultured as previously described (Trejo and Coughlin, 1999; Trejo et al., 2000; Stalheim et al., 2005). HeLa cells and Rat1 fibroblasts stably transfected with FLAG-tagged PAR2 wildtype and mutants were generated as previously published (Trejo and Coughlin, 1999; Trejo et al., 2000).

HeLa cells were transiently transfected for 72 h with 100 nM nonspecific siRNA or smart pool siRNAs targeting the clathrin heavy chain (CHC) or β-arrestin 1 and 2 using LipofectAMINE 2000 or DharmaFECT 2 according to manufacturer's protocol. All siRNAs were purchased from Dharmacon, Inc. (Lafayette, CO)

Phosphoinositide Hydrolysis

Cells stably expressing FLAG-tagged PAR2 wildtype or mutants were plated at 0.8 x 10^5 cells per well in 24-well plates and grown overnight. Cells were labeled with 1 μCi/ml *myo*-[^{3}H]inositol (American Radiolabeled Chemicals, St. Louis, MO) in serum- and inositol-free DMEM containing 1 mg/ml BSA overnight. [^{3}H]Inositol phosphates (IPs) were measured as previously described (Paing et al., 2002).

Receptor Immunoprecipitation, Phosphorylation and Immunoblot Analysis

Rat1 fibroblasts stably expressing FLAG-tagged PAR2 wildtype or mutants were plated at 3.5 x 10^5 cells per well in 6-well plates and grown for 48 h. Cells were labeled

with 200 μCi [^{32}P]orthophosphate in phosphate-free DMEM containing 1 mg/ml BSA for 3 h, washed and incubated in the absence or presence of 100 μM SLIGKV diluted in DMEM containing 1 mg/ml BSA and 10 mM HEPES for various times at 37°C. Cells were lysed with Triton lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 200 mM sodium orthovanadate, and 1% Triton-X-100) containing protease inhibitors. Cell lysates were immunoprecipitated with M2 anti-FLAG antibody, resolved by SDS-PAGE and transferred to membranes. Phosphorylated receptor was detected by autoradiography. The amount of PAR2 in immunoprecipitates was determined by immunoblotting with polyclonal anti-FLAG antibody and HRP-conjugated goat anti-rabbit antibody.

HeLa cells stably transfected with FLAG-tagged PAR2 wildtype or mutants were plated at 1.5 x 10^{6} cells per well in a 6-well plate and grown for 48 h at 37°C. Cells were incubated for various times with or without 100 μM SLIGKV diluted in DMEM containing 10 μM cycloheximide, 1 mg/ml BSA, and 10 mM HEPES. Cells were lysed, immunoprecipitated and immunoblotted as described above. Immunoblots were quantified using Image J software.

Cell Surface ELISA

Cells expressing FLAG-tagged PAR2 wildtype or mutants were plated on fibronectin-coated 24-well plates and grown for 48 h. After incubations, cells were washed and fixed with 4% paraformaldehyde (PFA). The amount of cell surface receptor was quantitated by incubation with M1 anti-FLAG antibody at 25°C for 1 h and HRP-conjugated goat anti-mouse antibody at 25°C for 1 h. The amount of bound secondary antibody was determined by incubating cells with 1-STEP ABTS (2,2'-azino-bis-3-

ethylbenzthiazoline-6-sulfonic acid) (Pierce, Rockford, IL) for 10-20 min at 25°C. An aliquot was taken to measure the optical density at 405 nm using a Molecular Devices microplate spectrophotometer.

Internalization Assay

Cells stably expressing FLAG-tagged PAR2 wildtype or mutants were plated on fibronectin-coated 24-well plates and grown for 48 h. Cells were prelabeled with M1 anti-FLAG antibody for 1 h at 4°C and then incubated with or without agonist for various times at 37°C. The loss of cell surface receptor was measured by ELISA.

Immunofluorescence Confocal Microscopy

HeLa cells stably expressing FLAG-tagged PAR2 wildtype or mutant were plated at 0.8 x 10^5 cells per well on fibronectin-coated glass cover slips in 12-well plates for 48 h at 37°C. Cells were washed with ice-cold DMEM and prelabeled with polyclonal anti-FLAG antibody on ice for 1 h. Cells were incubated in the absence or presence of 100 μM SLIGKV for 30 min at 37°C, washed and fixed with 4% PFA. Cells were permeabilized with ice-cold 100% methanol and washed with PBS containing 1% nonfat dry milk and 150 mM sodium acetate, pH 7.0. Cells were blocked with 1% nonfat dry milk in PBS and incubated with Alexa Fluor-488 conjugated goat anti-rabbit antibody. For lysosomal-associated membrane protein-1 (LAMP1) co-localization studies, cells were incubated in DMEM containing 2 mM leupeptin, a lysosomal protease inhibitor, for 37°C for 1 h. Cells were then processed as described above and immunostained for endogenous LAMP1, using a monoclonal anti-LAMP1 antibody for 1 h and Alexa Fluor-594 conjugated goat anti-mouse antibody. Cells were imaged by confocal microscopy as previously described (Gullapalli et al., 2006; Wolfe et al., 2007). For clathrin heavy

chain knockdown experiments, endogenous clathrin was detected using anti-clathrin monoclonal antibody and Alexa Fluor-594 conjugated goat anti-mouse antibody.

Receptor Recycling

HeLa cells stably expressing FLAG-tagged PAR2 wildtype or mutants were plated on fibronectin-coated 24-well plates and grown for 48 h at 37°C. Cells were washed and incubated with calcium-dependent M1 anti-FLAG antibody diluted in DMEM containing 1 mg/ml BSA, 1 mM $CaCl_2$, and 10 mM HEPES for 1 h. Cells were incubated in the absence or presence of 100 μM SLIGKV for 60 min at 37°C. Antibody bound receptor remaining on the cell surface was stripped by washing with ice cold PBS containing 0.4% EDTA to chelate calcium. Cells were incubated for various times to allow recovery of antibody bound receptor to the cell surface. Receptor recovery was measured by cell surface ELISA.

Data Analysis

Prism 3.0 Software (GraphPad) was used to analyze the data and statistical significance was determined using InStat 3.0 (GraphPad). Statistical analysis was determined using one-way ANOVA and Dunnett's Multiple Comparison Test.

2.4 Results

Phosphorylation of activated PAR2 occurs within the cytoplasmic tail

To determine the importance of phosphorylation in the regulation of PAR2 signaling and trafficking, we constructed a PAR2 mutant in which all serines (S) and threonines (T) in the C-tail region were mutated to alanines (A) (Fig. 2.1A). The PAR2 mutant was designated "PAR2 0P". We first directly examined the time course of activated PAR2 phosphorylation. Rat1 fibroblasts stably expressing FLAG-tagged PAR2

wildtype or 0P mutant labeled with [^{32}P]orthophosphate were incubated in the absence or presence of saturating concentrations of the PAR2 specific agonist peptide SLIGKV for various times at 37°C. PAR2 was immunoprecipitated from cell lysates, and phosphorylation was analyzed by autoradiography. In the absence of agonist, a minimal amount of wildtype PAR2 phosphorylation was detected (Fig. 2.1B, *lanes 1-2*). This phosphorylation was not observed in untransfected cells and likely represents basal PAR2 phosphorylation. The addition of agonist induced a rapid and significant peak in activated PAR2 phosphorylation at 2.5 min, and phosphorylation of PAR2 remained elevated for 30 min in the continued presence of agonist (Fig. 2.1B, *lanes 3-5*). In contrast, the PAR2 mutant lacking all serines and threonines in the C-tail, designated PAR2 0P, showed little to no phosphorylation over the 30 min time course of agonist stimulation (Fig. 2.1B, *lanes 6-9*). Thus, agonist induced rapid and sustained PAR2 phosphorylation predominantly within the C-tail domain.

Phosphorylation mediates PAR2 desensitization

Agonist-induced phosphorylation and binding of β-arrestins mediate desensitization and internalization of many reversibly activated GPCRs (Krupnick and Benovic, 1998). We previously showed that β-arrestins are essential for PAR2 desensitization (Stalheim et al., 2005); however, the contribution of phosphorylation to the termination of PAR2 signaling is not known. To test the importance of phosphorylation in PAR2 signal regulation we examined agonist-induced desensitization by quantitating the rate of inositol phosphate (IP) accumulation. Activated PAR2 elicits phosphoinositide (PI) hydrolysis in multiple cell types (Bohm et al., 1996a; Stalheim et al., 2005), an effect attributed to Gα$_q$ coupling to phospholipase C-β. We initially

compared the rates of agonist-induced PI hydrolysis in Rat1 fibroblasts and HeLa cells stably expressing comparable amounts of PAR2 wildtype and phosphorylation-deficient 0P mutant on the cell surface. Cells were incubated with or without a saturating concentration of trypsin for various times at 37°C, and total [^{3}H]IPs were measured. After 60 min of agonist exposure, a marked ~10-fold increase in PI hydrolysis was detected in wildtype PAR2 expressing Rat1 fibroblasts, whereas a substantially greater ~40-fold increase in signaling was measured in cells expressing the phosphorylation-deficient PAR2 0P mutant (Fig. 2.2A). Similarly, in HeLa cells, activation of phosphorylation-defective PAR2 0P mutant resulted in a ~10-fold increase in PI hydrolysis, a response considerably more robust than that observed in cells expressing comparable amounts of wildtype PAR2 (Fig. 2.2B). In both PAR2 wildtype and 0P mutant expressing HeLa cells, stimulation of endogenous muscarinic acetylcholine receptors with carbachol resulted in comparable changes in PI hydrolysis, indicating that there are no cell clone specific defects in G protein signaling (data not shown). These data suggest that agonist-induced PAR2 phosphorylation is critical for termination of G protein signaling.

Next, we examined whether the initial coupling of activated PAR2 wildtype and 0P mutant to Gα_q-promoted PI hydrolysis was affected. The concentration effect curves for trypsin at PAR2 wildtype and phosphorylation-defective 0P mutant were determined by incubating cells labeled with *myo*-[^{3}H]inositol and varying concentrations of trypsin for 10 min at 37°C, and accumulation of [^{3}H]IPs was measured. The effective concentration of trypsin needed to stimulate a half-maximal response after 10 min was similar for both PAR2 wildtype and 0P mutant expressed in HeLa cells and significantly

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different in Rat1 fibroblasts ($P<0.05$) (Fig. 2.2C and D). Moreover, activation of phosphorylation-defective PAR2 0P mutant caused an enhanced maximal signaling response compared with wildtype receptor (Fig. 2.2C) in Rat1 fibroblasts and HeLa cells (data not shown). These findings suggest that each activated PAR2 0P mutant coupled longer to PI hydrolysis before signaling was shut off, indicating a slower rate of desensitization.

To determine if specific phosphorylation sites are important for regulation of PAR2 signaling a series of receptor mutants were constructed in which clusters of serines and threonines within the C-tail were replaced with alanines (Fig. 2.3A). PAR2 S/T cluster mutants were stably expressed in Rat1 fibroblasts, and agonist promoted phosphorylation was determined. Wildtype PAR2 showed robust phosphorylation after 2.5 min of agonist activation, whereas phosphorylation of the PAR2 0P mutant lacking all C-tail serines and threonines was virtually abolished (Fig. 2.3B, lanes 1-4). Remarkably, activation of PAR2 S/T cluster mutants with SLIGKV for 2.5 min induced phosphorylation similar to that observed with wildtype PAR2 (Fig. 2.3B), suggesting that PAR2 phosphorylation occurs at multiple and/or redundant sites within the C-tail domain. To determine whether critical C-tail serine and threonine residues are important for desensitization, we examined agonist stimulated PI hydrolysis in cells expressing PAR2 S/T cluster mutants (Fig. 2.3C). Rat1 fibroblasts expressing similar amounts of cell surface PAR2 wildtype, 0P, or S/T cluster mutants were incubated in the absence or presence of a saturating concentration of trypsin for 30 min at 37°C, and total [^{3}H]IPs were measured. All PAR2 S/T cluster mutants exhibited an increase in agonist-induced PI hydrolysis comparable to wildtype PAR2, whereas the phosphorylation-defective

PAR2 0P mutant showed significantly enhanced signaling following agonist incubation. Taken together, these data suggest that multiple and/or redundant C-tail serine and threonine residues serve as critical sites for agonist-induced PAR2 phosphorylation and desensitization.

PAR2 0P mutant internalizes constitutively

To determine the role of phosphorylation in PAR2 intracellular trafficking, we first examined constitutive and agonist-induced receptor internalization. Rat1 fibroblasts stably expressing FLAG-tagged PAR2 wildtype or phosphorylation-deficient 0P mutant were incubated with M1 anti-FLAG antibody at 4^oC. Under these conditions only receptors residing on the cell surface binds antibody. Cells were then incubated in the absence or presence of a saturating concentration of SLIGKV for various times at 37^oC, and the amount of receptor remaining on the cell surface was quantified by ELISA. In wildtype PAR2 expressing cells, agonist induced rapid receptor internalization within 15 min, leading to a ~70% loss of cell surface receptor after 60 min (Fig. 2.4A). The rate of agonist-induced internalization of phosphorylation-deficient PAR2 0P mutant was comparable to wildtype receptor (Fig. 2.4A). We next examined constitutive internalization of wildtype PAR2 and observed a slow rate of internalization resulting in an ~5-10% loss of cell surface receptor after 60 min (Fig. 2.4B). In striking contrast, phosphorylation-deficient PAR2 0P mutant displayed an increased rate of constitutive internalization in which ~65% of receptor was lost from the cell surface after 60 min (Fig. 2.4B). Similar findings were also observed in HeLa cells (data not shown). Immunofluorescence microscopy studies of HeLa cells are consistent with enhanced constitutive internalization of phosphorylation-deficient PAR2 0P mutant (Fig. 2.4C).

HeLa cells stably expressing FLAG-tagged PAR2 wildtype or 0P mutant were incubated in the absence or presence of agonist for 30 min at 37°C, fixed, immunostained and analyzed by confocal microscopy. A substantial amount of internalized phosphorylation-deficient PAR2 0P mutant co-localized with early-endosomal antigen-1 (EEA1), a marker of early endosomes in the presence and absence of agonist stimulation or antibody prebinding (Fig. 2.4C). In contrast, wildtype PAR2 localized predominantly to the cell surface in the absence of agonist (Fig. 2.4C), whereas activated PAR2 wildtype showed robust internalization and co-localization with EEA1 (Fig. 2.4C). To determine whether PAR2 internalization requires specific serine and threonine residues, we examined the rates of constitutive and agonist-induced internalization of PAR2 S/T cluster mutants (Fig. 2.4D and E). Agonist-promoted PAR2 S/T cluster mutant internalization rates were comparable to wildtype PAR2 (Fig. 2.4D). Moreover, constitutive internalization rates of PAR2 wildtype and all S/T cluster mutants were similar, whereas phosphorylation-defective PAR2 0P mutant displayed an enhanced rate of constitutive internalization (Fig. 2.4E). Collectively, these findings indicate that serines and threonines in the PAR2 C-tail prevent constitutive internalization and suggest that basal phosphorylation and/or a distinct conformation of the PAR2 C-tail regulates cell surface retention.

We next examined whether phosphorylation-deficient PAR2 0P mutant internalized through a dynamin-dependent pathway like wildtype PAR2 (Roosterman et al., 2003). HeLa cells expressing PAR2 wildtype or 0P mutant were transiently transfected with GFP-tagged dynamin K44A mutant, and receptor internalization was examined by immunofluorescence microscopy. Constitutive internalization of

phosphorylation-deficient PAR2 0P mutant was substantially inhibited in dynamin K44A mutant expressing cells, which showed more receptor retained on the cell surface compared to adjacent cells not expressing dynamin K44A (Fig. 2.5A). Indeed, in cells lacking dynamin K44A expression, PAR2 0P mutant localized predominantly to endosomes and not at the cell surface (Fig. 2.5A, *arrowhead*). Internalization of activated PAR2 wildtype and 0P mutant were also markedly inhibited in dynamin K44A mutant expressing cells, which displayed more cell surface localized PAR2 than adjacent untransfected cells (Fig. 2.5A, *arrowheads*). These findings suggest that internalization of phosphorylation-deficient PAR2 0P mutant is mediated by dynamin similar to the wildtype receptor.

To determine the role of clathrin in phosphorylation-deficient PAR2 0P mutant internalization, we used siRNA targeting endogenous clathrin heavy chain as previously described (Wolfe et al., 2007). The expression of clathrin heavy chain was substantially reduced in cells transiently transfected with siRNA specifically targeting clathrin compared to non-specific siRNA control as detected by immunofluorescence microscopy (Fig. 2.5B, *bottom panels*). In cells lacking clathrin expression, activated PAR2 wildtype internalization was considerably impaired with more receptor retained at the cell surface compared to adjacent untransfected cells or cells transfected with non-specific control siRNA (Fig. 2.5B). In striking contrast, constitutive and agonist-induced internalization of phosphorylation-deficient PAR2 0P mutant occurred regardless of clathrin heavy chain expression (Fig. 2.5B). Thus, unlike wildtype receptor, phosphorylation- defective PAR2 0P mutant internalization occurs through a clathrin-independent pathway.

We previously reported that β-arrestins are rapidly recruited to activated PAR2 and are essential for agonist-promoted receptor internalization (Stalheim et al., 2005). To assess the importance of β-arrestins in phosphorylation-deficient PAR2 0P mutant internalization, we initially examined β-arrestin-1-GFP and β-arrestin-2-GFP redistribution following the addition of agonist. In untreated PAR2 wildtype expressing cells, β-arrestin-1-GFP and β-arrestin-2-GFP remained distributed throughout the cytoplasm, whereas stimulation with agonist peptide SLIGKV for 5 min resulted in β-arrestin-GFP rapid redistribution and co-localization with PAR2 wildtype (Fig. 2.6A and B). In contrast, β-arrestin-1-GFP and β-arrestin-2-GFP were not recruited to the cell surface in phosphorylation-deficient PAR2 0P mutant expressing cells but remained cytosolic under control, constitutive, and agonist-induced conditions (Fig. 2.6C and D).

Previous studies have demonstrated that β-arrestins were essential for wildtype PAR2 internalization (DeFea et al., 2000; Stalheim et al., 2005). To examine the function of β-arrestins in phosphorylation-deficient PAR2 0P mutant internalization, we transfected HeLa cells with siRNAs targeting endogenous β-arrestin 1 and 2. Immunoblot analysis showed a significant reduction in β-arrestin 1 and 2 protein expression in cells transfected with siRNAs targeting β-arrestins compared to non-specific siRNA control cells (Fig. 2.7A). Similar to previous results in β-arrestin 1 and 2 null MEFs, PAR2 wildtype failed to internalize in cells depleted of β-arrestins (Fig. 2.7B) (Stalheim et al., 2005). In contrast, loss of β-arrestin 1 and 2 had no effect on constitutive or agonist-induced internalization of phosphorylation defective PAR2 0P mutant (Fig. 2.7B). These findings suggest that β-arrestins are not essential for phosphorylation-deficient PAR2 0P mutant internalization.

Phosphorylation-deficient PAR2 0P mutant constitutively sorts to lysosomes and degrades and does not recycle to the cell surface

The increased accumulation of intracellular phosphorylation-deficient PAR2 0P mutant observed in the absence of agonist may result from an increased rate of constitutive internalization and/or an inability of the mutant receptor to recycle or sort to lysosomes for degradation. We therefore examined internalization and recycling of phosphorylation- deficient PAR2 0P mutant. HeLa cells expressing FLAG-PAR2 wildtype or 0P mutant were incubated with calcium-dependent M1 anti-FLAG antibody at 4°C to label cell surface receptors and then stimulated with or without agonist for 60 min at 37°C. In the absence of agonist, PAR2 wildtype showed a modest loss of cell surface receptor after 60 min of incubation at 37°C (t_1), whereas agonist addition resulted in an ~70% decrease in the level of PAR2-bound antibody on the cell surface (Fig. 2.8A). In contrast, phosphorylation-deficient PAR2 0P mutant expressing cells not exposed to agonist during the 60 min incubation showed ~70% internalization (t_1) (Fig. 2.8B), consistent with enhanced constitutive internalization. Exposure to agonist peptide SLIGKV during the 60 min incubation caused a similar ~70-80% decrease in cell surface PAR2 0P mutant (Fig. 2.8B).

After initial incubation with or without agonist, antibody was stripped from the cell surface and recovery of previously internalized receptor-bound antibody was measured at various times. In PAR2 wildtype expressing cells, little recovery of antibody was observed in cells irrespective of agonist incubation (Fig. 2.8A). Similarly, in phosphorylation-deficient PAR2 0P mutant expressing cells incubated in the absence or presence of agonist, there was minimal recovery of internalized antibody despite the large

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cohort of receptor-bound antibody that had been previously internalized (Fig. 2.8B). In contrast to PAR2, a chimeric PAR1 bearing the cytoplasmic tail of the substance P receptor (P/S C-tail) pretreated with the PAR1 specific agonist peptide TFLLRNPNDK showed substantial recovery of antibody on the cell surface with time (Fig. 2.8C), consistent with its internalization and recycling as previously reported (Trejo and Coughlin, 1999). These data are consistent with an enhanced rate of phosphorylation-deficient PAR2 0P mutant internalization and suggest a novel function for phosphorylation in negative regulation of PAR2 internalization.

To directly test whether phosphorylation-deficient PAR2 0P mutant constitutively sorts to lysosomes, we used confocal microscopy to assess whether receptors sort to a lysosomal-associated membrane protein-1 (LAMP1) positive compartment. LAMP1 is a specific marker of late endosomes and lysosomes. HeLa cells expressing PAR2 wildtype or 0P mutant were pretreated with or without leupeptin, a lysosomal protease inhibitor, and incubated in the absence or presence of peptide agonist SLIGKV for 2.5 h at 37°C. In the absence of agonist, PAR2 wildtype localized predominantly to the surface of cells treated with or without leupeptin (Fig. 2.9A). After prolonged agonist exposure, PAR2 was no longer detectable in control cells (Fig. 2.9A), whereas cells pretreated with leupeptin showed significant accumulation of receptor in vesicles that co-stained for LAMP1 (Fig. 2.9A). These findings are consistent with agonist-induced internalization and lysosomal degradation of wildtype PAR2. In the absence of leupeptin, little co-localization of phosphorylation-deficient PAR2 0P mutant and LAMP1 was observed in agonist treated or untreated cells (Fig. 2.9B), suggesting that internalized PAR2 0P mutant is sorted to lysosomes and rapidly degraded. In contrast to wildtype PAR2,

however, unactivated PAR2 0P mutant was found largely in LAMP1-positive vesicles in cells pretreated with leupeptin (Fig. 2.9B). Similarly, activated PAR2 0P mutant also localized predominantly to vesicles that co-localized with LAMP1 after prolonged agonist exposure in leupeptin treated cells (Fig. 2.9B). These results suggest that phosphorylation-deficient PAR2 0P mutant undergoes constitutive internalization and sorts from endosomes to lysosomes independent of phosphorylation.

We next directly compared the rates of PAR2 wildtype and phosphorylation-deficient 0P mutant degradation. HeLa cells expressing PAR2 wildtype or 0P mutant were incubated with or without agonist for various times at 37°C, and the amount of receptor protein remaining was determined by immunoblot analysis. PAR2 wildtype expressing cells not exposed to agonist showed a modest basal turnover when de novo receptor synthesis was inhibited with cycloheximide (Fig. 2.10A). The majority of PAR2 species migrated between ~36 and ~64 kDa, whereas higher molecular weight species are probably due to N-linked glycosylation and/or ubiquitination as previously reported (Compton et al., 2002; Jacob et al., 2005a). In cells exposed to agonist peptide SLIGKV for 2 hr, a significant decrease in the amount of PAR2 wildtype protein was observed and receptor protein was virtually abolished after 4 hr of agonist exposure (Fig. 2.10B, lanes 2-5). Interestingly, unlike wildtype receptor, unactivated phosphorylation-deficient PAR2 0P mutant protein was markedly decreased in the presence of cycloheximide and was comparable to that observed in agonist-treated cells (Fig. 2.10A and B). These findings are consistent with constitutive internalization and lysosomal degradation of phosphorylation-deficient PAR2 0P mutant.

2.5 Discussion

In the present study, we demonstrate that PAR2 signaling and endocytic trafficking are differentially regulated by phosphorylation. The PAR2 C-tail contains multiple serine and threonine residues that could serve as potential phosphorylation sites for GRKs or second messenger protein kinases. Previous studies showed that pharmacological inhibitors of protein kinase C (PKC) enhance PAR2-mediated calcium responses in KNRK and hBRIE 380 cells, suggesting a role for PKC in PAR2 desensitization (Bohm et al., 1996a). However, whether PAR2 is directly modified by phosphorylation and the function of such phosphorylation in regulation of PAR2 signaling and trafficking remains to be determined. We report here that activated PAR2 phosphorylation is critical for receptor desensitization and β-arrestin recruitment (Fig. 2.11). We found that PAR2 internalization can proceed independent of G protein activation and phosphorylation. These studies suggest that phosphorylation and/or a distinct conformation of PAR2 is essential for retaining the receptor at the cell surface. We further show that phosphorylation-deficient PAR2 0P mutant internalizes constitutively and sorts to lysosomes through a dynamin-dependent but clathrin- and β-arrestin-independent pathway (Fig. 2.11). Together these studies suggest that PAR2 internalization can be uncoupled from G protein activation and phosphorylation, indicating that distinct determinants control the capacity of PAR2 to signal *versus* recruitment of β-arrestin and endocytosis.

The mechanisms controlling GPCR desensitization are best characterized for the photoreceptor rhodopsin and β_2-AR (Pitcher et al., 1998). In this classic model, ligand-activated receptors are rapidly phosphorylated at multiple intracellular serine and

threonine residues by GRKs. Phosphorylation enhances binding of β-arrestins, which sterically hinders receptor interactions with G proteins. Thus, phosphorylation and β-arrestin binding function together to uncouple the receptor from G protein activation and thus, promote signal termination. We observed a similar mechanism of desensitization for PAR2 in which the phosphorylation-deficient PAR2 0P mutant exhibited a slowed rate of desensitization, suggesting a defect in uncoupling from G protein signaling. Moreover, we previously showed that PAR2 desensitization is severely impaired in β-arrestin 1 and 2 null mouse embryonic fibroblasts (MEFs), indicating that phosphorylation and β-arrestin binding are critical for definitively shutting-off PAR2 signaling (Stalheim et al., 2005).

Most activated and phosphorylated GPCRs bind to β-arrestins, which facilitates receptor recruitment to clathrin-coated pits and endocytosis. We previously showed that internalization of wildtype PAR2 is critically dependent on β-arrestins (Stalheim et al., 2005), like most classic GPCRs. In the present study, we found that phosphorylation-deficient PAR2 0P mutant was capable of internalization independent of β-arrestins. Interestingly, in the absence of agonist, PAR2 0P mutant displayed enhanced endocytosis, whereas unactivated PAR2 wildtype and S/T cluster mutants remained at the cell surface. In addition, constitutive and agonist-induced phosphorylation-defective PAR2 0P mutant internalization occurred normally in β-arrestin 1 and 2 siRNA depleted cells. In contrast, siRNA- targeted depletion of β-arrestins virtually ablated agonist-induced wildtype PAR2 internalization, consistent with our previous findings in which wildtype PAR2 internalization was completely abolished in β-arrestin 1 and 2 null MEFs (Stalheim et al., 2005). Collectively, these findings suggest that phosphorylation of

PAR2 is critical for specifying β-arrestin recruitment but is dispensable for receptor internalization. Thus, phosphorylation-deficient PAR2 0P has the capacity to engage the endocytic machinery and internalizes independent of β-arrestins even in the absence of agonist.

Similar to PAR2, previous studies have shown that phosphorylation is not required for agonist-induced internalization of CXCR4 or the δ-opioid receptor (Haribabu et al., 1997; Murray et al., 1998). β-arrestins are also dispensable for internalization of muscarinic acetylcholine receptors (mAChRs), PAR1, and the formyl peptide receptor (Lee et al., 1998; Paing et al., 2002; Vines et al., 2003). Studies of the m2 mAChR identified a S/T cluster mutant that displayed impaired desensitization of adenylyl cyclase signaling and binding to β-arrestins (Pals-Rylaarsdam et al., 1997; Pals-Rylaarsdam and Hosey, 1997), similar to phosphorylation-deficient PAR2 0P mutant. In addition, this m2 mAChR S/T cluster mutant internalized like wildtype receptor in HEK 293 cells, and overexpression of β-arrestin 1 or β-arrestin 2 had no effect on its internalization. The authors concluded that m2 mAChR predominantly uses an β-arrestin-independent pathway for receptor internalization and that phosphorylation may regulate both β-arrestin-dependent and β-arrestin-independent activities (Pals-Rylaarsdam et al., 1997). In the case of PAR1, unactivated receptor internalizes constitutively through clathrin-coated pits independent of phosphorylation and β-arrestin binding. Rather than β-arrestins, PAR1 constitutive endocytosis is mediated by adaptor protein complex-2 (AP-2), in which the μ2-subunit binds directly to a distal tyrosine-based motif within the receptor C-tail (Paing et al., 2006). The adaptor protein that mediates constitutive internalization of phosphorylation-deficient PAR2 0P mutant is not known. Finally, we

cannot exclude the possibility that mutating the PAR2 C-tail disrupts a conformation of the receptor important for cell surface retention and/or receptor endocytosis. Thus, our results suggest that phosphorylation and/or a distinct conformation of the C-tail domain of PAR2 are critical for negatively regulating receptor internalization and specifying endocytosis through an β-arrestin-dependent pathway.

PAR2 appears to internalize through a dynamin-dependent but clathrin- and β-arrestin-independent pathway in the absence of phosphorylation. Most classic GPCRs bind β-arrestins, which engages AP-2 and the clathrin heavy chain to promote receptor internalization through a dynamin- and clathrin-dependent pathway; however, some GPCRs utilize a dynamin-dependent but clathrin-independent mode of endocytosis (Marchese et al., 2008). Clathrin-independent endocytosis can occur through lipid rafts, membrane microdomains that are highly enriched in cholesterol and glycosphingolipids (Mayor and Pagano, 2007). A distinct subset of lipid rafts known as caveolae, form flask-shaped pits at the plasma membrane and are enriched in the structural scaffolding protein caveolin. Caveolin is thought to sequester membrane proteins, including GPCRs, into caveolae through protein-protein interactions with caveolin binding motifs. We have identified a putative caveolin-binding motif within the carboxyl-terminal portion of the seventh transmembrane domain of PAR2 (YYFVSHDF, φXφXXXXφ), which may confer an interaction with caveolin to facilitate movement into caveolar membranes and endocytosis. Indeed, in MDA-MB-231 cells, a fraction of PAR2 is present in caveolin-1 positive and detergent resistant lipid fractions and co-localizes with caveolin-1 as assessed by immunofluorescence microscopy (Awasthi et al., 2007). However, whether PAR2 and caveolin-1 directly interact has not been determined. The phosphorylation-

deficient PAR2 0P mutant may also utilize other clathrin-independent modes of endocytosis via non-caveolar pathways. In lymphocytes deficient in caveolin-1, the interleukin-2 receptor partitioned into detergent-resistant fractions and internalized through a clathrin-independent but dynamin- and RhoA- dependent pathway (Lamaze et al., 2001). It is unclear, however, whether RhoA activity mediates PAR2 redistribution into lipid rafts and/or facilitates receptor endocytosis.

Our studies further suggest that internalized PAR2 can sort from endosomes to lysosomes in the absence of receptor activation and phosphorylation. The β_2-AR and CXCR4 traffic from endosomes to lysosomes through an ubiquitin-dependent pathway (Marchese and Benovic, 2001; Shenoy et al., 2001). Ubiquitinated cargo interacts with the ubiquitin binding motif of hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) (Marchese et al., 2003). Hrs associates with early endosomal membranes and sorts ubiquitinated proteins from early endosomes to late endosomes. Hrs also directly binds to tumor suppressor gene product 101 (Tsg101) of the ESCRT complex, which is required for multivesicular body formation, lysosomal fusion, and subsequent degradation of proteins and lipids. A recent study showed that activated PAR2 is ubiquitinated by the E3 ligase Cbl, and a receptor mutant that is not ubiquitinated fails to degrade in the presence of agonist (Jacob et al., 2005a). In addition, post-endocytic sorting of PAR2 requires Hrs, but the role of Tsg101 and other members of the ESCRT machinery is currently unknown (Hasdemir et al., 2007). In contrast to wildtype PAR2, the δ-opioid receptor and PAR1 sort to lysosomes independent of ubiquitination (Tanowitz and Von Zastrow, 2002; Wolfe and Trejo, 2007). Interestingly, δ-opioid receptor still requires Hrs for lysosomal sorting, suggesting alternative sorting

mechanisms of Hrs for non-ubiquitinated cargo (Whistler et al., 2002). PAR1 sorts to lysosomes independent of Hrs and Tsg101 but requires SNX1, a protein that functions in vesicular trafficking (Gullapalli et al., 2006). Whether phosphorylation-deficient PAR2 0P mutant is ubiquitinated and sorts to lysosomes through an ESCRT-dependent or independent pathway remains to be determined.

Together our studies reveal a novel function for phosphorylation that differentially regulates PAR2 signaling and trafficking. However, whether GRKs or second messenger kinases phosphorylate PAR2 at distinct sites to differentially regulate PAR2 signaling and trafficking is not known and an important future pursuit. Moreover, the role of PAR2 phosphorylation in possibly regulating distinct functions of β-arrestins warrants further investigation. The binding of β-arrestins to activated and phosphorylated GPCRs appears to be independent of a consensus sequence or phosphorylation specific sites; however, recent studies suggest that phosphorylation by specific GRKs can have differential effects on β-arrestin function (Tobin, 2008). The depletion of GRK2/3 by siRNA knockdown reduced V2 vasopressin and AT_{1A} angiotensin receptor phosphorylation and inhibited receptor internalization and desensitization whereas loss of GRK 5/6 only prevented prolonged MAPK activation (Kim et al., 2005; Ren et al., 2005). Thus, it is possible that β-arrestins form a different active conformation when bound to GRK2/3- *versus* GRK5/6-phosphorylation sites and in turn, confer different β-arrestin-mediated functions (Tobin, 2008). Thus far, we have been unable to identify specific phosphorylation sites in PAR2 that might differentially regulate β-arrestin function, suggesting that other determinants may also contribute to the diverse regulatory mechanisms that control PAR2 signaling and trafficking.
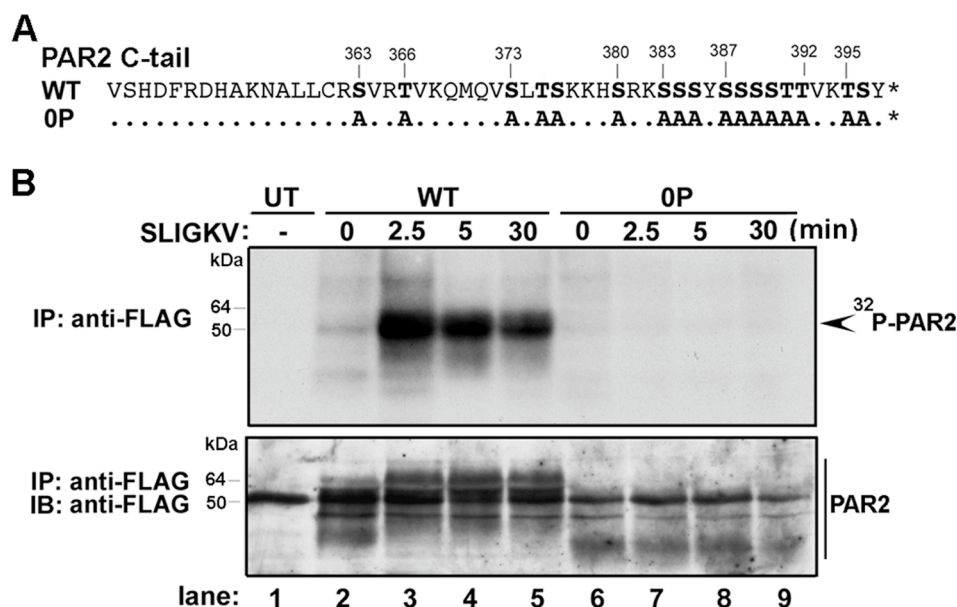
A

PAR2 C-tail

```
                       363 366        373      380 383   387     392 395
                        |   |          |        |   |     |       |   |
WT   VSHDFRDHAKNALLCRSVRTVKQMQVSLTSKKHSRKSSSYSSSSTTVKTSY*
0P   ................A..A......A.AA...A..AAA.AAAAAA..AA.*
```

B

Figure 2.1. **Agonist-induced phosphorylation of PAR2 occurs within the C-tail region.** (**A**) The PAR2 C-tail serines (S) and threonines (T) are shown in bold and were mutated to alanine (A) to generate the PAR2 0P mutant. (**B**) Rat1 fibroblasts stably expressing comparable amounts of FLAG-tagged PAR2 wildtype or 0P mutant labeled with $[^{32}P]$orthophosphate were stimulated with 100 μM SLIGKV for various times at $37^{o}C$. Equivalent amounts of cell lysates were immunoprecipitated with monoclonal M2 anti-FLAG antibody. Receptor immunoprecipitates were resolved by SDS-PAGE and analyzed by autoradiography. No phosphorylated proteins were detected in immunoprecipitates from untransfected (UT) Rat1 fibroblasts. Membranes were then probed with a polyclonal anti-FLAG antibody to detect total PAR2. The FLAG-positive band at 50 kDa in the UT lane is non-specific and also detected in all PAR2 wildtype and PAR2 0P lanes. The initial amounts (mean ± S.D.; *n*=3) of cell surface receptor expression measured by ELISA were 0.164 ± 0.033 and 0.112 ± 0.015 OD units for PAR2 wildtype and 0P mutant, respectively.
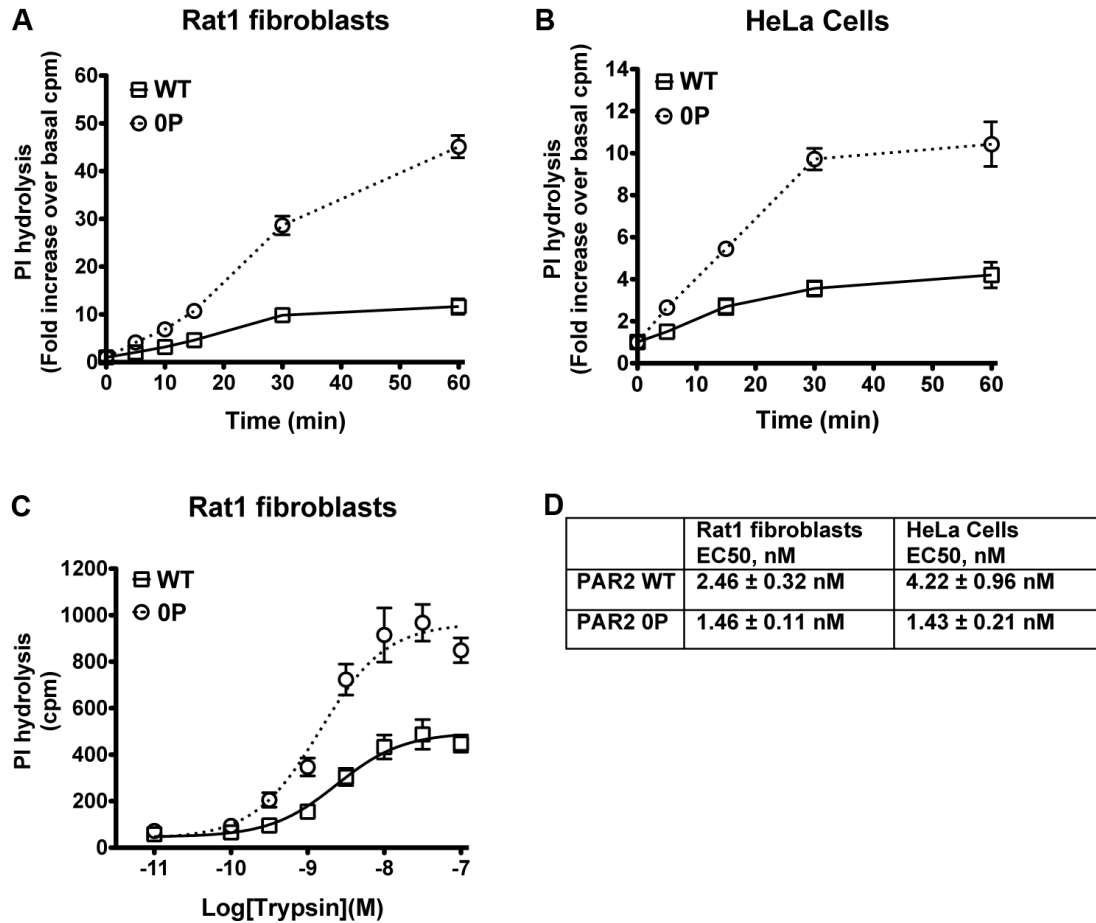
Figure 2.2. **Activated PAR2 phosphorylation is critical for desensitization.** (**A**) Rat1 fibroblasts and (**B**) HeLa cells stably expressing PAR2 wildtype and 0P mutant labeled with *myo*-[³H]inositol were incubated in the absence or presence of 10 nM trypsin for various times at 37°C, and total [³H]IPs were measured. The data (mean ± SEM; *n*=3) shown are expressed as -fold increase over basal [³H]IPs and averaged over three independent experiments. (**C**) The concentration effect curves of trypsin at PAR2 wildtype and 0P mutant were determined in Rat1 fibroblasts. Cells labeled with *myo*-[³H]inositol were incubated with varying concentrations of trypsin for 10 min at 37°C, and [³H]IPs were measured. The data (mean ± SEM; *n*=3) shown are averaged from three independent experiments. (**D**), The table presents the effective concentrations of trypsin at PAR2 wildtype and 0P mutant determined in Rat1 fibroblasts in Fig. 2.2C and in HeLa cells using the same experimental conditions. The data (mean EC50 ± SEM.; *n*=3) presented are averaged from three separate experiments. The initial cell surface receptor expression (mean ± SD; *n*=3) measured by ELISA in HeLa cells was 0.253 ± 0.022 and 0.196 ± 0.023 OD units for PAR2 wildtype and PAR2 0P mutant, respectively.
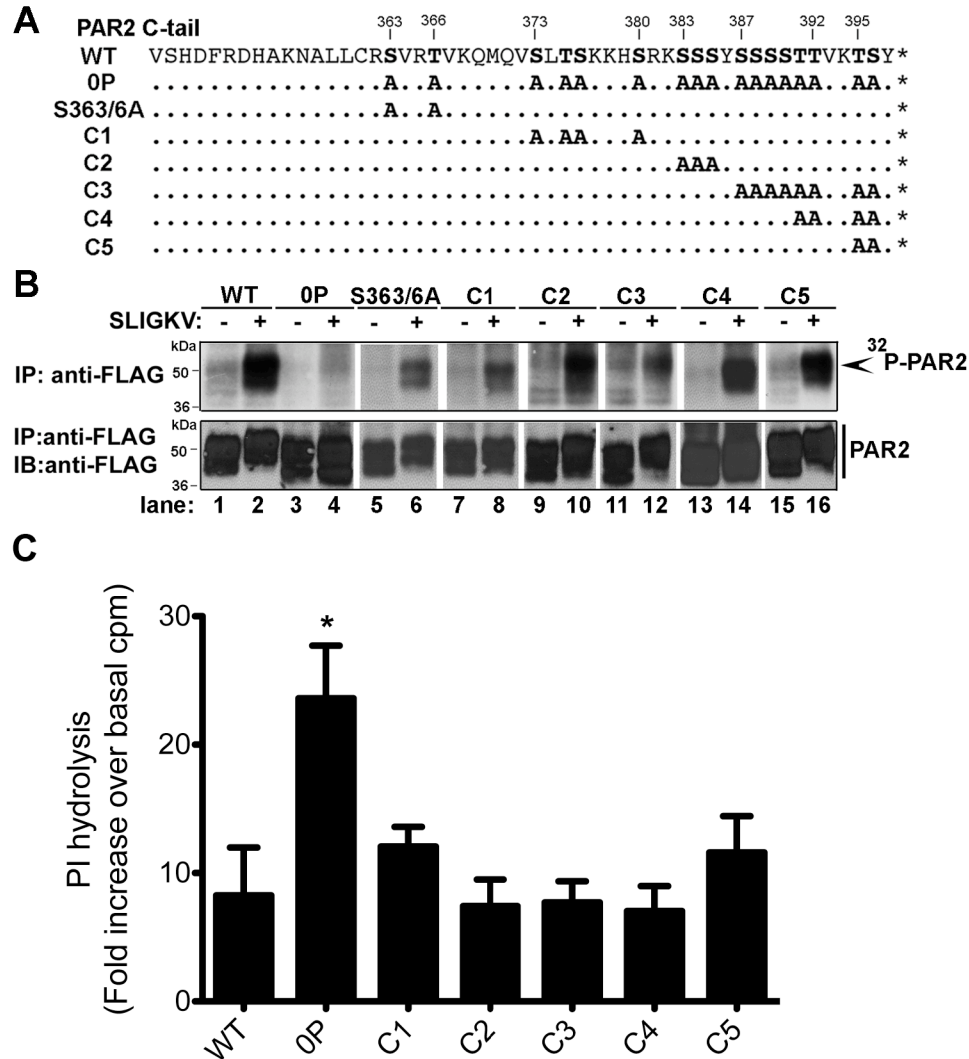
Figure 2.3. **Activated PAR2 exhibits multiple and/or redundant sites of phosphorylation.** (**A**) Several PAR2 C-tail serine (S) and threonine (T) cluster mutants were generated by replacing specific S/T with alanines (A) as depicted. Cluster mutants were designated as PAR2 S363/6A as previously described (DeFea et al., 2000; Stalheim et al., 2005) and C1-C5 for remaining cluster mutants. (**B**) Rat1 fibroblasts stably expressing FLAG-tagged PAR2 WT, 0P or S/T cluster mutants labeled with [^{32}P]orthophosphate were incubated in the absence or presence of 100 μM SLIGKV for 2.5 min at 37°C. Cell lysates were immunoprecipitated with M2 monoclonal anti-FLAG antibody, resolved by SDS-PAGE and analyzed by autoradiography. The membranes were stripped and reprobed with polyclonal anti-FLAG antibody to detect total PAR2. (**C**) Rat1 fibroblasts expressing FLAG-tagged PAR2 wildtype, 0P or S/T cluster mutants labeled with *myo*-[^{3}H]inositol were incubated in the absence or presence of 10 nM trypsin for 60 min at 37°C, and total [^{3}H]IPs were measured. The data (mean ± SEM; *n*=3) shown are expressed as -fold

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increase over basal [^{3}H]IPs and averaged from three independent experiments. A significant difference between PAR2 WT and PAR2 0P stimulated PI hydrolysis was detected (*, $P < 0.05$). The following are initial cell surface receptor amounts measured by ELISA (mean ± S.D.; n=3): WT 0.194 ± 0.027 OD units, 0P mutant 0.138 ± 0.004 OD units, C1 0.097 ± 0.013 OD units, C2 0.136 ± 0.010 OD units, C3 0.136 ± 0.010 OD units, C4 0.08 ± 0.015 OD units, C5 0.182 ± 0.004 OD units.
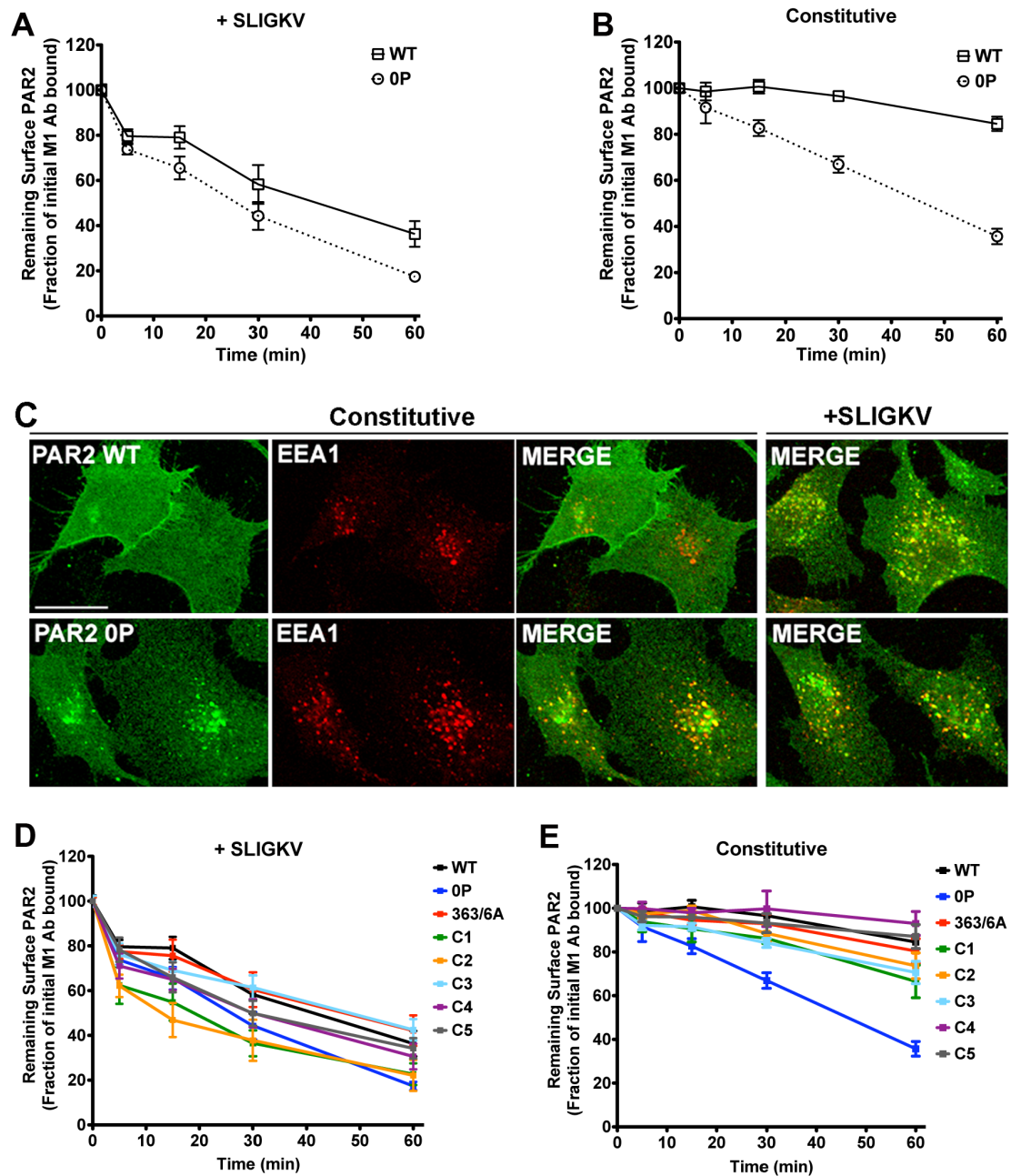
Figure 2.4. **Phosphorylation-deficient PAR2 0P mutant constitutively internalizes.**
Rat1 fibroblasts stably expressing similar amounts of cell surface PAR2 wildtype or
0P mutant were incubated with M1 anti-FLAG antibody at 4°C to label cell surface
receptors. Cells were then incubated in media with (**A**) or without (**B**) 100 μM
SLIGKV for various times at 37°C. Cells were fixed, and the amount of antibody
bound receptor remaining on the cell surface was quantitated by ELISA. The data are
shown as the fraction of initial M1 anti-FLAG antibody bound receptor at time 0 min.
The data (mean ± SEM.; *n*=4) is averaged from four independent experiments. These

cell lines were also used in analyzing PAR2 signaling, and initial cell surface receptor expression was reported in Fig. 1. (**C**) HeLa cells expressing PAR2 wildtype or 0P mutant were incubated in the absence or presence of 100 μM SLIGKV for 30 min at 37°C, fixed, permeabilized and immunostained for PAR2 (*green*) using polyclonal anti-FLAG antibody and for the early endosomal marker EEA1 (*red*) using monoclonal anti-EEA1 antibody. The cells were imaged by confocal microscopy. Co-localization of PAR2 with EEA1 is shown as yellow in the merged image. The initial cell surface receptor expression in HeLa cells was the same as in Fig. 2.2. Scale bar, 10 μm. (**D** and **E**) Rat1 fibroblasts expressing PAR2 wildtype, PAR2 0P mutant, or PAR2 S/T cluster mutants were examined for receptor internalization as described above with (**D**) or without (**E**) 100 μM SLIGKV for various times. The data (mean ± SEM; *n*=4) shown are averaged from four separate experiments. Initial cell surface expression was determined by ELISA and reported in Fig. 2.3 legend.
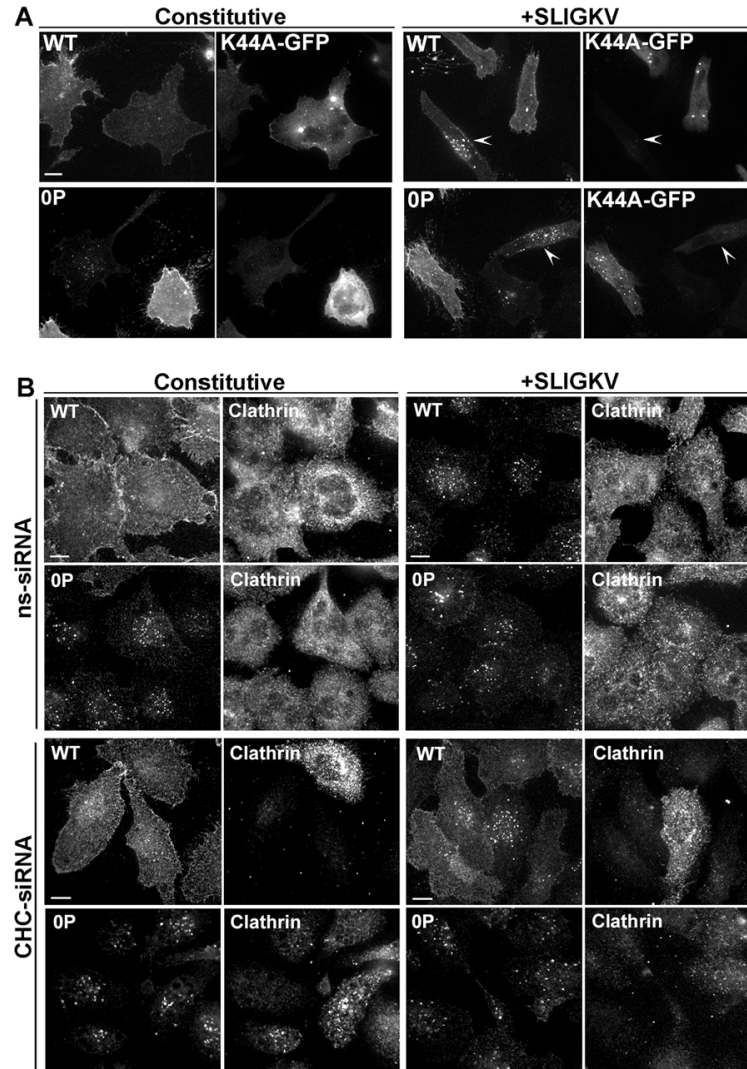
Figure 2.5. **Constitutive internalization of PAR2 0P mutant requires dynamin but not clathrin.** HeLa cells were transiently transfected with FLAG-tagged PAR2 wildtype or 0P mutant and GFP-tagged dynamin K44A. (**A**). Cells were labeled with M1 monoclonal anti-FLAG antibody for 1 h at 4°C and stimulated with or without 100 μM SLIGKV for 30 min at 37°C. Cells were fixed and processed for confocal microscopy. In GFP-tagged dynamin K44A expressing cells, PAR2 wildtype and 0P endocytosis was inhibited, whereas in cells lacking dynamin K44A, receptor internalization was partially impaired (*arrowheads*). (**B**) HeLa cells stably expressing FLAG-tagged PAR2 wildtype or 0P were transiently transfected with clathrin heavy chain (CHC) or non-specific (ns) siRNA for 72 h. Cells were labeled with M1 monoclonal anti-FLAG antibody for 1 h at 4°C and then stimulated with or without 100 μM SLIGKV for 30 min at 37°C. Cells were fixed and processed for confocal microscopy. Cells depleted of endogenous clathrin were immunostained using anti-clathrin monoclonal antibody X22. The images shown are representative of at least three separate experiments. Scale bar, 10 μm.

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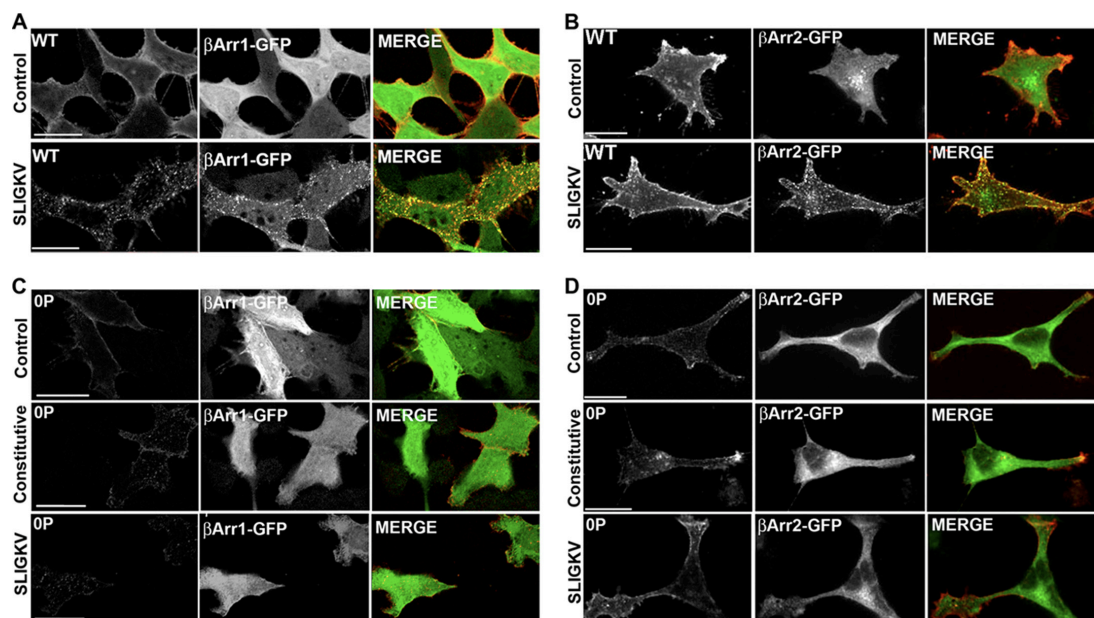
Figure 2.6. **PAR2 0P mutant fails to induce β-arrestin-1 or -2-GFP translocation.**
(**A**) HeLa cells stably expressing FLAG-tagged PAR2 wildtype or 0P were transiently transfected with β-arrestin 1 and 2 or non-specific siRNAs for 72 h. The depletion of β-arrestin 1 and 2 expression was determined by immunoblot analysis using polyclonal anti-β-arrestin antibody A1CT. Membranes were stripped and reprobed with an anti-actin antibody as a control for loading. (**B**) Cells were labeled with polyclonal anti-FLAG antibody for 1 h at 4°C. Cells were then treated with or without 100 μM SLIGKV for 30 min at 37°C and processed for confocal microscopy. Confocal microscopy images are representative of results from three independent experiments. Scale bar, 10 μm.

Figure 2.7. **Constitutive internalization of PAR2 0P mutant occurs independent of β-arrestins.** HeLa cells stably expressing FLAG-tagged PAR2 wildtype or 0P were transiently transfected with β-arrestin 1 and 2 or non-specific siRNAs for 72 h. Cells were labeled with polyclonal anti-FLAG antibody for 1 h at 4°C. Cells were then treated with or without 100 μM SLIGKV for 30 min at 37°C and processed for confocal microscopy. Images are representative of results from three independent experiments. The depletion of β-arrestin 1 and 2 e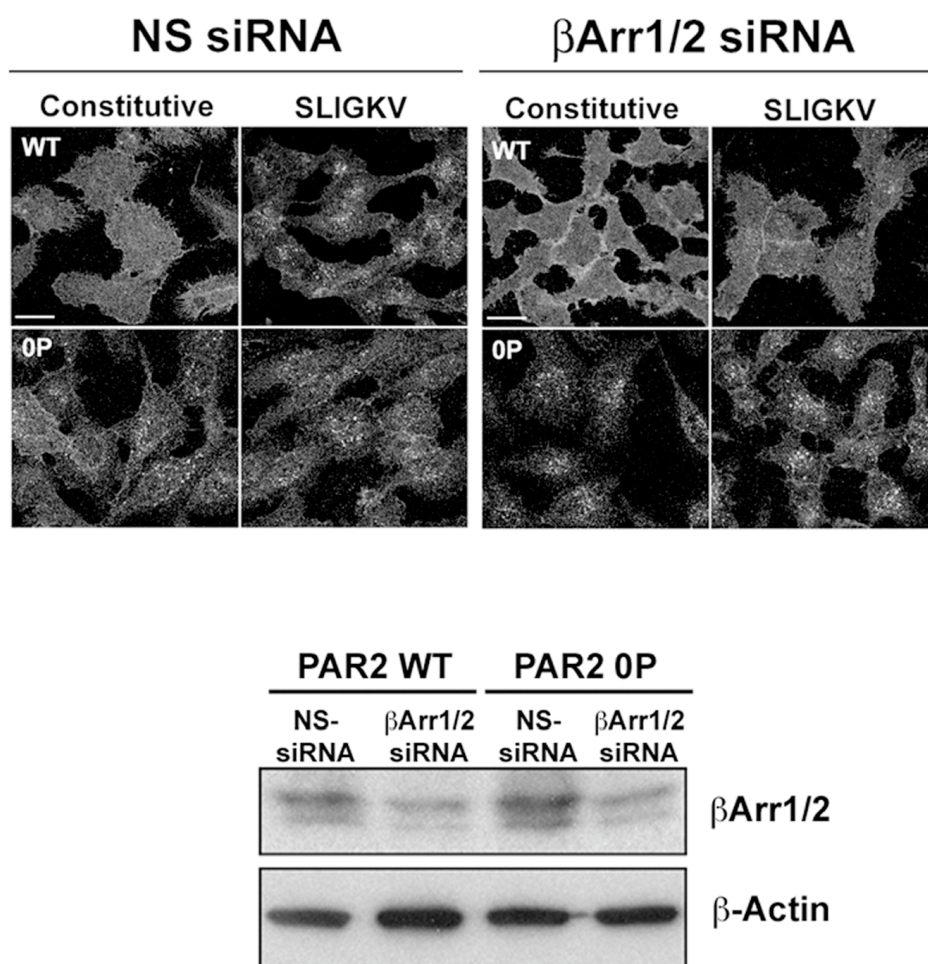xpression was determined by immunoblot analysis using polyclonal anti-β-arrestin antibody A1CT. Membranes were stripped and reprobed with an anti-actin antibody as a control for loading. Scale bar, 10 μm.
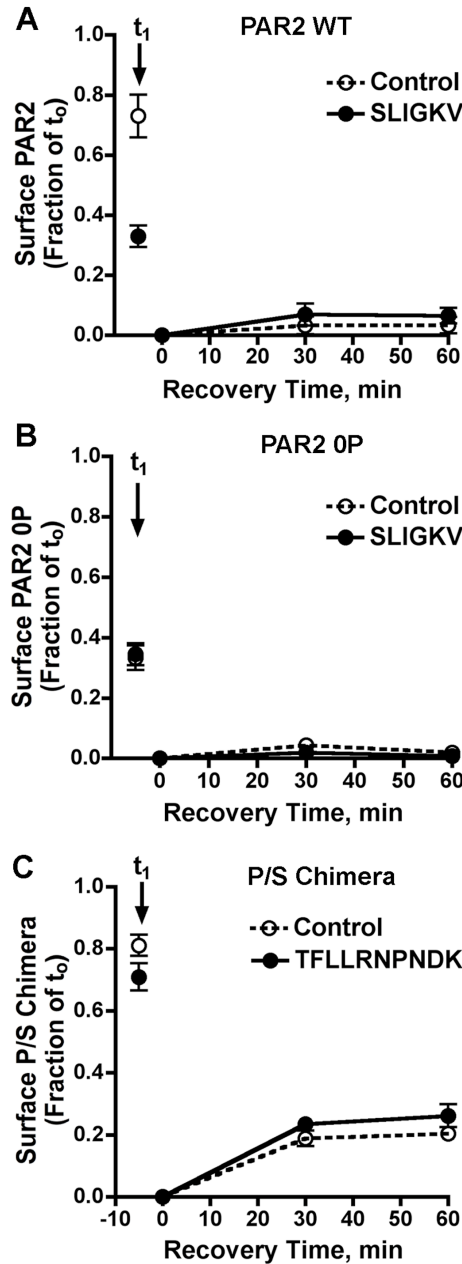
Figure 2.8. **Phosphorylation-deficient PAR2 0P mutant constitutively internalizes and does not recycle.** HeLa cells stably expressing FLAG-tagged PAR2 wildtype (**A**), 0P mutant (**B**), or the P/S chimera (**C**) were labeled with M1 anti-FLAG antibody for 1 h at 4 $^{\circ}$C. At t_o, cells were washed to remove unbound antibody and stimulated with or without 100 μM SLIGKV (PAR2-specific) or 100 μM TFLLRNPNDK (PAR1-specific) for 60 min at 37 $^{\circ}$C. Cells were washed with PBS/EDTA to strip away any antibody bound receptor remaining on the cell surface and warmed for various times at 37 $^{\circ}$C to allow recovery of internalized receptor to the cell surface. Cells were then fixed and recovery of antibody bound receptor was quantitated by ELISA. The data (mean ± S.D.; n=3) is represented as a fraction of total M1 antibody bound at time t_o, and representative of three independent experiments.
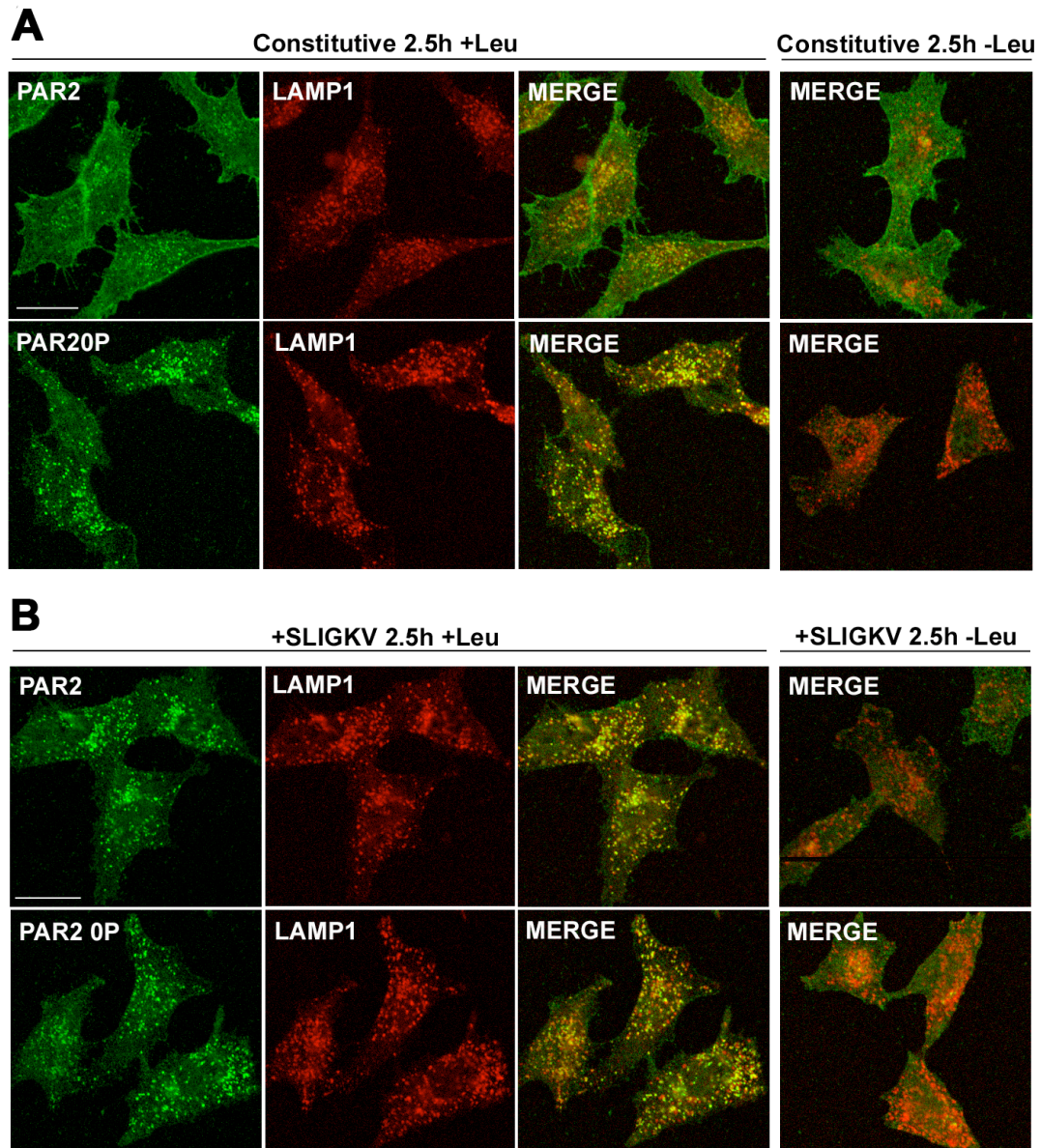
Figure 2.9. **Co-localization of PAR2 wildtype and 0P mutant with LAMP1.** HeLa cells stably expressing FLAG-tagged PAR2 wildtype or 0P mutant were pre-treated with or without 2 mM leupeptin for 1 h at 37°C. Cells were incubated with polyclonal anti-FLAG antibody for 1 h at 4°C to label cell surface receptors and then incubated in the absence (**A**) or presence (**B**) of 100 μM SLIGKV 2.5 h at 37°C. Cells were fixed, processed and immunostained for PAR2 (*green*) and for LAMP1 (*red*) and imaged by confocal microscopy. Co-localization of PAR2 with LAMP1 is shown as yellow in the merged imaged and the cells shown are representative of many cells visualized in three independent experiments. Scale bar, 10 μm.
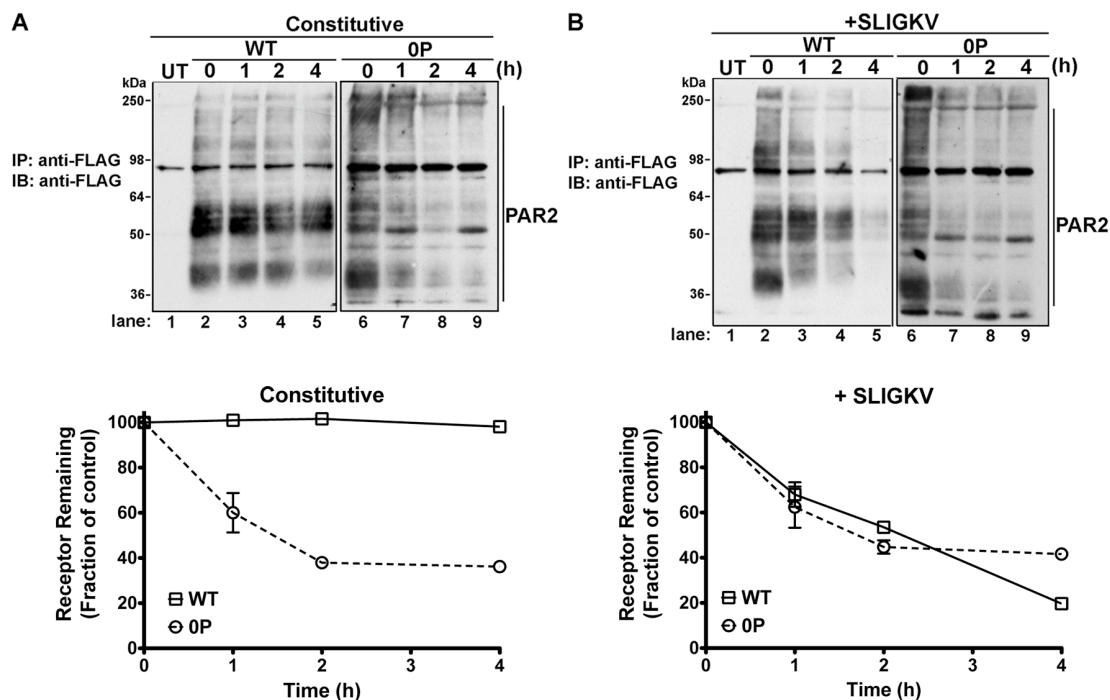
Figure. 2.10. **Phosphorylation-deficient PAR2 0P mutant is constitutively sorted to lysosomes and degraded.** HeLa cells expressing FLAG-tagged PAR2 wildtype (**A**) or 0P mutant (**B**) were incubated with or without 100 μM SLIGKV for various times at 37°C in presence of 10 μM cycloheximide (CHX) to prevent new receptor synthesis. Equivalent amounts of cell lysates were immunoprecipitated with M2 anti-FLAG antibody, and the amount of PAR2 remaining was detected by immunoblot analysis. Untransfected (UT) HeLa cells are shown in the adjacent lanes. The band below 98 kDa is non-specific and is also observed in untransfected control cells. Immunoblot data (mean ± SEM; *n*=2) was quantified using Image J software and plotted as a fraction of untreated control cells.
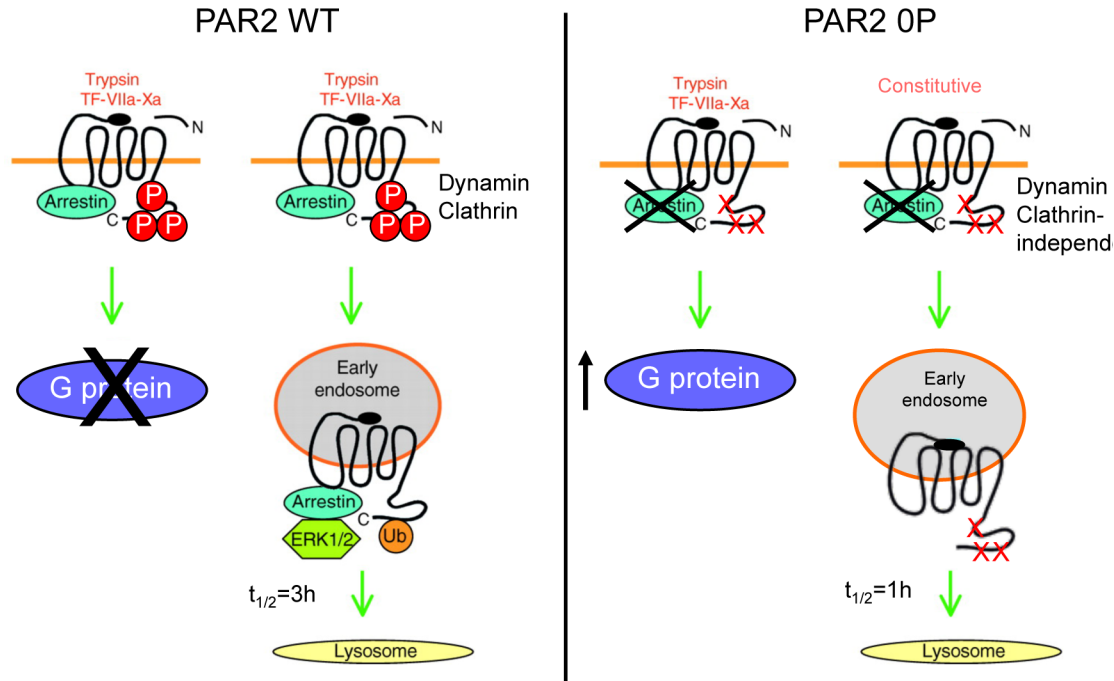
Figure 2.11. **Model of PAR2 signaling and trafficking.** Upon agonist exposure, PAR2 is rapidly phosphorylated on its cytoplasmic tail, and arrestins are recruited to the cell surface to uncouple the receptor from heterotrimeric G proteins and facilitate receptor internalization through a dynamin and clathrin-dependent pathway. PAR2 also signals independent of heterotrimeric G proteins through its interaction with arrestins. Internalized PAR2 is sorted to lysosomes and degraded through a ubiquitin-dependent pathway. In contrast, the PAR2 0P mutant is deficient in phosphorylation and exhibits a defect in desensitization. PAR2 0P fails to recruit arrestins to the cell surface and constitutively internalizes in a dynamin-dependent but clathrin- and arrestin-independent pathway. PAR2 0P is also constitutively sorted to lysosomes and degraded. Collectively, these results show the PAR2 phosphorylation differentially regulates receptor desensitization and endocytic trafficking.

CHAPTER 3

INTRODUCTION – PHOSPHOLIPASE C-β ISOZYMES

3.1 Phospholipase C Isozymes

Phospholipase C (PLC) isozymes hydrolyze PtdIns(4,5)P_2 to second messengers, diacylglycerol and Ins(1,4,5)P_3 in response to numerous extracellular stimuli, including hormones, peptides, proteases, and growth factors (Rhee, 2001; Harden and Sondek, 2006). Membrane-bound diacylglycerol activates various protein kinase C isozymes, and Ins(1,4,5)P_3 binds to receptors on the endoplasmic reticulum to mobilize intracellular calcium. Generation of these two second messengers and activation of downstream signaling pathways leads to a host of cellular responses such as cell growth, proliferation, differentiation, contraction, and secretion.

The PLC family is divided into of six major classes, PLC-β, -γ, -δ, -ε, -ζ, and -η, based on sequence homology, and each class has both common and unique structural motifs and distinct tissue distribution (Rhee, 2001; Harden and Sondek, 2006). PLC isozymes have a low overall sequence homology, but all share the same core domain architecture typified in PLC-δ (Fig. 3.1). PLC-δ contains a pleckstrin homology (PH) domain, four tandem elongation factor (EF) hands, a catalytic triose phosphate isomerase (TIM) barrel, and a C2 domain. While this core structure is shared by PLC isozymes, other PLCs contain additional regulatory domains that allow diversity in intracellular signaling. For example, PLC-β isozymes contain a long C-terminal region following the

C2 domain that is reported to be required for membrane binding, dimerization, and activation by the $G\alpha_q$ family of heterotrimeric G proteins (Blank et al., 1993; Park et al., 1993b; Wu et al., 1993; Singer et al., 2002). In addition, PLC-β isozymes are also activated by $G\beta\gamma$ dimers and members of the Rac family of small GTPases (Boyer et al., 1992; Camps et al., 1992; Katz et al., 1992; Illenberger et al., 1998). PLC-γ isoforms contain an additional split PH domain, two SH2 domains, and an SH3 domain (Fig. 3.1). PLC-γ1 isozymes bind receptor and nonreceptor tyrosine kinases through their N-terminal SH2 domain, resulting in tyrosine phosphorylation and intramolecular interaction with the C-terminal SH2 domain to activate the phospholipase (Poulin et al., 2005). Recent studies have expanded regulation of PLC-γ2 to include Rac GTPases, which interact with the split PH domain and promote activation of PLC-γ2 (Piechulek et al., 2005; Walliser et al., 2008). PLC-ε contains regulatory domains for activation by both Rho and Ras GTPase family members (Shibatohge et al., 1998; Kelley et al., 2001; Song et al., 2001; Wing et al., 2003; Seifert et al., 2004). Thus, PLC isozymes coordinate signaling from distinct cellular inputs to convert PtdIns(4,5)P$_2$ into second messengers and to elicit diverse physiological responses.

3.2 Structure of PLC isozymes

3.2.1 PH domain

Three-dimensional crystal structures of two separate PLC-δ1 fragments provide structural insight into the core domains of all PLC isozymes. The first structure consists of the N-terminal rat PLC-δ1 PH domain bound to Ins(1,4,5)P$_3$, and the second structure contains the remaining PLC domains (Ferguson et al., 1995; Essen et al., 1996). The structure of PLC-δ1 PH domain is similar to the architecture of other known PH domains

and consists of ~120 amino acids. The PH domain forms a β sandwich of seven β strands with a C-terminal α-helix at one end (Ferguson et al., 1995). PH domains are small protein modules found in molecules that function in cell signaling, cytoskeletal rearrangement, protein trafficking, and other cellular processes (Lemmon and Ferguson, 2000).

PH domains were initially reported to bind specifically to certain phosphoinositides with high affinity to direct membrane targeting similar to the PH domain of PLC-$\delta 1$, which binds tightly and selectively to PtdIns(4,5)P_2. Recent advances, however, suggest that this is not always the case. A genome-wide screen of 33 *S. cerevisiae* PH domains showed that 67% of PH domains exhibited no specificity for different phosphoinositides tested and bound phospholipids with low affinity (Yu et al., 2004). Six PH domains completely lacked phosphoinositide binding, and only one specifically associated with PtdIns(4,5)P_2 (Yu et al., 2004). In the human genome, ~10% of the ~230 known PH domains bind specifically and with high affinity to phosphoinositides, and the function of the majority of PH domains are not clearly known. (Lemmon, 2008). For some PH domains, binding to protein substrates is a mechanism for regulating cellular processes. For example, the PH domain of GRK2 and PLC-β isozymes interact with G$\beta\gamma$, and the PH domains of PLC-β and PLC-γ bind Rho GTPases for membrane targeting and activation (Pitcher et al., 1992; Wang et al., 1999b; Wang et al., 2000; Illenberger et al., 2003a; Illenberger et al., 2003b; Walliser et al., 2008).

In the case of PLC-$\delta 1$, its N-terminal PH domain has a polarized electrostatic surface opposite the C-terminal helix for specifically binding PtdIns(4,5)P_2 and Ins(1,4,5)P_3 (Ferguson et al., 1995). In the crystal structure, negatively charged

Ins(1,4,5)P$_3$ binds predominantly to positively charged β/β loops of the PH domain (Ferguson et al., 1995). In cells, the PH domain binds PtdIns(4,5)P$_2$ to aid in membrane association of PLC-δ1 and to promote its contact with phospholipid substrates (Cifuentes et al., 1993; Garcia et al., 1995; Lemmon et al., 1995). Thus, the PH domain enables PLC-δ isozymes to interact with PtdIns(4,5)P$_2$-containing membranes and catalyze hydrolysis of many phospholipid substrates before dissociating from the membrane, a process known as membrane scooting (Cifuentes et al., 1993; Lomasney et al., 1996). The Ins(1,4,5)P$_3$ product competes with PtdIns(4,5)P$_2$ substrate for binding to the PH domain, inhibits the association of PLC-δ1 with cell membranes, and acts as a negative feedback loop for enzymatic activity.

Other PLC isozymes also contain PH domains at their N-terminus. However, comparisons of the PH domain of PLC-δ1 with other PLC isozymes reveal that the polarized electrostatic surface and the positively charged residues needed for PtdIns(4,5)P$_2$ binding are not well conserved (Singh and Murray, 2003; Jezyk et al., 2006). Instead, the PH domains of PLC-β isozymes form protein-protein interactions with their regulators, Gβγ and Rac GTPases (Wang et al., 1999b; Wang et al., 2000; Illenberger et al., 2003a; Illenberger et al., 2003b). Rac GTPases bind exclusively to the PH domain of PLC-β2 and PLC-β3, recruiting the enzymes to the cell surface and orienting them for contact with phosphoinositide substrates (Illenberger et al., 2003a; Illenberger et al., 2003b; Snyder et al., 2003). Thus, unlike PLC-δ1, PLC-β isozymes use Rac GTPases and possibly other regulators to associate with membranes to control enzymatic activity instead of phosphoinositides. A second PLC isozyme, PLC-γ, also lacks residues in its PH domain needed for binding PtdIns(4,5)P$_2$ and Ins(1,4,5)P$_3$ and

instead preferentially binds PtdIns(3,4,5)P_3-containing membranes in response to PI3 kinase activity (Falasca et al., 1998). Thus, while PLC isozymes share the same core architecture, the mechanisms that regulate membrane binding and enzyme activity differ between the PLC family members, providing complexity and diversity in PLC signaling.

3.2.2 EF hands

The second three-dimensional structure of PLC-δ1 consists of the remaining core architectural motifs including a series of EF hands, a catalytic TIM barrel, and a C-terminal C2 domain (Essen et al., 1996). PLC-δ1 and other PLC isozymes contain four tandem EF hands following the N-terminal PH domain. EF hand motifs are composed of a helix-loop-helix domain found in calcium binding proteins such as calmodulin and troponin C (Strynadka and James, 1989). Positively charged calcium binds within the loop region of the EF hand motif to acidic residues, Asp and Glu, as well as to the peptide backbone.

The EF hands of PLC isozymes form four consecutive helix-loop-helix arrangements. This domain does not interact with the C-terminal catalytic TIM barrel but does make extensive contacts with the C2 domain (Essen et al., 1996; Jezyk et al., 2006). While other characterized EF hands are known to bind calcium, the analogous regions of PLC isozymes are not well conserved. In the crystal structure, PLC-δ1 lacked calcium binding within its EF hands (Essen et al., 1996). However, subsequent studies showed that PLC-δ1 EF hands are capable of binding calcium and that this contributes to the affinity of the PH domain for PtdIns(4,5)P_2-containing membranes (Yamamoto et al., 1999).

3.2.3 TIM barrel

TIM barrel proteins predominantly function as enzymes for cellular metabolism and are ubiquitously expressed in nature (Wierenga, 2001). The TIM barrel structure was first observed for triose-phosphate isomerase, which forms a barrel structure composed of eight βα repeats. The eight β-strands fold into parallel β-sheets surrounded by eight α-helices, and the catalytic domain is located near the C-terminal end of the barrel. This three-dimensional fold is highly conserved in nature and found in proteins with diverse enzymatic functions.

The crystal structure of PLC-δ1 revealed that the catalytic domain of this enzyme forms a distorted TIM barrel, consisting of two highly conserved regions, an X and Y box separated by a long flexible linker (Essen et al., 1996). The X and Y box form two halves of the catalytic TIM barrel. The X box encompasses the N-terminal half, forming a classic βα repeat arrangement of other known TIM barrels. The Y box comprises the C-terminal portion and adopts a typical βα repeat topology except for a disordered loop between Tβ5 and Tβ6. The active site is located at the C-terminal end of the barrel and forms a cleft that accommodates calcium and phosphoinositides. Structural and mutational analysis of rat PLC-δ1 illustrates the importance of Lys438, Lys440, Ser522, and Arg549 in binding and stabilizing the 4- and 5-phosphate groups of the phosphoinositide ring (Fig. 3.2) (Ellis et al., 1998). These residues are highly conserved amongst all PLC isozymes from bacteria to mammals. In addition, the active site is also surrounded by several hydrophobic loops between Tβ1/Tα1, Tβ2/Tα2, and Tβ7/Tα6 that form a hydrophobic ridge, possibly important for insertion into membranes and localization of the active site with phosphoinositide substrates (Essen et al., 1996).

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All PLC isozymes use the same mechanism to hydrolysis PtdIns(4,5)P_2 to diacylglycerol and Ins(1,4,5)P_3. The residues required for substrate and calcium binding and acid-base catalysis are conserved in other PLC isoforms. The catalysis reaction requires calcium, which is located at the bottom of the active site (Fig. 3.2A). In rat PLC-δ1, Asn312, Asp343, Glu341, and Glu390 coordinate the calcium ion and also make contacts with hydroxyl groups and the 1-phosphate on the inositol ring (Essen et al., 1996). The basic imidazole rings of His311 and His356 facilitate acid-base catalysis of phosphoinositide substrates (Fig. 3.2B). The 2-hydroxyl group is deprotonated and subsequently undergoes nucleophilic attack of the 1-phosphoryl group on the inositol ring, forming a 1,2-cyclic-inositolphosphate intermediate and diacylglycerol. A water molecule hydrolyzes the cyclic inositol phosphate intermediate to give the linear Ins(1,4,5)P_3 product, which is then released from the active site into the cytosol.

3.2.4 X/Y linker

The highly conserved X and Y boxes of the catalytic core are split by a flexible region known as the X/Y linker. This region varies in length and amino acid sequence across PLC isozymes and lacks conserved protein domains with the exception of PLC-γ. The X/Y linker of PLC-γ contains various modular domains, including a split PH domain, two SH2 domains, and a SH3 domain. The N-terminal SH2 domain is phosphorylated by receptor and non-receptor tyrosine kinases and intramolecularly binds to the C-terminal SH2 domain for PLC-γ activation (Poulin et al., 2005). The split PH domain of PLC-γ2 forms one functional unit and binds to Rac GTPases to stimulate PLC activity (Piechulek et al., 2005; Walliser et al., 2008).

Studies of the X/Y linkers of various PLC isozymes suggest that this region plays an autoinhibitory role and prevents basal PLC activity. Limited trypsin digestion of the PLC-δ1 X/Y linker showed that proteolytic fragments of the highly conserved X and Y box formed a stable complex and displayed enhanced catalytic activity compared to full length PLC-δ1 (Ellis et al., 1993). Similar proteolysis studies of PLC-β2 and PLC-γ also demonstrated that the separate X and Y box form one functional unit and exhibit higher basal activity (Fernald et al., 1994). These results were further validated for PLC-β2 and PLC-γ1 when individual polypeptides of the X and Y regions were coexpressed in cells, and a significant increase in PLC activity was observed. No activity was detected when these regions were expressed individually (Horstman et al., 1999; Zhang and Neer, 2001). Using sequential deletion, removal of the SH array in the X/Y linker of PLC-γ1 was found to specifically increase basal activity, suggesting that the SH domains play a negative regulatory role in modulating basal PLC-γ1 activity (Horstman et al., 1999).

Two high-resolution crystal structures of PLC-β2 in complex with Rac1 and the unbound PLC-β2 holoenzyme provided both structural and biochemical evidence to explain why proteolysis or deletion of X/Y linkers enhances basal PLC activity. Both of these structures revealed that an ordered 20 amino acid portion of the X/Y linker blocked the active site and formed hydrogen bonds with several residues on the TIM barrel (Jezyk et al., 2006; Hicks et al., 2008). Removal of this ordered region increased basal activity in intact cells by 5-20-fold compared to wildtype PLC-β2, and deletion of the remaining portion of the X/Y linker increased basal activity to a much greater extent (Hicks et al., 2008). These results were confirmed with purified proteins reconstituted into phospholipid vesicles containing [^{3}H]PtdIns(4,5)P$_2$. Purified PLC-β2 lacking the ordered

region of the X/Y linker displayed a 8-fold increase in PLC activity compared to wildtype PLC-β2. In addition, PLC-β2 mutants lacking the X/Y linker were still sensitive to further stimulation by Gα$_q$, Gβγ, or constitutively active Rac3 in intact cells and in reconstitution experiments (Hicks et al., 2008). Removing the X/Y linkers of PLC-β1 or divergent isozymes PLC-δ1 and PLC-ε also resulted in constitutively active enzymes (Hicks et al., 2008). Collectively, these observations supported a general mechanism for occlusion of the active site by X/Y linkers and autoinhibition of PLC activity.

The X/Y linkers of PLC isozymes differ greatly in length and amino acid sequence, but they all contain a large portion of negatively charged residues. Hicks et al. proposed that negatively charged X/Y linkers are repelled from negatively charged phospholipid membranes, and autoinhibition of PLC isozymes is released (Hicks et al., 2008). Comparison of the three-dimensional structures of PLC-β2 in complex with Rac1 to unbound PLC-β2 revealed little to no change in conformation of the catalytic domain (Jezyk et al., 2006; Hicks et al., 2008). As a whole, these results indicated that regulators of PLC isozymes such as Rac and possibly Gα$_q$ and Gβγ recruit and orient PLC isoforms to lipid bilayers, and removal of the autoinhibitory X/Y linker by negatively charged membranes leads to PtdIns(4,5)P$_2$ binding and hydrolysis. In the basal state, the X/Y linker blocks the active site of PLC isozymes and prevents spurious substrate hydrolysis.

3.2.5 C2 domain

C2 domains are protein modules of ~130 amino acids that are found in many proteins involved in signal transduction and membrane trafficking (Cho and Stahelin, 2006; Lemmon, 2008). C2 domains form an anti-parallel β-sandwich topology consisting of eight β-strands (Sutton et al., 1995; Perisic et al., 1998; Sutton and Sprang,

1998). For some C2 domains, calcium binding facilitates association with phospholipid membranes. Calcium binds to conserved loops between β-strands and changes the overall electrostatic potential of the membrane-binding surface from negative to positive (Shao et al., 1997; Murray and Honig, 2002). Calcium ions can also "bridge" C2 domains to anionic phospholipids. A crystal structure of the PKCα C2 domain showed phosphatidylserine contacting a calcium ion and amino acids within the conserved β/β loops (Verdaguer et al., 1999). In some cases, C2 domain function is calcium-independent, and a subgroup of C2 domains use a separate positively-charged region on the surface of the β-sandwich to attach to phospholipid membranes (Cho and Stahelin, 2006; Lemmon, 2008). C2 domains are also known to form protein-protein interactions, which can be calcium-dependent or independent (Cho and Stahelin, 2006; Lemmon, 2008).

Inspection of the PLC-δ1 structure showed that its C2 domain ligates calcium at three or four sites through its β/β loops to facilitate membrane attachment (Essen et al., 1996). The C2 domain functions together with the PH domain to bind lipid bilayers (Essen et al., 1996; Essen et al., 1997). In the case of PLC-β isozymes, the C2 domain has no apparent affinity for phospholipid membranes in the presence of calcium (Wang et al., 1999a). PLC-β and other PLC isozymes lack the residues needed for calcium binding and use other mechanisms to associate with lipid bilayers. Instead, PLC-β2 C2 domains were found to interact with activated $G\alpha_q$ in a nucleotide-dependent manner and contribute to PLC-β activation by $G\alpha_q$ (Wang et al., 1999a).

3.3 PLC-β isozymes

PLC-β isozymes contain a long C-terminal extension of approximately 400 amino acids following their C2 domain, making these enzymes unique from other PLC family members (Fig. 3.3). The C-terminal region is proposed to be required for membrane association and for binding and activation by $G\alpha_q$ subunits (Blank et al., 1993; Park et al., 1993b; Wu et al., 1993). In addition, PLC-β isozymes are also directly activated by Rac GTPases and $G\beta\gamma$ subunits released from the $G\alpha_{i/o}$ family of heterotrimeric G proteins (Boyer et al., 1992; Camps et al., 1992; Katz et al., 1992; Illenberger et al., 1998). Activation by these diverse protein regulators allows for modulation of phosphoinositide signaling from various extracellular stimuli acting on both heterotrimeric and small monomeric G proteins (Fig. 3.3).

3.4 Physiology of PLC-β isozymes

PLC-β isozymes are arguably the major effector proteins downstream of the $G\alpha_q$ signaling pathway. Extracellular stimuli including hormones, peptides, and proteases activate ~40% of GPCRs that either couple partly or exclusively to the $G\alpha_q$ family of heterotrimeric G proteins and regulate cellular processes through the $G\alpha_q$/PLC-β signaling axis (Harmar et al., 2009). These cellular processes include platelet activation and regulation of memory, motor coordination, appetite, and sleep (Offermanns and Simon, 1998; Wettschureck and Offermanns, 2005; Hubbard and Hepler, 2006). Indeed, $G\alpha_q$ signaling in the cardiovascular system controls smooth muscle tone and blood pressure through activation of PLC-β isozymes. $Ins(1,4,5)P_3$-promoted release of intracellular calcium leads to phosphorylation of myosin light chain, which mediates actin contractility and vasoconstriction.

Studies of PLC-β knockout mice have revealed isoform-specific functions of PLC-β isozymes in physiological and pathological processes. Each member of the PLC-β family (PLC-β1, -β2, -β3, -β4) has distinct expression profiles in various tissues and cell types. PLC-β1 is highly expressed in the hippocampus and cerebral cortex whereas PLC-β2 is mostly restricted to hematopoietic cell types (Ross et al., 1989; Park et al., 1992; Sun et al., 2007). PLC-β3 is present in the brain, liver, and parotid gland, and PLC-β4 is highly expressed in regions of the brain and retina (Jhon et al., 1993; Tanaka and Kondo, 1994; Adamski et al., 1999). Mice deficient in PLC-β1 exhibit epilepsy possibly due to a loss of mAChR signaling in the hippocampus (Kim et al., 1997). PLC-β4$^{-/-}$ mice display ataxia, which may be due to a defect in metabotropic glutamate receptor (mGluR) signaling in Purkinje cells in the cerebellum (Kim et al., 1997). Consistent with this conclusion, mGluR1 is predominantly expressed in Purkinje cells, and mGluR1$^{-/-}$ mice also display an ataxia phenotype (Aiba et al., 1994).

Neutrophils isolated from PLC-β2$^{-/-}$ and PLC-β2$^{-/-}$;PLC-β3$^{-/-}$ mice are impaired in chemokine signaling, specifically showing a decrease in inositol phosphate accumulation, intracellular calcium levels, superoxide production, and MAC-1 expression on the cell surface (Jiang et al., 1997; Li et al., 2000). Despite this loss in chemokine signaling, neutrophils isolated from PLC-β2$^{-/-}$ and PLC-β2$^{-/-}$;PLC-β3$^{-/-}$ mice show no defects in chemotaxis and in some leukocyte populations display an enhanced chemotactic response (Jiang et al., 1997; Li et al., 2000). In contrast, PLC-β2$^{-/-}$;PLC-β3$^{-/-}$ lymphocytes are impaired in chemotaxis, suggesting that the function of PLC-β isozymes in cell migration is dependent on enzyme expression in particular cell types and can have a positive, negative, or no effect on chemotactic activity (Bach et al., 2007).

PLC-β3$^{-/-}$ mice display an increased sensitivity to μ-opioids in behavioral assays measuring nociception responses, indicating that PLC-β3 negatively regulates μ-opioid function (Xie et al., 1999). These results were recapitulated in dorsal root ganglia neurons where PLC-β3 deficiency enhanced μ-opioid-induced regulation of voltage-gated calcium channels (Xie et al., 1999). Another study showed a role for PLC-β3 in mediating signaling in sensory neurons. Dorsal root ganglia neurons derived from PLC-β3$^{-/-}$ mice showed a significant decrease in histamine-induced calcium efflux whereas other agonists such as ATP, UTP, and bradykinin stimulated similar calcium responses in both wildtype and PLC-β3$^{-/-}$ cells (Han et al., 2006). Moreover, PLC-β3$^{-/-}$ mice displayed a significant decrease in scratching induced by the histamine H1 receptor, demonstrating that PLC-β3 is required for the histamine-mediated itch response (Han et al., 2006).

PLC-β3 is also implicated in pathological responses. For example, a recent study showed that PLC-β3 might potentiate atherosclerosis by promoting cell survival of macrophages in atherosclerotic plaques (Wang et al., 2008). Loss of PLC-β3 increases the sensitivity of macrophages to apoptotic stimuli. Furthermore, in an atherosclerosis model, PLC-β3$^{-/-}$;apoE$^{-/-}$ mice had significantly less macrophages in atherosclerotic plaques and were protected from atherosclerotic lesions compared to apoE$^{-/-}$ mice, suggesting a negative role for PLC-β3 in progression of atherosclerosis (Wang et al., 2008). PLC-β3 may also have tumor suppressor activity (Xiao et al., 2009). Xiao et al. found that PLC-β3$^{-/-}$ mice developed a variety of tumors including myeloproliferative disease, lymphoma, and skin and lung carcinomas within an age of 16 months, which was not observed with PLC-β2$^{-/-}$ mice (Xiao et al., 2009). PLC-β3$^{-/-}$ mice had an increased number of hematopoietic stem cells that hyperproliferated, evaded apoptosis, and were

capable of myeloid differentiation, suggesting that PLC-β3 may act as a tumor suppressor in hematopoietic tissues (Xiao et al., 2009).

PLC-β isoforms have overlapping expression profiles in some tissues, but only one isoform mediates a particular response. For example, in mouse dorsal root ganglia, PLC-β3 is the predominant PLC-β isoform, but PLC-β4 and PLC-β1 are also expressed at lower levels (Han et al., 2006). Loss of PLC-β3 had a significant effect on histamine-induced signaling but no effect on ATP, UTP, or bradykinin-stimulated signaling, indicating that other PLC-β isoforms elicit responses to these ligands (Han et al., 2006). The selectivity of PLC-β isoforms can occur through several different mechanisms. In addition to distinct tissue distribution and varying expression levels, PLC-β isozymes also have different sensitivities to protein regulators. PLC-β1 and PLC-β3 are the most responsive to $G\alpha_q$ stimulation (Hepler et al., 1993; Jhon et al., 1993; Park et al., 1993a; Smrcka and Sternweis, 1993). In contrast, PLC-β2 and PLC-β3 are the most responsive to $G\beta\gamma$ subunits released from $G\alpha_i$ whereas PLC-β1 shows little to no activation (Park et al., 1993a; Smrcka and Sternweis, 1993). PLC-β2 is the most sensitive to activation by Rac GTPases (Illenberger et al., 2003a; Snyder et al., 2003). Thus, differences in sensitivity to protein regulators provide some level of PLC-β specificity for a particular signaling pathway.

In addition, PLC-β isozymes have PSD-95/Dlg/ZO-1 (PDZ) ligands on their C-terminal regions and interact with scaffolding molecules containing PDZ domains (Hwang et al., 2000; Tang et al., 2000). In *Drosophila*, PDZ-containing protein, INAD facilitates formation of a multiprotein complex composed of NorpA (PLC-β), PKC, $G\alpha_q$, and light-sensitive ion channels TRP and TRPL. INAD targets these proteins to

photoreceptor cell membranes to promote phototransduction, and disruption of the PLC and INAD interaction leads to abnormal termination of light responses (Chevesich et al., 1997; Shieh et al., 1997; Tsunoda et al., 1997). In mammalian systems, PLC-β1 and PLC-β2 were found to interact with the first PDZ domain of Na^+/H^+ exchanger regulator factor 1 (NHERF1) and PLC-β3 with the second PDZ domain on NHERF2 (Hwang et al., 2000; Tang et al., 2000). NHERF proteins promote formation of large signaling complexes and modulate the function of ion channels, signaling molecules, transcription factors, and cytoskeletal proteins (Weinman et al., 2005). When NHERF2 was exogenously expressed in COS-7 and HeLa cells, PLC activity was potentiated in the presence of carbachol (Hwang et al., 2000). The molecular mechanism that controls this additive effect of NHERF2 is not currently known. Nonetheless, these observations suggest that under certain conditions PLC-β isozymes may engage PDZ domain-containing proteins, which facilitate formation of large signaling complexes of specific PLC-β isoforms and regulators and provide a mechanism for eliciting particular signaling responses (Hwang et al., 2000; Suh et al., 2001).

3.5 Activation of PLC-β isozymes by heterotrimeric G proteins

3.5.1 Heterotrimeric G proteins

Heterotrimeric G proteins coordinate signals from cell surface G protein-coupled receptors (GPCRs) to intracellular effectors through a regulated cycle of activation and deactivation steps. Heterotrimeric G proteins are composed of three subunits, α, β, and γ. G proteins are inactive in a GDP-bound state and associate with G$\beta\gamma$ dimers. Extracellular stimuli bind to cell surface GPCRs and cause a conformational change that promotes coupling to heterotrimeric G proteins. GPCRs catalyze exchange of GTP for

GDP on the Gα subunit. Consequently, GTP-bound Gα subunits have a lower affinity for the Gβγ dimers, and both GTP-bound Gα and Gβγ dimers interact with downstream effectors to transmit intracellular signaling. Signaling is terminated by the intrinsic GTPase activity of the Gα subunit, converting active GTP-bound Gα to an inactive GDP-bound state. GTP hydrolysis is accelerated by GTPase activating proteins (GAPs) present in some effector molecules or in regulator of G protein signaling (RGS) proteins to complete the activation and deactivation cycle.

The heterotrimeric G protein family is divided into four classes, $G\alpha_s$ ($G\alpha_s$, $G\alpha_{olf}$), $G\alpha_i$ ($G\alpha_{i1-3}$, $G\alpha_o$, $G\alpha_t$, $G\alpha_z$) $G\alpha_q$ ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15/16}$), and $G\alpha_{12/13}$ based on sequence homology and function (Simon et al., 1991; Oldham and Hamm, 2006). $G\alpha_s$ proteins bind and stimulate adenylyl cyclases, which convert ATP to cAMP. Augmented cAMP levels regulate the activities of several molecules including protein kinase A and ion channels. Cyclic AMP also directly binds and stimulates GEF proteins known as exchange proteins activated by cAMP (EPAC) to regulate the activity of Ras GTPase homologues Rap1 and Rap2 (Kozasa et al., 1993; de Rooij et al., 1998). In addition, $G\alpha_s$ may also directly stimulate cardiac calcium channels and Src tyrosine kinases (Yatani et al., 1987; Mattera et al., 1989; Ma et al., 2000). $G\alpha_{i1-3}$ and $G\alpha_z$ proteins directly bind and inhibit some adenylyl cyclases to reduce cAMP levels and modulate activity of voltage-gated ion channels through release of Gβγ subunits (Simon et al., 1991). $G\alpha_t$ regulates phototransduction in retinal rods and cones by interacting with the inhibitory γ subunit of cGMP phosphodiestrase (PDE). All $G\alpha_q$ proteins activate the PLC-β family of isozymes, which hydrolyze PtdIns(4,5)P$_2$ to Ins(1,4,5)P$_3$ and diacylglycerol (Exton, 1996). Ins(1,4,5)P$_3$ binds receptors on the endoplasmic reticulum to mobilize intracellular

calcium, and diacylglycerol activates PKC. In recent studies, $G\alpha_q$ was also shown to directly engage p63RhoGEF to stimulate GEF activity on RhoA (Lutz et al., 2005; Rojas et al., 2007). $G\alpha_{12/13}$ proteins activate the Rho pathway by binding to Rho-specific GEFs p115RhoGEF, PDZ-RhoGEF, and LARG (Hart et al., 1998; Fukuhara et al., 1999; Fukuhara et al., 2000). $G\alpha_{12/13}$ proteins were also shown to bind and stimulate activity of Bruton's tyrosine kinase and rasGAP (Jiang et al., 1998). In addition, $G\alpha_{12/13}$ interact with cadherin and radixin, proteins involved in cell-cell adhesion, cytoskeletal rearrangement, and gene transcription (Vaiskunaite et al., 2000; Meigs et al., 2001).

All $G\alpha$ subunits have a similar three-dimensional structure consisting of a GTPase domain and a helical domain. The GTPase domain is structurally homologous to small, monomeric G proteins of the Ras superfamily and composed of six β-strands enclosed by five α-helices (Noel et al., 1993; Coleman et al., 1994; Lambright et al., 1994). The GTPase domain contains highly conserved regions for guanine nucleotide and Mg^{2+} binding, hydrolyzing GTP, and interactions with Gβγ, GPCRs, and RGS proteins. The GTPase domain contains three highly conserved flexible loops referred to as Switch I, Switch II, and Switch III (Noel et al., 1993; Coleman et al., 1994; Lambright et al., 1994; Sondek et al., 1994; Mixon et al., 1995). The switch regions undergo structural changes when bound to GDP, GTP or in the GTP-bound transition state, forming a more open conformation in the GDP-bound state and a collapsed conformation when bound to GTP. The guanine nucleotide-binding pocket within the GTPase domain is capped by the six α-helices of the helical domain, burying the nucleotide in the cleft of the protein. The N-terminal and C-terminal regions of $G\alpha$ subunits form α-helices and contain sequences for lipid modifications and insertion into cell membranes (Oldham and

Hamm, 2006). In addition, these domains are critical for receptor selectivity and G protein activation.

There are six β subunits encoded in the mammalian genome, and each consists of seven WD40 repeats that form a seven-bladed β-propeller (Sondek et al., 1996). A portion of the N-terminal domain contributes to the last β-strand of the final blade, and the remaining portion forms an α-helix that binds to the helix-loop-helix of the Gγ subunit through coiled-coiled interactions. Gγ subunits are small 7-8 kDa proteins and make up a family of 12 subunits (Downes and Gautam, 1999). The Gγ subunits contain a C-terminal CaaX box motif for isoprenylation and membrane association of Gβγ subunits. As a stable complex, Gβγ subunits directly interact with and activate downstream effectors including adenylyl cyclases, PLC-β isozymes, and voltage-gated ion channels to elicit cellular responses.

3.5.2 Activation of PLC-β isozymes by Gα subunits

In the 1950's, Hokin and Hokin first discovered that addition of hormones to secretory tissues caused rapid turnover of membrane phospholipids (Hokin and Hokin, 1953). Twenty years later, research by Berridge, Mitchell, and others found a link between phospholipid turnover and calcium signaling. Their work showed that hormone stimulation of secretory cells and other cell types increased generation of $Ins(1,4,5)P_3$ and that this second messenger was responsible for release of intracellular calcium from the endoplasmic reticulum (Berridge, 1987; Berridge, 1993). In addition, work by Nishizuka and colleagues found that generation of DAG activated protein kinase C to elicit a host of cellular responses (Nishizuka, 1992). By the 1980's, several studies had demonstrated a molecular link between hormone stimulation of cell surface receptors to phosphoinositide

turnover and generation of second messengers, but a key component directly coupling receptors to phosphoinositide turnover remained elusive.

Studies in the 1980's and early 1990's were the first to provide direct evidence that guanine nucleotide binding proteins regulated phosphoinositide hydrolysis. Cockcroft and Gomperts showed that treatment of human peripheral neutrophil membranes with GTPγS or other non-hydrolyzable GTP analogues increased hydrolysis of polyphosphoinositides (Cockcroft and Gomperts, 1985). In addition, Litosch et al. demonstrated that stimulating homogenates and cell membranes of blowfly salivary glands with 5-hydroxytryptamine and non-hydrolyzable GTP analogues increased inositol phosphate accumulation (Litosch et al., 1985). Collectively, these results implicated a guanine nucleotide binding protein in regulating hormone-stimulated phosphoinositide turnover and provided a molecular link between cell surface receptors and phospholipase activity (Cockcroft and Gomperts, 1985; Litosch et al., 1985).

Several studies demonstrated that hormone-activated phosphoinositide hydrolysis occurred through both a pertussis toxin-sensitive and insensitive pathway depending on the tissue type or stimulating hormone. These studies alluded to the existence of an unknown heterotrimeric G protein in activating the pertussis toxin-insensitive pathway. Taylor et al. first identified a toxin-insensitive activator of PLC in bovine liver plasma membranes (Taylor et al., 1990). In this study, a GTPγS analog stimulated PLC activity in cholate-solubilized extracts. The PLC activity was absorbed on heparin-Sepharose matrix and only restored when recombined with supernatant proteins. The PLC activator was purified and identified as a unique 42-kDa Gα subunit using immunoblot analysis and antibodies specific and common for known Gα subunits (Taylor et al., 1990).

In a concurrent study, a 42-kDa G protein was purified from bovine brain using immobilized Gβγ subunits attached to an affinity matrix (Pang and Sternweis, 1990). Amino acid sequencing of tryptic protein fragments revealed that the novel Gα subunit was identical to $G\alpha_q$ and $G\alpha_{11}$, two G proteins with high sequence homology recently cloned by Strathmann and Simon (Strathmann et al., 1989; Pang and Sternweis, 1990; Strathmann and Simon, 1990). Further characterization of purified $G\alpha_q$ and $G\alpha_{11}$ showed activation of a partially purified PLC preparation only in the presence of Gα subunits and AlF_4^- (Smrcka et al., 1991). Immunoblot analysis of the PLC preparations identified PLC-β isozymes as the activated PLC.

In a separate study, Harden and colleagues purified a 150-kDa PLC-β from turkey erythrocytes and reconstituted PLC activity in phospholipid vesicles containing radiolabeled $PtdIns(4,5)P_2$ (Morris et al., 1990b; Morris et al., 1990a). Using this assay, they purified the PLC-activating protein from turkey erythrocyte membranes and identified it as a member of the G_q family of heterotrimeric G proteins by immunoblot analysis (Waldo et al., 1991). The PLC-activating G protein was later identified as $G\alpha_{11}$ using amino acid sequencing and immunoblotting techniques (Maurice et al., 1993).

The G_q family of heterotrimeric G proteins includes $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$, and each Gα subunit directly binds and activates PLC-β isozymes but not other PLCs (Taylor et al., 1991; Taylor and Exton, 1991; Lee et al., 1992; Wu et al., 1992). Studies using purified proteins established that $G\alpha_q$ and $G\alpha_{11}$ activate PLC-β isozymes with the following relative efficacies: PLC-β1≥PLC-β3>>PLC-β2 (Hepler et al., 1993; Jhon et al., 1993; Smrcka and Sternweis, 1993). PLC-β4 is also stimulated by $G\alpha_q$ subunits (Jiang et al., 1994). $G\alpha_q$ family members bind directly to PLC-β isozymes, and

PLC-β lacking the ~50 kDa C-terminal region was not activated by Gα$_q$ but retained activation by Gβγ (Blank et al., 1993; Park et al., 1993b; Wu et al., 1993). A 100-kDa proteolytic fragment of PLC-β1 missing the long C-terminal region was purified from bovine extracts and was catalytically active under basal conditions but was no longer stimulated by Gα$_q$ and AlF$^-_4$ (Park et al., 1993b). Sequential deletion of the C-terminal region of PLC-β1 suggested that a highly basic region consisting of residues 903-1030 is needed for membrane association and residues 1030-1142 were needed for activation by Gα$_q$ (Wu et al., 1993). Further analysis of PLC-β isozymes indicated that the C2 domain also contributes to activation by Gα$_q$. Isolated PLC-β1 and PLC-β2 C2 domains bind selectively to Gα$_q$ in a GTPγS specific manner whereas no binding occurs between C2 domains and Gβγ or Gα$_i$ (Wang et al., 1999a).

The PLC-β binding domain of Gα$_q$ was determined using small synthetic peptides corresponding to the entire Gα$_q$ sequence (Arkinstall et al., 1995). Peptides corresponding to regions of the α3 and α4 helices of Gα$_q$ inhibited activation of PLC-β by GTPγS-bound Gα$_q$. A separate study used chimeric Gα$_q$/Gα$_s$ proteins consisting of constitutively active Gα$_q$ altered with analogous regions of Gα$_s$ to identify domains required for PLC-β activation (Venkatakrishnan and Exton, 1996). Using these chimeric proteins, the authors pinpointed a region consisting of residues Ile217-Lys276 located within the α3 helix and α3/β5 linker of Gα$_q$. Alanine scanning mutagenesis of this region in constitutively active Gα$_q$ revealed the importance of five residues (Asp243, Asn244, Glu245, Arg256 and Thr257) in activation of PLC-β isozymes. Collectively, these two independent studies demonstrated an important role for the α3 helix of Gα$_q$ but

published conflicting results for the role of the α4 helix (Arkinstall et al., 1995; Venkatakrishnan and Exton, 1996).

3.5.3 GAP activity of PLC-β isozymes

In vitro studies of purified Gα subunits revealed that GTP hydrolysis was significantly slower than physiological rates of signal termination, suggesting that additional regulatory proteins are required to accelerate GTP hydrolysis *in vivo* (Vuong and Chabre, 1990; Vuong and Chabre, 1991). A similar mechanism is essential for deactivating monomeric G proteins of the Ras superfamily in which GTPase accelerating proteins (GAPs) increase the rate of GTP hydrolysis approximately 1000-fold. PLC-β isozymes were the first GAPs identified for heterotrimeric G proteins (Berstein et al., 1992). PLC-β isozymes are not only effector molecules activated by GTP-bound $G\alpha_q$ but also accelerate GTP hydrolysis on their activating G protein. When purified PLC-β1 was reconstituted into phospholipid vesicles containing purified M1 mAChR and $G\alpha_q/_{11}$, addition of carbachol accelerated the rate of GTP hydrolysis over 1000-fold (Berstein et al., 1992; Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999). This effect was rapidly blocked in the presence of muscarinic receptor antagonist, illustrating the importance of receptor-catalyzed GTP exchange and PLC-β GAP activity in regulating the cycle of activation and deactivation of $G\alpha_q$ and therefore, PLC activity.

Further analysis suggested that the region of PLC-β1 GAP activity was located in the C-terminal extension, a region previously reported as the binding domain of $G\alpha_q$. Purified peptide fragments of PLC-β1 C-terminus exhibited steady-state GAP activity in reconstitution assays with purified $G\alpha_q/_{11}$ and M1 mAChR (Paulssen et al., 1996). The peptide fragments accelerated steady-state GTP hydrolysis by 4-fold, exhibiting only

about 15-20% of activity observed with full length PLC-β1. The authors suggested that this difference in GAP activity is probably due to lower affinity of the peptides for Gα_q and a lack of membrane binding for each of the peptide fragments. In addition, they suggested that a second binding site on Gα_q could contribute to the remaining GAP activity of PLC-β1.

The importance of PLC-β GAP activity in phototransduction was demonstrated *in vivo* using *Drosophila* as a model organism (Cook et al., 2000). In invertebrates and vertebrates, phototransduction is stimulated through a heterotrimeric G protein signaling pathway. Light activates a GPCR, rhodopsin, that catalyzes GTP exchange on the α subunit of a heterotrimeric G protein. In *Drosophila*, rhodopsin stimulates Gα_q, which activates its effector NorpA (PLC-β) to induce phototransduction (Montell, 1999). Cook and colleagues used mutant alleles of *norpA* that express reduced levels of PLC-β isozymes. In flies expressing lower amounts of PLC-β, response termination was significantly slower when light stimulus was removed compared to wildtype flies (Cook et al., 2000). In addition, the *norpA* mutants showed a reduction in GTPase activity with no effect on GTPγS binding. GTPase activity was rescued when head membranes from PLC-β-deficient flies were fused with membranes from flies expressing normal PLC-β levels. Using flies expressing varying amounts of PLC-β, a strong correlation was observed between low expression of PLC-β and reduced GAP activity. The authors concluded that in *norpA* mutants, slow response termination is due to low PLC-β levels, and therefore, reduced GTPase activity on Gα_q. Consequently, active GTP-bound Gα_q continues to elicit downstream signaling in the dark until limited PLC-β eventually accelerates GTP hydrolysis and signal termination.

3.5.4 Molecular consequences of effector GAP activity

Biochemical and kinetic analysis has provided insight into how effector GAP proteins, like PLC-β isozymes, maintain signal amplitude despite the presence of deactivating GAP activity. At steady-state, signal amplitude is sustained by a balance of activation and deactivation steps in the GTPase cycle (Ross, 2008). This balance allows for a fraction of Gα subunits to remain GTP-bound and active and for rapid deactivation when agonist is removed. Ross and colleagues demonstrated this effect for PLC-β1 using a phospholipid vesicle system and purified proteins (Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999). Purified PLC-β1 was reconstituted into proteoliposomes containing purified M1 mAChR and heterotrimeric G_q. Despite an increase in GTP hydrolysis at steady-state, a significant fraction of GTP-bound $G\alpha_q$ stimulated PLC activity approximately 1000-fold (Mukhopadhyay and Ross, 1999). Analysis of GTP exchange activity revealed that the fraction of GTP-bound $G\alpha_q$ was maintained in the presence of GAP activity as a consequence of enhanced receptor-catalyzed exchange activity in the presence of PLC-β1 (Mukhopadhyay and Ross, 1999; Turcotte et al., 2008). A similar effect on exchange activity was demonstrated when purified RGS4 was added to phospholipid vesicles containing heterotrimeric $G\alpha_i$ and M2 mAChR (Posner et al., 1999).

Ross and colleagues proposed a model for kinetic scaffolding to explain these observations (Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999; Ross, 2008). In this model, a stable complex of $G\alpha_q$, GPCR, and PLC-β is formed at steady-state because GTP hydrolysis occurs much faster than the rate of receptor and Gα dissociation (Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999; Ross, 2008; Turcotte et al.,

2008) (Fig. 3.4). Consequently, agonist-bound receptor catalyzes multiple rounds of GDP/GTP exchange on $G\alpha_q$, and $G\alpha_q$ proceeds through the GTPase cycle numerous times (Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999; Ross, 2008). Therefore, a sufficient fraction of GTP-bound $G\alpha_q$ is available for activating PLC-β1, and only when agonist is removed is signal transduction terminated (Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999; Ross, 2008).

3.5.5 Activation of PLC-β isozymes by G$\beta\gamma$ subunits

PLC-β isozymes are also regulated by G$\beta\gamma$ subunits through a pertussis toxin-sensitive pathway. Direct evidence showed that G$\beta\gamma$ purified from bovine brain and turkey erythrocytes stimulated avian PLC-β but not bovine PLC-β1 (Boyer et al., 1992). In a separate study, Katz et al. found that transient transfection of COS-7 cells with G$\beta\gamma$ and PLC-β2 significantly increased phosphoinositide hydrolysis whereas no effect was observed with cells transfected with PLC-β1 (Katz et al., 1992). The response was inhibited when cells were transfected with $G\alpha_i$ to scavenge free G$\beta\gamma$ dimers and prevent PLC-β2 activation. Receptor-promoted PLC activity was also observed in cells transfected with M2 mAChR, which couples to pertussis toxin-sensitive $G\alpha_i$ proteins, and the response was blocked by pertussis toxin treatment (Katz et al., 1992). In a concurrent study, reconstitution of purified G$\beta\gamma$ subunits and cell extracts expressing PLC-β2 into PtdIns(4,5)P$_2$-containing phospholipid vesicles increased inositol phosphate accumulation, but little activity was observed with PLC-β1 expressing extracts (Camps et al., 1992). PLC activity was inhibited with GDP-bound $G\alpha_t$, which prevented free G$\beta\gamma$ subunits from activating PLC-β2 (Camps et al., 1992). Using purified proteins, G$\beta\gamma$ dimers have been shown to directly bind and activate PLC-β2 and PLC-β3. PLC-β2 is

the most sensitive to Gβγ stimulation whereas PLC-β1 is the least responsive (Park et al., 1993a; Smrcka and Sternweis, 1993).

One region of Gβγ binding was mapped to the catalytic TIM barrel of PLC-β2. A 116 amino acid fragment of PLC-β2 encompassing the X and Y box was co-transfected with Gβγ and full-length PLC-β2 in COS-7 cells and prevented Gβγ-stimulated PLC activity (Kuang et al., 1996). In addition, the peptide fragment also hindered activation of PLC-β2 in response to the Gα$_i$-coupled complement C5a receptor but not a Gα$_q$-coupled receptor, suggesting that this peptide fragment interferes with Gβγ signaling but not Gα$_q$. A subsequent study used 20 amino acid peptides to narrow down the region of interaction between PLC-β2 and Gβγ identified by Kuang et al. Two overlapping peptides containing a 10 amino acid region (Glu574-Lys583) in the PLC-β2 Y box inhibited Gβγ-stimulated PLC-β2 activity *in vitro* and interacted with Gβγ dimers in crosslinking experiments (Sankaran et al., 1998). A second Gβγ binding site occurs within the PH domain of PLC-β2. Wang et al. generated a chimeric protein in which the PH domain of PLC-δ1 was replaced with the analogous PH domain of PLC-β2. The PLC-β2/δ1 chimera was activated by Gβγ subunits whereas wild-type PLC-δ1 was not, suggesting that the PH domain is needed for Gβγ binding and activation of PLC-β isozymes (Wang et al., 1999b; Wang et al., 2000).

3.6 Activation of PLC-β isozymes by small monomeric G proteins

In the late 1980's and 1990's, a role for heterotrimeric G proteins in regulating the activity of PLC-β isozymes was well established. In 1997, Gierschik and colleagues reported that PLC-β isozymes are also effectors for small monomeric G proteins, revealing that PLC isozymes coordinate signaling from several cellular inputs to create

diversity and complexity in phosphoinositide signaling (Illenberger et al., 1997). Illenberger et al. showed that soluble fractions of HL-60 granulocytes and bovine neutrophils contained a heterodimeric complex that stimulated recombinant PLC-β2 in the presence of GTP and non-hydrolyzable GTP analogues but not PLC-β1 or PLC-δ1 (Illenberger et al., 1997). Furthermore, a PLC-β2 mutant lacking the C-terminal region required for activation by Gα$_q$ was also activated in the presence of the soluble PLC-stimulating factor, suggesting that the soluble factor was not a member of the Gα$_q$ family and also binds to a distinct region on PLC-β2 to activate the enzyme (Illenberger et al., 1997). Amino acid sequencing and immunoblot analysis determined that the components of the heterodimeric complex were a Rho GTPase and a guanine nucleotide dissociation inhibitor, LyGDI (Illenberger et al., 1998). In a reconstitution experiment, PLC-β2 was directly activated by purified, recombinant Cdc42Hs but not RhoA. In addition, Rac1, Rac2, and Rac3 were also reported to directly stimulate PLC-β2 in a guanine nucleotide-dependent manner and are significantly more potent than Cdc42Hs (Illenberger et al., 1998; Illenberger et al., 2003a; Snyder et al., 2003). Rac GTPases also activate PLC-β1 and PLC-β3 although to a lesser extent and at a much lower potency (Snyder et al., 2003).

A three-dimensional crystal structure of PLC-β2 in a GTPγS-dependent complex with Rac1 was solved to determine the molecular mechanism of PLC-β activation by Rac GTPases (Jezyk et al., 2006). Rac1 binds exclusively to the PH domain of PLC-β2, confirming biochemical and cell biological data that Rac GTPases make no other contacts with the remaining regions of PLC-β2 (Illenberger et al., 2003a; Illenberger et al., 2003b). Similar to other small GTPases, Rac1 interacts with PLC-β2 entirely through its

switch regions and primarily makes hydrophobic contacts that are supported by hydrogen bonds and electrostatic interactions (Jezyk et al., 2006). Comparison of the PLC-β2·Rac1 structure to a structure of unbound PLC-β2 revealed that the isozyme undergoes little to no conformational change when bound to Rac1, and the residues in the PLC-β2 active site are in the same position regardless of Rac1 binding (Jezyk et al., 2006; Hicks et al., 2008). Collectively, these results suggest that active, membrane bound Rac GTPases enhance translocation of PLC-β isozymes to the membrane and position them in the correct orientation for phosphoinositide binding and hydrolysis (Jezyk et al., 2006; Hicks et al., 2008).

3.7 Research Goals

PLC-β isozymes hydrolyze PtdIns(4,5)P_2 to second messengers, diacylglycerol and Ins(1,4,5)P_3, in response to activation by $G\alpha_q$, $G\beta\gamma$, and Rac GTPases. In addition, PLC-β isozymes also accelerate GTP hydrolysis on their activating G proteins, and therefore, deactivate phospholipid signaling. Despite numerous studies examining regulation of PLC-β activity, the question remains: how do heterotrimeric G proteins activate PLC-β isozymes. To better understand the mechanisms of PLC-β activation, the Harden lab has recently generated a three-dimensional crystal structure of an AlF_4^--dependent complex of PLC-β3 and its activator $G\alpha_q$. The lab has identified three novel contacts within the PLC-β3·$G\alpha_q$ interface that we hypothesize are important for $G\alpha_q$ binding and GTPase accelerating activity of PLC-β3. The goal of this research is to use structure/function analysis of the PLC-β3·$G\alpha_q$ complex to understand the mechanisms of PLC-β activation by $G\alpha_q$ subunits and the mechanisms of PLC-β GAP activity. We will use mutational analysis to determine the importance of key residues and domains in the

PLC-β3·Gα_q interface and examine PLC activity in intact cells and directly using *in vitro* biochemical studies. The results from this work will further our knowledge of the molecular mechanisms required for the canonical Gα_q signaling pathway which couples activation of cell surface receptors to intracellular phospholipid signaling and various cellular responses.
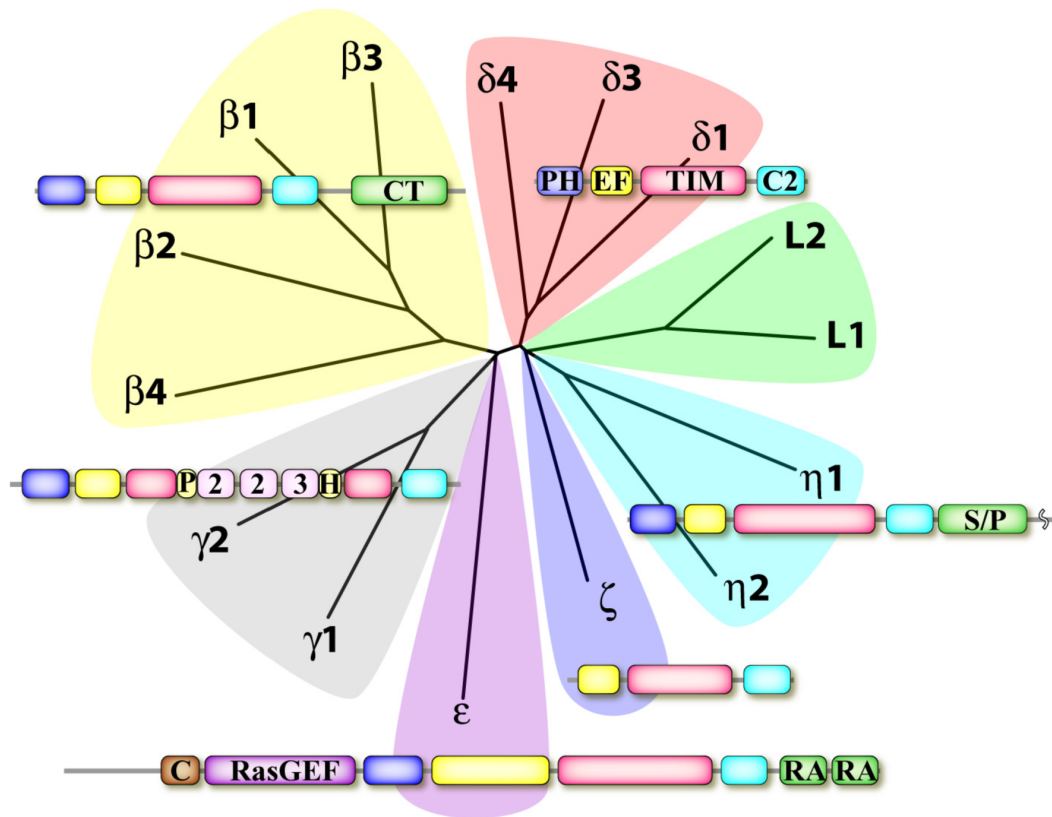
Figure 3.1. **Mammalian phospholipase C isozymes**. The mammalian genome encodes six major classes of PLC isozymes (PLC-β, -γ, -δ, -ε, -ζ, -η) and two catalytic dead PLC-like molecules (PLC-L1, -L2). PLC isozymes share the same core domain architecture exemplified in PLC-δ. PLC-δ isozymes contain a N-terminal PH domain, four tandem EF hands, a catalytic TIM barrel, and a C-terminal C2 domain. Other PLC isozymes have regulatory domains outside of this core that provide complexity in phospholipid signaling. PLC-β isozymes have a long C-terminal extension (CT) following the C2 domain. The C-terminal domain is reported to be important for membrane association, dimerization, and binding $G\alpha_q$. PLC-γ isozymes contain an additional split PH domain, two Src homology 2 (SH2) domains and an SH3 domain flanked by regions of the TIM barrel. Additional domains in PLC-ε include a N-terminal cysteine (C)-rich region that may be needed for membrane binding, a Ras guanine nucleotide exchange factor (RasGEF) domain, and two tandem Ras association (RA) domains. PLC-η isozymes contain a serine/proline (S/P)-rich region that has no known function. PLC-ζ isozymes are the simplest PLC in that they only consist of four EF hands, a TIM barrel, and a C2 domain. Figure is from Harden and Sondek, 2006.
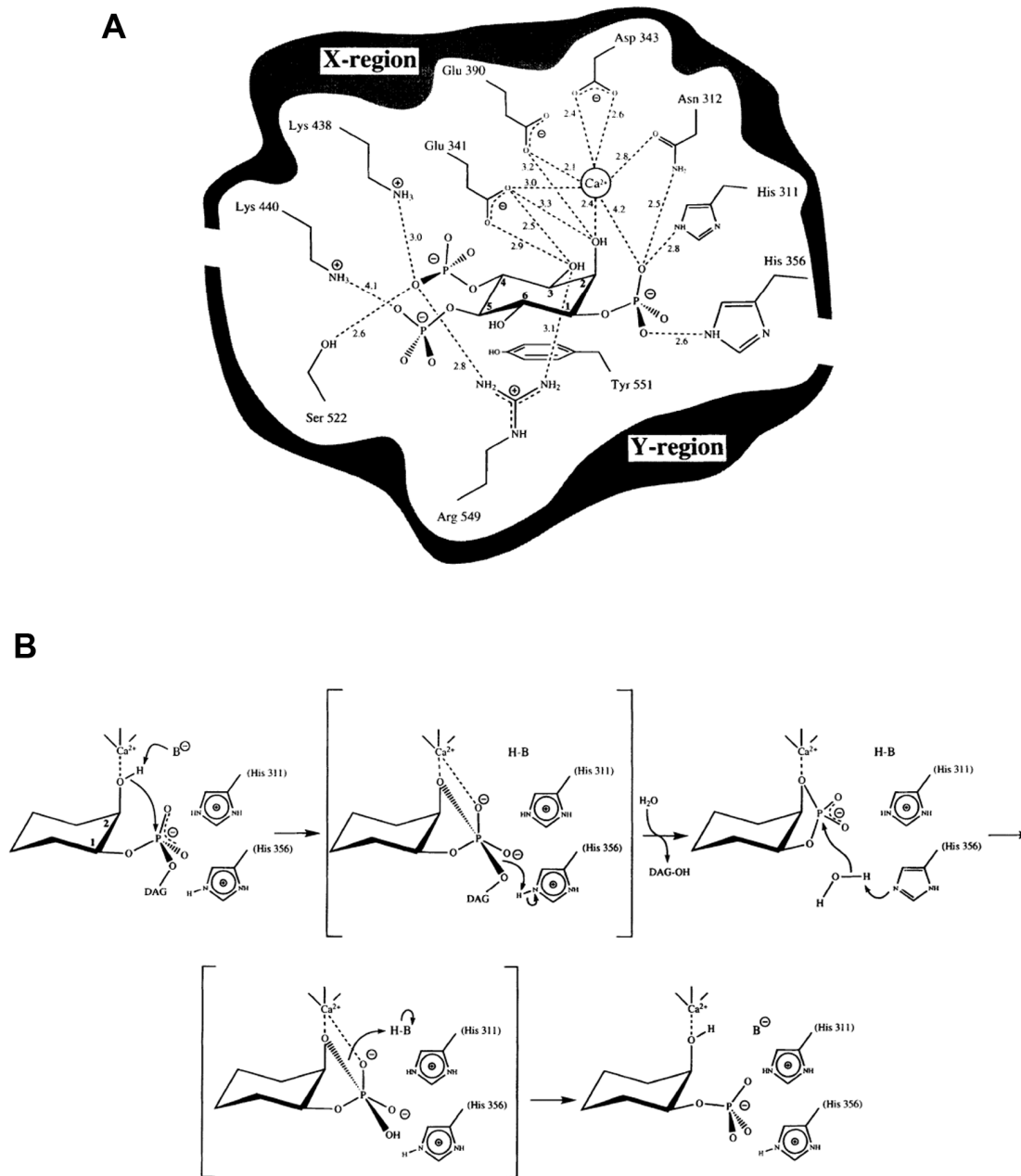
A

B

Figure 3.2. **Model of the catalytic domain of rat PLC-δ1.** (**A**) Amino acids and their side chains that interact with Ca^{2+} at the bottom of the active site and $Ins(1,4,5)P_3$ are depicted. (**B**) Proposed mechanism of PLC-catalyzed $PtdIns(4,5)P_2$ hydrolysis. See text for description of acid-base catalysis reaction. Figure is from Essen et al., 1996.
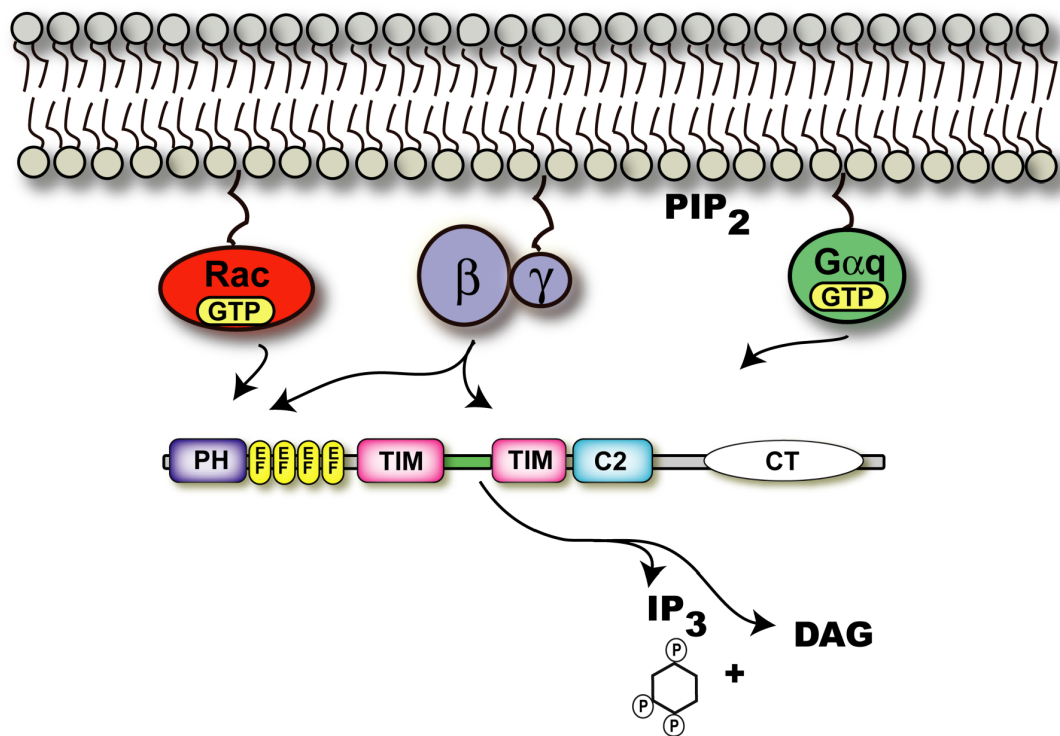
Figure 3.3. **Activators of PLC-β isozymes**. PLC-β isozymes bind to phospholipid membranes and hydrolyze PtdIns(4,5)P_2 (PIP$_2$) to Ins(1,4,5)P_3 (IP$_3$) and diacylglycerol (DAG). All PLC-β isozymes are stimulated by the Gα_q family of heterotrimeric G proteins. Historical data suggests that GTP-bound Gα_q binds to the basic C-terminal region of PLC-β isozymes and that this region is important for Gα_q-dependent activation. Gβγ dimers activate PLC-β2 and PLC-β3 and have been reported to bind to the PH domain and regions of the catalytic TIM barrel. Some PLC-β isozymes are also stimulated by GTP-bound Rac GTPases, which bind exclusively to the PH domain to recruit PLC-β isozymes to lipid bilayers and orient them for PIP$_2$ hydrolysis.
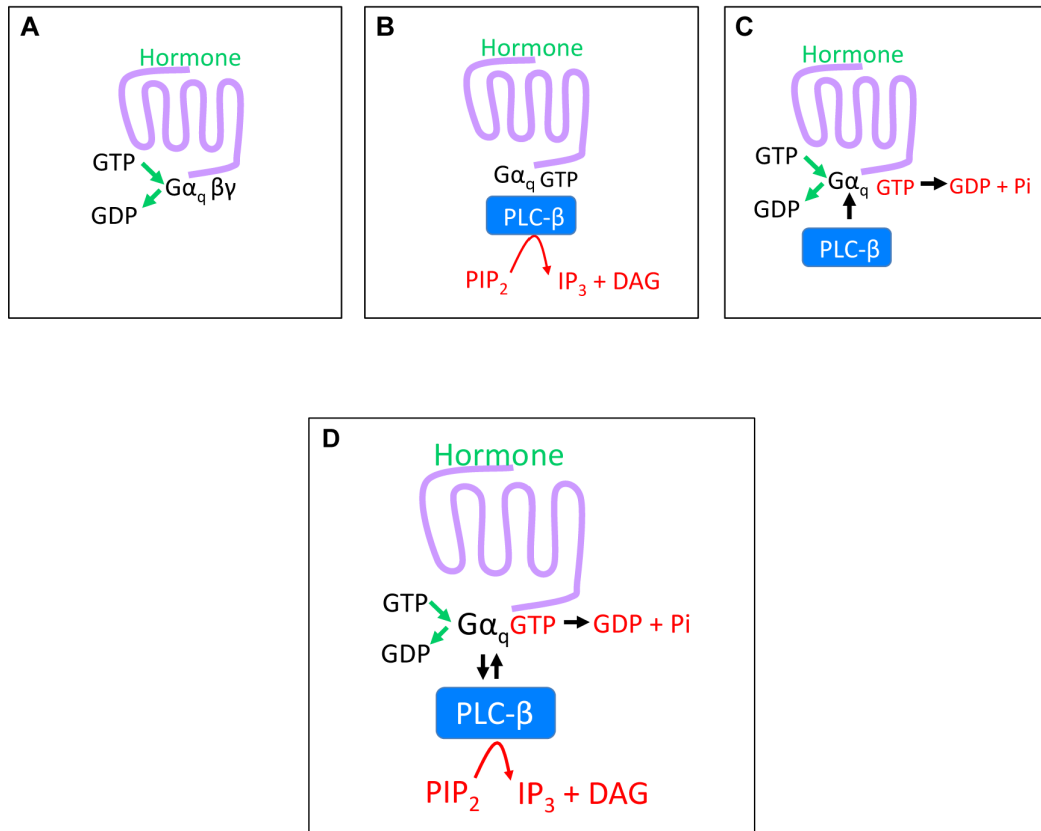
Figure 3.4. **Basic model of kinetic scaffolding**. (**A**) The GTPase cycle of heterotrimeric G proteins is a regulated balance of activation and deactivation steps. Hormone-bound GPCRs catalyze GDP for GTP exchange on $G\alpha_q$, causing dissociation of $G\beta\gamma$. (**B**) GTP-bound $G\alpha_q$ binds and activates PLC-β isozymes, which hydrolysis PIP_2 to IP_3 and DAG. (**C**) PLC-β isozymes also accelerate GTP hydrolysis on their activator $G\alpha_q$, and therefore, deactivate phospholipid signaling. (**D**) A model for kinetic scaffolding was proposed to reconcile how PLC-β signaling is sustained despite deactivating GTPase accelerating activity. In the kinetic scaffolding model, a stable complex of $G\alpha_q$, GPCR, and PLC-β is formed at steady-state in the presence of hormone. This is a consequence of GTP hydrolysis occurring much faster than the rate of GPCR and $G\alpha_q$ dissociation. Thus, hormone-bound receptor catalyzes many rounds of GDP/GTP exchange on $G\alpha_q$ and promotes many GTPase cycles before dissociating from $G\alpha_q$. Consequently, a fraction of GTP-bound $G\alpha_q$ is available despite the presence of PLC-β GTPase accelerating activity.

CHAPTER 4

MECHANISMS OF Gα$_q$-DEPENDENT ACTIVATION AND DEACTIVATION OF PHOSPHOLIPASE C-β

4.1 Abstract

Phospholipase C-β (PLC-β) isozymes hydrolyze PtdIns(4,5)P$_2$ to second messengers Ins(1,4,5)P$_3$ and diacylgylcerol in response to extracellular stimuli. PLC-β isozymes are regulated by all members of the G$_q$ family of heterotrimeric G proteins and Gβγ dimers. PLC-β isozymes also accelerate the intrinsic GTPase activity of Gα$_q$ subunits, and therefore, deactivate their activating G protein. Despite numerous studies investigating regulation of PLC isozymes by heterotrimeric G proteins, the mechanisms of Gα$_q$-promoted activation of PLC-β isozymes and termination of phospholipid signaling are not clearly understood. The Harden lab has generated a three-dimensional crystal structure of PLC-β3 in an AlF$^-_4$-dependent complex with Gα$_q$. The binding interface of the PLC-β3/Gα$_q$ complex contains three novel interactions. A 20 amino acid region following the C2 domain adopts a helix-turn-helix topology and engages the hydrophobic effector binding domain of Gα$_q$. Gα$_q$ makes additional electrostatic interaction with a small region preceding the C2 domain of PLC-β3. A third region of contact occurs between a short loop connecting the third and fourth EF hands of PLC-β3 and the nucleotide binding pocket of Gα$_q$. Deletion of the EF hand loop or single point mutations markedly impaired the GTPase accelerating activity of PLC-β3. Furthermore,

a GAP-deficient PLC-β3 mutant displayed a much slower rate of deactivation when agonist was removed. As a result, PLC activity was virtually unchanged compared to the rapid termination of wildtype PLC-β3 signaling. Thus, our studies define the essential domains within the PLC-β3/Gα$_q$ interface that are required for Gα$_q$-stimulated activation of PLC-β isozymes and deactivation of phospholipid signaling.

4.2 Introduction

Phospholipase C (PLC) isozymes hydrolyze PtdIns(4,5)P$_2$ to second messengers Ins(1,4,5)P$_3$ and diacylglycerol (DAG) in response to various extracellular stimuli including hormones, peptides, and proteases (Rhee, 2001; Harden and Sondek, 2006). Soluble Ins(1,4,5)P$_3$ binds to receptors on the endoplasmic reticulum to mobilize intracellular calcium, and membrane-bound DAG and calcium activate protein kinase C. Generation of these second messengers and activation of downstream signaling pathways leads to various cellular responses including cell growth, proliferation, differentiation, contraction, and secretion.

The four mammalian PLC-β isozymes (PLC-β1, -β2, -β3, -β4) have the same core domain architecture observed in other PLC isozymes (PLC-γ, -δ, -ε, -ζ, and -η) encoded in the mammalian genome (Rhee, 2001; Harden and Sondek, 2006). PLC-β isozymes contain an N-terminal PH domain, four EF hands, a catalytic TIM barrel, and a C2 domain. PLC-β isozymes also have a long C-terminal region following the C2 domain not present in other PLC isoforms. PLC-β isozymes are regulated downstream of G protein-coupled receptors through direct interaction with GTP-bound Gα$_q$ and Gβγ dimers (Pang and Sternweis, 1990; Smrcka et al., 1991; Taylor et al., 1991; Taylor and Exton, 1991; Waldo et al., 1991; Boyer et al., 1992; Camps et al., 1992; Katz et al.,

1992). Historical data suggests that $G\alpha_q$ engages the long C-terminal extension of PLC-β for $G\alpha_q$-dependent activation (Blank et al., 1993; Park et al., 1993b; Wu et al., 1993). A proteolytic fragment of PLC-β1 lacking the C-terminal domain was no longer activated by $G\alpha_q$ and AlF_4^- but retained activation by Gβγ (Park et al., 1993b). Sequential deletion of the C-terminal region of PLC-β1 suggested that residues 903-1030 were needed for membrane association and residues 1030-1142 were required for binding to and activation by $G\alpha_q$ (Wu et al., 1993). In addition, the C2 domains of PLC-β isozymes were also implicated in binding $G\alpha_q$. Purified C2 domains of PLC-β1 and PLC-β2 were found to bind selectively to $G\alpha_q$ in a GTPγS-dependent manner but not to Gβγ or $G\alpha_i$ (Wang et al., 1999a).

PLC-β isozymes were the first GTPase activating proteins (GAPs) identified for heterotrimeric G proteins and greatly accelerate the intrinsic GTPase activity of $G\alpha_q$ (Berstein et al., 1992). Thus, PLC-β isozymes are not only effector molecules of $G\alpha_q$ but also act as GAPs on their activating G protein, converting $G\alpha_q$ to its inactive GDP-bound state. GAP activity of PLC-β1 was first demonstrated using purified proteins in a reconstitution system (Berstein et al., 1992). Purified M1 mAChR, $G\alpha_q$, and $G\beta_1\gamma_2$ were reconstituted into phospholipid vesicles, and addition of carbachol and PLC-β1 greatly increased GTP hydrolysis up to 1000-fold (Berstein et al., 1992; Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999). Studies examining the kinetics of PLC-β1 activation and PLC-β1-promoted GAP activity have provided some insight into the molecular consequences of a molecule harboring an effector and GAP domain that both activates and terminates signaling. Ross and colleagues found that under steady-state conditions, addition of PLC-β1 accelerated GTP hydrolysis on $G\alpha_q$ and also increased the rate of

receptor-promoted GTP exchange (Mukhopadhyay and Ross, 1999). The authors proposed that a fraction of GTP-bound $G\alpha_q$ was maintained for activating PLC-β1 isozymes as a consequence of a stable complex of receptor, $G\alpha_q$, and PLC-β1. This stable complex is maintained at steady-state because GTP hydrolysis occurs much faster than the rate of receptor and $G\alpha_q$ dissociation, and hormone-bound receptor can quickly catalyze reactivation of $G\alpha_q$. In the absence of agonist, PLC-β1 rapidly accelerates GTP hydrolysis and terminates phospholipid signaling.

Towards better understanding the mechanisms of $G\alpha_q$-dependent PLC-β activation and PLC-β GAP activity, we present a three-dimensional crystal structure of PLC-β3 in an AlF_4^--dependent complex with its activator $G\alpha_q$. We have identified three novel interactions in the PLC-β3/$G\alpha_q$ binding interface. PLC-β3 engages the highly conserved effector binding domain of $G\alpha_q$ using a small kinked α-helical region following the C2 domain. PLC-β3 makes additional contacts with $G\alpha_q$ through charged residues in a region preceding the C2 domain. In addition, a short loop between the third and fourth EF hands of PLC-β3 interacts with the nucleotide binding pocket of $G\alpha_q$ and is required for stimulating the intrinsic GTPase activity on $G\alpha_q$. When PLC activity was measured, GAP-deficient PLC-β3 exhibited a much slower rate of deactivation when agonist was removed because its activating G protein was still GTP bound. These results extend our knowledge of the complex dynamics of the $G\alpha_q$/PLC-β signaling axis and the regulatory mechanisms of PLC-β activation and deactivation.

4.3 Materials and Methods

Reagents and Antibodies

Myo-[³H]inositol was purchased from American Radiolabeled Chemicals (St. Louis, MO). [*Inositol*-2-³H]phosphatidylinositol 4,5-bisphosphate substrate and [γ-³²P]GTP were purchased from Perkin-Elmer (Boston, MA). Brain L-α-phosphatidylinositol 4,5-bisphosphate and bovine liver L-α-phosphatidylethanolamine were from Avanti Polar Lipids (Alabaster, AL). Inositol-free Dulbecco's Modified Eagle's medium (DMEM) was from ICN Biochemicals (Costa Mesa, CA). Polyclonal rabbit anti-PLC-β1, PLC-β3, and PLC-δ1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal anti-β-actin antibody was from Sigma-Aldrich (St. Louis, MO).

Mutagenesis and Cloning

To generate mutations across the helix-turn-helix and EF hand loop of PLC-β3, small DNA cassettes containing the desired mutations were inserted into PLC-β3 cDNA (Fig 4.1, A and B). For helix-turn-helix mutants, silent mutations were first introduced into PLC-β3 cDNA to add unique HindIII and XbaI sites to the 5′ and 3′ ends of a ~550 bp region of PLC-β3 (Fig. 4.1A). This region encompasses the helix-turn-helix domain of PLC-β3. To generate DNA cassettes, PCR was used to amplify the same ~550 bp region in PLC-β3 with a forward primer containing tandem 5′ KpnI and HindIII sites and a reverse primer containing a 3′ XbaI site (Fig. 4.1C). The PCR fragments were digested using KpnI and XbaI restriction enzymes and ligated into a pBS SK vector. QuikChange mutagenesis was used to introduce single point mutations into the helix-turn-helix region, and the mutated cassettes were then digested with HindIII and XbaI restriction enzymes and ligated into the modified PLC-β3 plasmid. To generate mutations in the EF hand of PLC-β3, a similar strategy was employed except that unique ClaI and BsrGI sites were

added to the 5′ and 3′ ends of a ~400 bp region of the PLC-β3 EF hand (Fig. 4.1B). To generate EF hand DNA cassettes, PCR was used to amplify the same ~400 bp region with a forward primer containing a 5′ ClaI site and a reverse primer containing tandem 3′ BsrGI and BamHI sites (Fig. 4.1D). After digesting with ClaI and BamHI restriction enzymes and ligating into pBS SK vector, the DNA cassettes were mutated, digested with ClaI and BsrGI, and ligated into the modified PLC-β3 plasmid.

All other mutations of PLC-β isozymes were generated using QuikChange mutagenesis according to the manufacturer's protocol. All mutations were confirmed by dideoxy sequencing.

Quantification of Phospholipase C activity

COS-7 cells were plated at 6.5 x 10^4 cells per well in a 12-well plate and grown overnight in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C and 10% CO_2/90% air. Cells were transfected with mammalian expression plasmids and empty vector equaling 700 ng total DNA using Fugene6 (Roche) transfection reagent according to manufacturer's protocol. After incubating for 24 h, culture medium was removed and replaced with inositol-free DMEM containing 1 μCi/ml *myo*-[^{3}H]inositol to metabolically label membrane phospholipids. Cells were incubated overnight and then treated for 1 h at 37°C with 10 mM LiCl to inhibit inositol monophosphatases. The reaction was stopped by removing culture medium, adding cold 50 mM formic acid, and neutralizing with 150 mM ammonium hydroxide. Accumulation of [^{3}H]inositol phosphates were quantified by anion exchange chromatography and scintillation counting.

Immunoblotting

COS-7 cells were transfected as described above and lysed on ice with 200 μl RIPA buffer. Cell lysates were scraped into microcentrifuge tubes and centrifuged at 8,000 x g for 10 min at 4°C. The supernatant was transferred to a microcentrifuge tube containing 2X sample buffer and boiled at 100°C. Cell lysates were subject to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 3% bovine serum albumin in TBS-Tween before incubating with the appropriate primary and secondary antibodies.

In Vitro GTPase assay

Purified P2Y$_1$-R, and lipidated Gα_q and G$\beta_1\gamma_2$ were reconstituted into phospholipid vesicles as previously described (Cunningham et al. 2001; Waldo and Harden 2004). Briefly, 15 pmol purified P2Y$_1$-R, 50 pmol Gα_q, and 150 pmol G$\beta_1\gamma_2$ were reconstituted into proteoliposomes using Sephadex G-50 chromatography. Phospholipid vesicles consisted of L-α-phosphatidylethanolamine from liver, L-α-phosphatidylserine from brain, and cholesteryl hemisuccinate. Wildtype and mutant PLC-β3 were reconstituted into proteoliposomes, and GTPase activity was quantified by measuring hydrolysis of [γ-^{32}P]GTP in the presence of 10 μM P2Y$_1$-R agonist 2MeSADP. The reaction mixture contained 20 mM HEPES, pH 8.0, 50 mM NaCl, 2 mM MgCl$_2$, 1 mM EDTA, and 2 μM [γ-^{32}P]GTP (~4500 cpm/pmol) in a final volume of 50 μl. Proteoliposomes were incubated for 30 min at 30°C, and the reaction was stopped with 950 μl cold 5% activated charcoal in 20 mM H$_3$PO$_4$. Soluble [^{32}P]Pi was quantified by scintillation counting.

In Vitro Phosphoinositide Hydrolysis

Purified P2Y$_1$-R and lipidated Gα_q and G$\beta_1\gamma_2$ were reconstituted into proteoliposomes containing 50 μM [^{3}H]PtdIns(4,5)P$_2$ as described above for the *in vitro* GTPase assay. The reaction mixture (60 μl final volume) contained 20 mM HEPES, pH 7.4, 70 mM KCl, 3 mM EGTA, 2mM dithiothreitol, 0.16 mg/ml fatty acid-free bovine serum albumin, 10 μM free calcium, and 0.03% sodium cholate. The reaction mixture was centrifuged, and accumulation of soluble [^{3}H]inositol phosphates were quantified by scintillation counting.

4.4 Results

Structure of PLC-β3 in an AlF$^-_4$-dependent complex with Gα_q

The six major isoforms of the PLC family share the same core domain architecture, consisting of an N-terminal PH domain, four tandem EF hands, a catalytic TIM barrel, and a C2 domain. PLC-β isozymes are unique in that they also contain a long C-terminal extension following the C2 domain (Fig. 4.2A). Previous studies have reported that the C-terminal extension is needed for membrane association and docking of Gα_q subunits for PLC-β activation (Blank et al., 1993; Park et al., 1993b; Wu et al., 1993). In initial studies, however, the Harden lab found that a truncated mutant of PLC-β3 (PLC-β3 ΔCT), lacking ~350 amino acids of the C-terminal region, still formed a high affinity complex with Gα_q. Both full-length PLC-β3 and PLC-β3 ΔCT have essentially the same affinity for Gα_q by surface plasmon resonance analysis, suggesting that the C-terminal region is dispensable for high affinity binding to Gα_q (data not shown). Based on these observations, the Harden lab solved a three-dimensional crystal structure of the PLC-β3 ΔCT·Gα_q complex at a resolution of 2.7Å (Fig. 4.2B and Table 4.1).

Gα_q engages PLC-β3 at three distinct interfaces (Fig. 4.2B). The primary region of contact is observed between the hydrophobic effector binding domain of Gα_q and a 20 amino acid extension (residues 852-870) following the C2 domain of PLC-β3 (Fig. 4.2B). The C2 domain extension forms a ~70° helix-turn-helix that packs in between the α2 helix (Switch II) and α3 helix of Gα_q. This PLC-β3/Gα_q binding interface consists mostly of nonpolar contacts and is reminiscent of interactions between other Gα subunits and their effectors, including interactions between Gα_q and its effectors, GRK2 and p63RhoGEF (Tesmer et al., 1997b; Slep et al., 2001; Tesmer et al., 2005; Lutz et al., 2007). A second region of contact precedes the C2 domain and contains several charged residues that form salt bridges with amino acids in Gα_q (Fig. 4.2B). The third region of contact consists of a loop between the third and fourth EF hands of PLC-β3 and the nucleotide binding site of Gα_q, implicating the EF hand region of PLC-β3 in facilitating GTPase accelerating activity on Gα_q (Fig. 4.2B).

Comparison of the Gα_q interaction domain of PLC-β3 with another Gα_q effector, p63RhoGEF, (Lutz et al., 2007) reveals a similar helix-turn-helix domain binding in the hydrophobic effector binding pocket of Gα_q (Fig. 4.3). The PH domain extension of p63RhoGEF forms an α-helix that adopts a ~70° angle and lies between the α2 and α3 helices of Gα_q similar to the C2 domain extension of PLC-β3 (Fig 4.3). In biochemical studies of p63RhoGEF, mutation of five specific residues, F471, L472, L475, P478, and I479, prevented binding to Gα_q and abolished Gα_q-stimulated exchange activity *in vitro* but had no effect on basal activity (Lutz et al., 2007; Rojas et al., 2007). These hydrophobic residues form nonpolar interactions with Gα_q and are required for high affinity binding (Lutz et al., 2007). Sequence alignment of the helix-turn-helix of

p63RhoGEF with that of PLC-β3 and other PLC-β isozymes highlights conservation of key amino acids required for effector binding in the hydrophobic pocket of $G\alpha_q$ (Fig 4.3, C and D). Based on structural and biochemical data of the p63RhoGEF·$G\alpha_q$ complex, we hypothesized that the corresponding residues in PLC-β3 are also required for high affinity $G\alpha_q$ binding and activation of PLC-β3.

Quantification of PLC-β activity

Residue L859 of PLC-β3 is completely conserved in all PLC-β isoforms and in the helix-turn-helix domain of p63RhoGEF (Fig. 4.3, C and D). In the crystal structure of PLC-β3 ΔCT·$G\alpha_q$, L859 docks into the hydrophobic cleft formed between the α2 and α3 helices of $G\alpha_q$, making nonpolar interactions with L254, I258, and Y261 of $G\alpha_q$ (Fig. 4.6, B and C). Mutation of the corresponding Leu residue in p63RhoGEF completely abrogates binding to $G\alpha_q$ and $G\alpha_q$-stimulated exchange activity (Lutz et al., 2007; Rojas et al., 2007). To examine the importance of L859 in $G\alpha_q$-stimulated PLC activity, COS-7 cells were cotransfected with $G\alpha_q$ and varying concentrations of wildtype or mutant PLC-β3 DNA, and accumulation of $[^3H]$inositol phosphates was quantified (Fig. 4.4). Expression of $G\alpha_q$ or PLC-β3 alone resulted in a negligible increase in phosphoinositide hydrolysis compared to empty vector control. As expected, expression of $G\alpha_q$ greatly stimulated wildtype PLC-β3 activity at all DNA concentrations tested (Fig. 4.4B). In contrast, the PLC-β3(L859E) mutant was completely impaired in $G\alpha_q$-stimulated PLC activity (Fig. 4.4B). Comparable levels of protein expression were observed for both wildtype PLC-β3 and the PLC-β3(L859E) mutant. The deficiency in PLC activity was not a consequence of misfolding or disruption of the catalytic domain because $G\beta_1\gamma_2$ dimers activated both wildtype PLC-β3 and the PLC-β3(L859E) mutant to a similar

extent over a broad range of DNA concentrations (Fig. 4.4). Gβγ dimers engage regions of PLC-β isozymes that are distinct from Gα$_q$ binding, and therefore, still interact with PLC-β3 to activate the enzyme despite mutation of the helix-turn-helix (Kuang et al., 1996; Sankaran et al., 1998; Wang et al., 1999b; Wang et al., 2000). These results suggest that the PLC-β3(L859E) mutant is specifically impaired in Gα$_q$-stimulated activity, and the conserved leucine residue in the helix-turn-helix is required for Gα$_q$ binding (Fig. 4.3D and 4.4).

PLC-β3 and PLC-β1 have a similar binding affinity for Gα$_q$ and exhibit similar efficacies *in vitro* (Hepler et al., 1993; Jhon et al., 1993; Smrcka and Sternweis, 1993). To determine if the corresponding leucine residue is also required for Gα$_q$ activation of another PLC-β isoform, PLC activity was also quantified for PLC-β1(L810E). Similar to the PLC-β3 mutant, Gα$_q$ failed to activate the PLC-β1(L810E) mutant despite similar amounts of protein expression for both wildtype and mutant PLC-β1 at all DNA concentrations tested (Fig. 4.5). Thus, the highly conserved leucine residue within the helix-turn-helix of PLC-β3 is essential for Gα$_q$-stimulated activity of PLC-β isozymes as well as the Gα$_q$ effector, p63RhoGEF (Lutz et al., 2007; Rojas et al., 2007).

To examine the importance of the remaining residues in the helix-turn-helix of PLC-β3 in Gα$_q$-stimulated PLC activity, PLC-β3 was mutated at single amino acids and cotransfected with Gα$_q$ or Gβ$_1$γ$_2$ into COS-7 cells before measuring accumulation of [^{3}H]inositol phosphates (Fig. 4.6). Cells transfected with Gα$_q$ or PLC-β3 alone exhibited little increase in [^{3}H]inositol phosphate accumulation over cells transfected with empty vector. Mutation of Y855, L859, N861, P862, and I863 in PLC-β3 resulted in a significant loss of Gα$_q$-promoted PLC activity whereas Gβ$_1$γ$_2$ activated each of these

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mutants similar to wildtype PLC-β3 (Fig. 4.6A). Residues C-terminal to I863 in the second α-helix were also slightly impaired in $G\alpha_q$-stimulated activity (Fig. 4.6A). Immunoblot analysis showed similar protein expression for wildtype and each mutant PLC-β3 tested in the presence of $G\alpha_q$ and $G\beta_1\gamma_2$ (Fig. 4.6A). As shown in Figure 4.6B, Y855 makes extensive contacts with Y261, P262, and W263 in the α3-helix of $G\alpha_q$. Residue P862 facilitates the turn between the two α-helices in PLC-β3, and this turn is supported by N861, which makes hydrogen bonds with the peptide backbone. Alanine substitution at either of these residues would disrupt the conformation of the $G\alpha_q$ binding domain in PLC-β3. Collectively, these results indicate that the primary residues for $G\alpha_q$ binding and stabilizing the helix-turn-helix domain are in the first α-helix of PLC-β3, and the remaining residues in the second α-helix further facilitate PLC-β3 binding to $G\alpha_q$ (Fig. 4.6).

Inspection of the PLC-β3·$G\alpha_q$ interface reveals additional contact sites between $G\alpha_q$ and a region before the C2 domain of PLC-β3 (Fig. 4.2 and 4.7). These PLC-β3 residues (R707, D709, K710, and D721) form salt bridges with amino acids in $G\alpha_q$ (Fig. 4.7B). Specifically, R707 and K710 make electrostatic interactions with E191 in Switch I of $G\alpha_q$ (Fig. 4.7B). In addition, D709 and D721 in PLC-β3 form salt bridges with R202 and K41 in $G\alpha_q$, respectively (Fig. 4.7B). To examine whether these four charged residues are also required for $G\alpha_q$ activation of PLC-β3, single alanine mutations were generated in PLC-β3, and the mutants were tested for $G\alpha_q$-stimulated PLC activity in COS-7 cells (Fig. 4.7A). Mutation of each charged residue significantly impaired PLC activation by $G\alpha_q$ but not by $G\beta_1\gamma_2$. Similar amounts of protein expression were detected for wildtype PLC-β3 and each PLC-β3 mutant. Multiple sequence alignment of PLC-β

isozymes shows that these charged residues are completely conserved, suggesting that the electrostatic interactions between the C2 domain and $G\alpha_q$ are necessary for $G\alpha_q$ binding and activation of PLC-β isozymes (Fig. 4.7C).

EF hand loop of PLC-β3 accelerates GTP hydrolysis of $G\alpha_q$

PLC-β isozymes were the first GTPase activating proteins identified for heterotrimeric G proteins and greatly accelerate the intrinsic GTP hydrolysis of $G\alpha_q$ (Berstein et al., 1992). Thus, PLC-β isozymes are not only stimulated by $G\alpha_q$ but also act as GAPs on their activating G protein. Analysis of the PLC-β3 ΔCT·$G\alpha_q$ complex reveals that a loop between the third and fourth EF hands of PLC-β3 interacts with the guanine nucleotide binding site of $G\alpha_q$ and may mediate PLC-β3 GAP activity (Fig. 4.2B). Multiple sequence alignment of PLC-β isoforms and PLC-δ1 reveals that this region contains an eight amino acid insert that is not present in PLC-δ1 or other PLC family members (Fig. 4.8, data not shown). As shown in Figure 4.9, a conserved Asn residue in PLC-β3 interacts with Q209 in Switch II, a highly conserved amino acid in Gα subunits required for polarizing the nucleophilic water for GTP hydrolysis. Hydrogen bonding occurs between the side chain nitrogen of N260 and the carbonyl oxygen of Q209 and stabilizes the GTP transition state of $G\alpha_q$ (Fig. 4.9B). Therefore, we hypothesized that the EF hand loop is required for accelerating GTP hydrolysis on $G\alpha_q$.

Several PLC-β3 mutants were generated to determine the importance of the EF hand loop in accelerating GTP hydrolysis. Single point mutations were introduced at N260 in PLC-β3. An additional PLC-β3 mutant contained the EF hand loop of PLC-δ1 in place of the longer PLC-β3 loop. This mutant was designated PLC-β3 δEF. In collaboration with Gary Waldo, a phospholipid vesicle assay was used in which purified

P2Y$_1$ receiver (P2Y$_1$-R), Gα_q and G$\beta_1\gamma_2$ subunits were reconstituted into phospholipid vesicles. Purified wildtype or mutant PLC-β3 was added to proteoliposomes, and GTPase activity was quantified by measuring hydrolysis of [γ-^{32}P]GTP in the presence of the P2Y$_1$-R agonist, 2MeSADP (Fig. 4.10). As expected, addition of 2MeSADP and increasing concentrations of wildtype PLC-β3 robustly stimulated GTP hydrolysis with a potency of ~3 nM (Fig. 4.10A). In contrast, PLC-β3 δEF was markedly impaired in stimulating agonist-induced GTPase activity (Fig. 4.10A). Alanine, glycine, and serine substitutions at N260 also inhibited PLC-β3-promoted GTPase activity whereas mutating V262 had no effect (Fig. 4.10A). Inspection of the EC50 values for each PLC-β3 mutant showed that although these mutants are defective in accelerating GTP hydrolysis, there is no effect on the apparent affinity, indicating that the reduction in PLC-β3-promoted GAP activity is not a consequence of decreased binding to Gα_q (Fig. 4.10B).

GAP-deficient PLC-β3 displays a much slower rate of deactivation in the presence of P2Y$_1$-R antagonist

Activation and deactivation of heterotrimeric G proteins occurs through a series of tightly regulated catalytic steps. Ligand-bound receptors catalyze GTP exchange to activate G proteins, and GTP-bound Gα subunits and G$\beta\gamma$ dimers bind and activate downstream effectors. GAP proteins accelerate the intrinsic GTP hydrolysis of the Gα subunit to convert the enzymes to their GDP-bound state. When agonist is removed, the entire catalytic cycle shuts off, and the Gα subunit remains in its inactive GDP-bound state. We hypothesize that a GAP-deficient PLC-β3 mutant will deactivate at a greatly reduced rate when agonist is removed because its activating G protein remains GTP-bound. To test this hypothesis, deactivation of Gα_q was first measured in a reconstitution

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assay in collaboration with Gary Waldo. Purified P2Y$_1$-R, Gα_q, and G$\beta_1\gamma_2$ were reconstituted into phospholipid vesicles. As show in Figure 4.11, little GTPase activity was observed in the presence of PLC-β3 alone. In contrast, addition of both 2MeSADP and PLC-β3 greatly accelerated steady-state GTP hydrolysis, and this effect was rapidly blocked with a saturating concentration of P2Y$_1$-R competitive antagonist MRS2500, reducing GTPase activity to basal levels (Fig. 4.11).

PLC activity of GAP-deficient PLC-β3 was quantified in [^{3}H]PtdIns(4,5)P$_2$-containing phospholipid vesicles reconstituted with purified P2Y$_1$-R, Gα_q, and G$\beta_1\gamma_2$. Addition of 2MeSADP activated P2Y$_1$-R to catalyze GTP exchange on Gα_q. PLC-β3 isozymes were subsequently added to proteoliposomes, and accumulation of [^{3}H]inositol phosphates was quantified (Fig. 4.12). Wildtype PLC-β3 containing vesicles displayed a significant increase in steady-state PtdIns(4,5)P$_2$ hydrolysis in the presence of 2MeSADP (Fig. 4.12A). Addition of antagonist rapidly inhibited this effect, reducing agonist-promoted PLC activity to basal levels. In phospholipid vesicles containing GAP-deficient PLC-β3(N260A) mutant, stimulation with 2MeSADP promoted PtdIns(4,5)P$_2$ hydrolysis similar to wildtype PLC-β3 (Fig. 4.12B). In contrast, antagonist addition had no effect on agonist-stimulated steady-state PLC activity, and the rate of [^{3}H]PtdIns(4,5)P$_2$ hydrolysis was virtually unchanged. These results illustrate that the GTPase accelerating activity of PLC-β isozymes is critical for rapid termination of PLC activity and maintaining the fidelity of phospholipid signaling.

We next examined PLC activity of GAP-deficient PLC-β3 mutants in intact cells. We hypothesized that loss of PLC-β3 GAP activity would prevent GTP hydrolysis, and therefore, retain Gα_q in an active GTP-bound state. Consequently, GTP-bound Gα_q

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would increase PLC activity compared to wildtype PLC-β3. To test this hypothesis, wildtype PLC-β3 and PLC-β3 δEF were cotransfected into COS-7 cells with Gα_q or G$\beta_1\gamma_2$, and accumulation of [^{3}H]inositol phosphates was measured after incubating with LiCl for 1h (Fig. 4.13). Expression of Gα_q greatly increased activity of wildtype PLC-β3 at all DNA concentrations tested. Interestingly, Gα_q stimulated activity of PLC-β3 δEF to a level similar to that of wildtype despite a loss of PLC-β3 GAP activity as shown in Figure 4.10A (Fig. 4.13A). As expected, expression of G$\beta_1\gamma_2$ resulted in activation of both wildtype PLC-β3 and PLC-β3 δEF over a broad range of DNA concentrations (Fig. 4.13B). Comparable expression of wildtype PLC-β3 and PLC-β3 δEF was confirmed by immunoblot analysis. Gα_q and G$\beta_1\gamma_2$ also stimulated activity of GAP-deficient PLC-β3(N260G) and PLC-β3(N260S) mutants similar to wildtype PLC-β3 (data not shown). These results suggest that loss of PLC-β3 GAP activity has no effect on the overall extent of PLC activity under the conditions tested. Alternatively, it is possible that this particular assay is not sensitive enough to measure differences in PLC activity between wildtype and GAP-deficient PLC-β3. Quantification of intracellular calcium is a more sensitive technique, and kinetic experiments are in progress to examine how loss of PLC-β3 GAP activity affects PLC-β3-promoted calcium release at various times.

The intact cell assays were performed in the absence of receptor stimulation, which may be needed to observe any steady-state increases in PLC activity and calcium mobilization when GAP activity is absent. Several attempts were made to develop an assay to measure receptor-promoted PLC activity in COS-7, HeLa, and HEK293 cells by stimulating various endogenous or recombinant GPCRs. Unfortunately, robust activation of endogenous PLC-β isozymes masked any effects of exogenous expression of wildtype

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and mutant PLC-β3 in these cell lines (data not shown). Experiments are currently underway to knockdown endogenous PLC-β isozymes in HEK293 cells using short hairpin RNAs (shRNAs) and to reintroduce siRNA resistant forms of GAP-deficient PLC-β3 mutants. Using these cell lines, we can better evaluate how loss of PLC-β3 GAP activity affects $G\alpha_q$-stimulated PLC activity and release of intracellular calcium. We can examine the rates of receptor-promoted signal initiation and termination as well as quantify signal magnitude and duration.

PLC-β3 engages both the conserved effector and RGS binding domains of $G\alpha_q$

Three-dimensional crystal structures have been solved for each of the four families of heterotrimeric G proteins in complex with their effectors (Posner et al., 1999; Slep et al., 2001; Chen et al., 2005; Tesmer et al., 2005; Lutz et al., 2007; Chen et al., 2008). Comparison of these structures shows that distinct effector proteins bind to the same localized region of Gα subunits. The α2 (Switch II) and α3 helices of each Gα subunit form a hydrophobic cleft, and effector proteins use nonpolar residues to dock between these two conserved helices (Fig. 4.14, A and C). RGS proteins also bind to a localized region of Gα subunits (Posner et al., 1999; Slep et al., 2001), making contacts with all three switch regions to stabilize the Gα backbone for GTP hydrolysis (Fig. 4.14, A and D). RGS proteins and effectors bind to distinct sites on Gα and can engage Gα subunits simultaneously to modulate signaling (McEntaffer et al., 1999; Skiba et al., 1999; Slep et al., 2001; Tesmer et al., 2005; Shankaranarayanan et al., 2008). PLC-β isozymes are both effectors and GAPs and engage the effector and RGS binding surface of $G\alpha_q$ (Fig. 4.14B). PLC-β3 and other G protein effectors share common Gα contact sites and bind to the same hydrophobic cleft of Gα. The EF hand loop of PLC-β3

promotes GTPase accelerating activity and interacts with Gα residues that are required for RGS binding (Fig. 4.14, B and D).

4.5 Discussion

In the present study, we have identified three novel contacts between PLC-β3 and Gα$_q$ based on analysis of a three-dimensional crystal structure of a C-terminally truncated form of PLC-β3 in an AlF$^-_4$-dependent complex with Gα$_q$. Previous biochemical and mutational analysis suggested that the long C-terminal region of PLC-β isozymes engages Gα$_q$ and is needed for PLC-β activation (Blank et al., 1993; Park et al., 1993b; Wu et al., 1993). A PLC-β1 fragment lacking the C-terminal region was impaired in Gα$_q$-promoted PLC activity but retained activation by Gβγ (Park et al., 1993b). Sequential deletion of this C-terminal portion suggested that residues 903-1030 are needed for membrane association and residues 1030-1142 contact Gα$_q$ for activation of PLC-β1 (Wu et al., 1993). Preliminary studies in the Harden lab, however, found that a PLC-β3 fragment truncated at residue 886 and missing the analogous C-terminal region still formed a high affinity complex with Gα$_q$, suggesting that this region is dispensable in Gα$_q$ binding. Inspection of the PLC-β3 ΔCT·Gα$_q$ structure revealed that a 20 amino acid region following the C2 domain is a major site of Gα$_q$ binding (Fig. 4.2). This region adopts a helix-turn-helix domain and docks in the hydrophobic pocket of Gα$_q$ between the α2 and α3 helices, forming nonpolar interactions with Gα$_q$ (Fig. 4.6B). Mutation of key residues (Y855, L859, N861, P862, I863) significantly decreased Gα$_q$-stimulated PLC activity but had no effect on activation by Gβγ subunits. Furthermore, mutation of the highly conserved Leu residue in PLC-β1 also prevented activation by Gα$_q$. Multiple sequence alignment of PLC-β isozymes revealed complete conservation

of L859 and P862 and partial conservation for the remaining residues, suggesting that PLC-β isozymes use the same mechanism to engage $G\alpha_q$ subunits for PLC activation (Fig. 4.4C). A second region of PLC-β3 preceding the C2 domain was also found to interact with $G\alpha_q$. Four charged residues (R707, D709, K710, D721) in the C2 domain make electrostatic interactions with amino acids in Switch I and the β1 and β3 strands of $G\alpha_q$ (Fig. 4.7B). Mutation of these four amino acids in PLC-β3 inhibits $G\alpha_q$-promoted PLC activity, and these charged residues are completely conserved in PLC-β isozymes from worms to mammals, suggesting that the electrostatic interactions between PLC-β and $G\alpha_q$ are required for PLC-β activation (Fig. 4.7).

Representative crystal structures from each heterotrimeric G protein family ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$) in complex with their effector molecules have provided insight into the interaction domains on $G\alpha$ subunits. These crystal structures include complexes of $G\alpha_s$·adenylyl cyclase, $G\alpha_t$·PDEγ·RGS9, $G\alpha_q$·GRK2, $G\alpha_q$·p63RhoGEF, $G\alpha_{13}$·p115RhoGEF, and $G\alpha_{13}$·PDZRhoGEF (Tesmer et al., 1997b; Slep et al., 2001; Chen et al., 2005; Tesmer et al., 2005; Lutz et al., 2007; Chen et al., 2008). Similar to other effector proteins, PLC-β3 binds the same conserved hydrophobic pocket of $G\alpha$ between the α2-helix of Switch II and α3-helix. These results confirm earlier mutational analysis demonstrating that the α3-helix of $G\alpha_q$ interacts with PLC-β1 (Arkinstall et al., 1995; Venkatakrishnan and Exton, 1996). Small synthetic peptides corresponding to the α3- and α4- helices of $G\alpha_q$ were shown to inhibit PLC-β activation by GTPγS-bound $G\alpha_q$ (Arkinstall et al., 1995). In a separate study, regions of constitutively activated $G\alpha_q$ were replaced with analogous regions of $G\alpha_s$ to determine the PLC-β binding domain (Venkatakrishnan and Exton, 1996). The α3 helix and α3/β5 linker of $G\alpha_q$ but not the

α4-helix were identified as regions needed for $G\alpha_q$ activation of PLC-β isozymes in agreement with the structure of PLC-β3 ΔCT·$G\alpha_q$ (Venkatakrishnan and Exton, 1996).

Structural and biochemical analysis also suggests that interactions occur between the α4/β6 loop of $G\alpha_s$ and adenylyl cyclase, $G\alpha_t$ and PDEγ, as well as $G\alpha_q$ and p63RhoGEF (Berlot and Bourne, 1992; Rarick et al., 1992; Tesmer et al., 1997b; Liu et al., 1999; Tesmer et al., 2005; Lutz et al., 2007). In addition, the C-terminal region of $G\alpha_q$ engages the DH domain of p63RhoGEF (Lutz et al., 2007). While the structure of PLC-β3 ΔCT·$G\alpha_q$ does not indicate contacts between PLC-β3 and the α4/β6 loop or the C-terminus of $G\alpha_q$, additional contacts may occur between $G\alpha_q$ and the C-terminal region of PLC-β3 deleted in this crystal structure. However, these potential interactions presumably would be less significant based on the high affinity binding of PLC-β3 ΔCT and $G\alpha_q$. Indeed, surface plasmon resonance studies showed that PLC-β3 ΔCT has virtually the same binding affinity for $G\alpha_q$ as full-length PLC-β3, suggesting that the C-terminal region of PLC-β3 is dispensable in $G\alpha_q$ binding.

PLC-β isozymes were the first GTPase activating proteins identified for heterotrimeric G proteins (Berstein et al., 1992). PLC-β isozymes are $G\alpha_q$-specific GAPs and accelerate GTP hydrolysis on GTP-bound $G\alpha_q$ by ~1000-fold (Berstein et al., 1992; Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999). C-terminal peptides of PLC-β1 displayed GAP activity and accelerated GTP hydrolysis approximately 4-fold, suggesting that a portion of the C-terminal region was required for PLC-β1 GAP activity (Paulssen et al., 1996). The authors concluded that the difference in GAP activity between full-length PLC-β1 and the C-terminal peptides was possibly due to the peptides

binding Gα_q at a much lower affinity or due to the requirement of other regions of PLC-β1 in promoting GTP hydrolysis.

We have identified a highly conserved loop between the third and fourth EF hands of PLC-β3 in accelerating PLC-β3 GAP activity. As shown in Figure 4.9, the EF hand loop is located near the nucleotide binding pocket, and N260 interacts with Q209 of Gα_q, positioning the residue for stabilizing nucleophilic water for GTP hydrolysis. The model shown in Figure 4.9B is analogous to the mechanism used by RGS proteins to accelerate GTP hydrolysis of heterotrimeric G proteins (Fig. 4.15). RGS proteins bind Gα subunits in the GTP-bound transition state and stabilize the flexible switch regions to accelerate the intrinsic GTP hydrolysis of Gα (Tesmer et al., 1997a; Slep et al., 2001). Comparison of Gα_i-bound RGS4 to Gα_q-bound PLC-β3 illustrates that the Asn residues are in the same location and position Q209 for stabilizing nucleophilic water for GTP hydrolysis. Mutation of N260 in PLC-β3 significantly impaired PLC-β3 GAP activity (Fig. 4.10). Furthermore, deletion of the entire EF hand loop and replacing it with the analogous region of PLC-δ1 also reduced PLC-β3 GAP activity whereas mutation of V262 had no effect (Fig. 4.10). While mutation of the EF hand loop prevented PLC-β3 GAP activity, no effect was observed on the apparent affinity for Gα_q. When PLC activity was quantified *in vitro*, GAP-deficient PLC-β3 deactivated much more slowly than wildtype PLC-β3 when agonist was removed because activated Gα_q was still GTP-bound. Consequently, the rate of [^{3}H]PtdIns(4,5)P$_2$ hydrolysis was largely unchanged (Fig. 4.12B). These results illustrate the importance of PLC-β GAP activity in accelerating signal termination when agonist is removed to maintain the fidelity of intracellular phospholipid signaling.

GAP proteins work to sharpen intracellular signaling in the presence of agonist (Ross, 2008). At steady-state, signal amplitude is maintained by a balance of receptor-catalyzed GTP binding on heterotrimeric G proteins and GAP-accelerated GTP hydrolysis. This balance allows for a fraction of Gα subunits to remain GTP-bound and active and provides a mechanism for rapid deactivation when agonist is removed. Using a phospholipid vesicle system, Ross and colleagues demonstrated this point for PLC-β1 (Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999). GTP hydrolysis or PLC activity was quantified for purified PLC-β1 reconstituted into proteoliposomes containing purified M1 mAChR and heterotrimeric G$_q$. Despite a rapid rate of GTP hydrolysis in the presence of agonist, sufficient GTP-bound Gα_q was available for activation of PLC-β1 at steady-state. This was a consequence of an increased rate of receptor-promoted GDP/GTP exchange in the presence of PLC-β1 (Mukhopadhyay and Ross, 1999; Turcotte et al., 2008). Similarly, Posner et al. also observed an increased rate in receptor-catalyzed exchange activity when RGS4 was added to phospholipid vesicles containing heterotrimeric G$_i$ and M2 mAChR (Posner et al., 1999).

Ross and colleagues proposed a model for kinetic scaffolding to explain how PLC-β1 activity is maintained despite continuous GAP activity (Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999). In this model, a stable complex of receptor, Gα, and GAP is formed at steady-state since GTP hydrolysis occurs much faster than the time needed for receptor and Gα to dissociate (Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999; Ross, 2008; Turcotte et al., 2008). Therefore, agonist-occupied receptor catalyzes multiple rounds of GDP/GTP exchange and promotes many GTPase cycles. In this way, GAP proteins indirectly increase G protein activation and accelerate

deactivation to sharpen signaling without attenuating the magnitude of signaling at steady-state (Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999; Ross, 2008).

Analysis of GAP-deficient PLC-β3 δEF in intact cells revealed that Gα$_q$ activated PLC-β3 δEF to the same extent as wildtype PLC-β3 despite the loss of deactivating GAP activity. In these experiments, Gα$_q$ and either wildtype PLC-β3 or PLC-β3 δEF are cotransfected into COS-7 cells, and accumulation of inositol phosphates is quantified after 1h LiCl treatment. Results from these experiments suggest that under these conditions loss of PLC-β3 GAP activity has no effect on overall accumulation of inositol phosphates. Future studies are underway to determine the effect of PLC-β3 GAP activity on the rate of receptor-promoted signal initiation and termination as well as signal amplitude and duration.

PLC-β isozymes were the first effector GAPs identified for heterotrimeric G proteins, and subsequent studies identified p115RhoGEF, a Gα$_{13}$-specific effector and GAP (Berstein et al., 1992; Kozasa et al., 1993). Structural comparison of PLC-β3 with known effector·Gα and RGS·Gα complexes revealed that PLC-β3 engages both the conserved effector and GAP binding domains of Gα$_q$, indicating a similar mechanism of binding to Gα subunits for proteins that contain these domains separately or as a single molecular unit (Fig. 4.14). It is not completely understood why an effector protein would evolve to terminate its own signaling. Based on kinetic analysis, an effector GAP may provide more efficient temporal control that would be lacking if additional proteins were required to bind and deactivate Gα subunits (Mukhopadhyay and Ross, 1999). In addition, as a GAP for heterotrimeric G$_q$, PLC-β isozymes specifically modulate phospholipid signaling pathways whereas RGS proteins regulate signaling for several G

protein pathways (Ross and Wilkie, 2000). Effector GAP proteins also ensure that signal transduction occurs before signaling is prematurely terminated (Ross and Wilkie, 2000). Effector GAPs require newly activated $G\alpha$ subunits to bind and activate a signaling pathway before deactivation proceeds (Ross and Wilkie, 2000). Therefore, in the absence of agonist, deactivation occurs rapidly upon effector binding to prevent basal signaling. If agonist is present, signaling is sustained by a balance of activation and deactivation steps in the GTPase cycle.

Analysis of the three-dimensional crystal structure of the PLC-$\beta3\Delta$CT·$G\alpha_q$ complex enhances our understanding of the molecular mechanisms of $G\alpha_q$-dependent activation and deactivation of PLC-β isozymes. Identification of regions specifically required for $G\alpha_q$-stimulated PLC activity will now allow us to interrogate the importance PLC-β activation by $G\alpha_q$ vs. $G\beta\gamma$ in cells and in *in vivo* models. In addition, we can determine the importance of GAP activity in regulating the rates of PLC-β activation and deactivation as well as the amplitude and duration of PLC-β signaling. Results from these studies will further our knowledge of the molecular mechanisms regulating the canonical PLC-β/$G\alpha_q$ signaling axis.

DATA COLLECTION	
Space group	C2
Cell dimensions	
a, b, c (Å)	204.93, 91.62, 92.78
α, β, γ (°)	90.00, 101.56, 90.00
Resolution (Å) (anisotropic)	50 - 2.38
Rsym	0.086
I/σ	21.2
Redundancy	4.4
REFINEMENT	
Resolution (Å)	2.68
Completeness for range	91%
No. reflections	40,874
Rwork/Rfree	0.20/0.26
No. atoms	8813
Mean B value	55.4
R.m.s. deviations:	
Bond length (Å)	0.02
Bond angles (°)	1.9

Table 4.1. **Data collection and current refinement statistics of PLC-β3 ΔCT·Gα_q structure**.
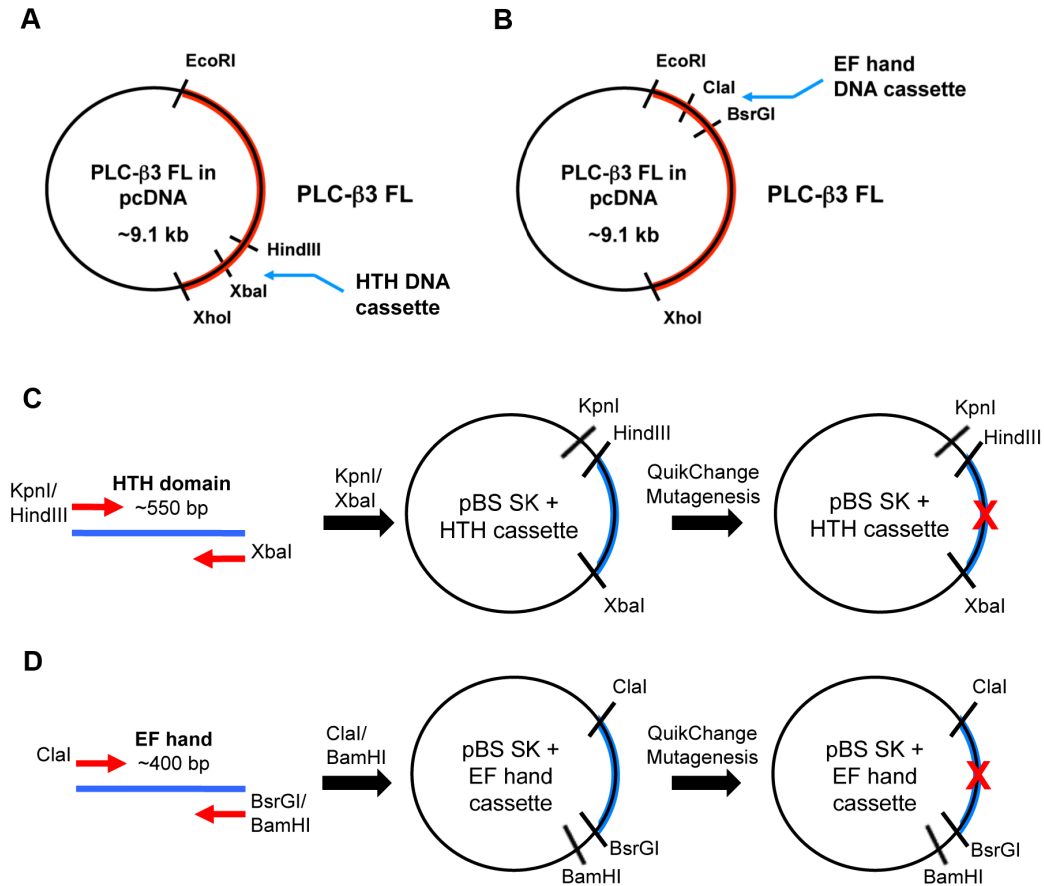
Figure 4.1. **Mutagenesis strategy**. (**A**) Using QuikChange mutagenesis, silent mutations were introduced into PLC-β3 cDNA to add HindIII and XbaI restriction sites to the 5′ and 3′ ends of a ~550 bp region. These restriction sites encompass the helix-turn-helix (HTH) region of PLC-β3. A ~550 bp DNA cassette of the HTH region containing the appropriate mutations was digested with HindIII and XbaI and ligated into the modified PLC-β3 plasmid. The PLC-β3 gene is cloned into pcDNA using EcoRI and XhoI restriction sites. (**B**) Mutations in the EF hands of PLC-β3 were made using a similar strategy. ClaI and BsrGI sites were introduced into PLC-β3 and enclose a region of ~400 bp. A DNA cassette consisting of the same region and desired mutations was digested with ClaI and BsrGI and ligated into PLC-β3. (**C**) To generate the DNA cassettes, the HTH region was PCR amplified using a forward primer containing tandem 5′ KpnI and HindIII sites and a reverse primer consisting of a 3′ XbaI sites. The PCR fragment was digested with KpnI and XbaI and ligated into pBS SK vector. The HTH cassette was mutated using QuikChange mutagenesis according to the manufacturer's protocol. (**D**) The EF hand was PCR amplified using a forward primer containing a 5′ ClaI site and a reverse primer consisting of tandem 3′ BsrGI and BamHI sites. The PCR product was digested with ClaI and BamHI, ligated into pBS SK, and mutated.
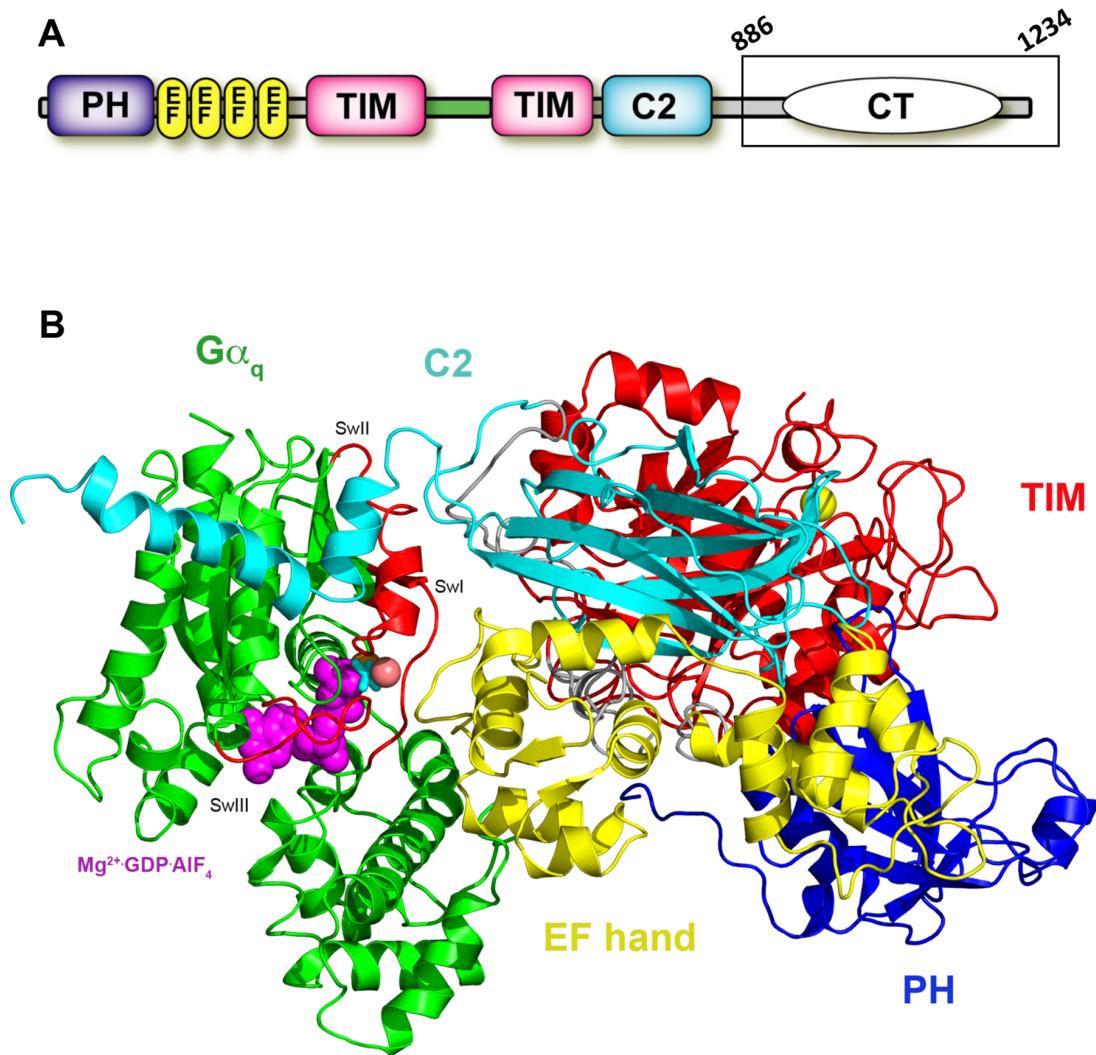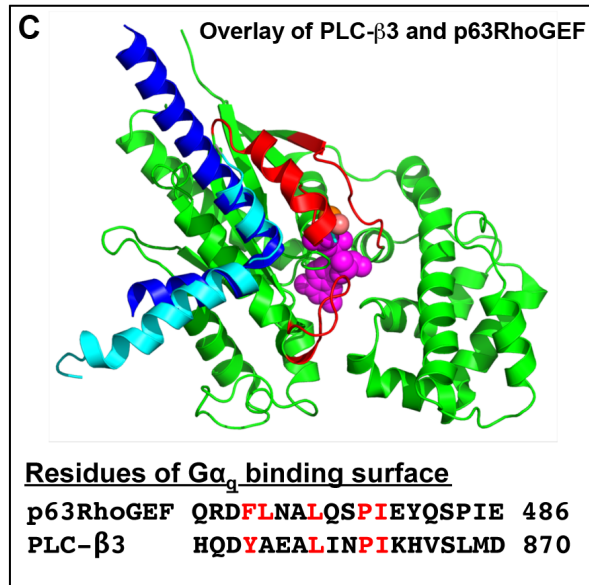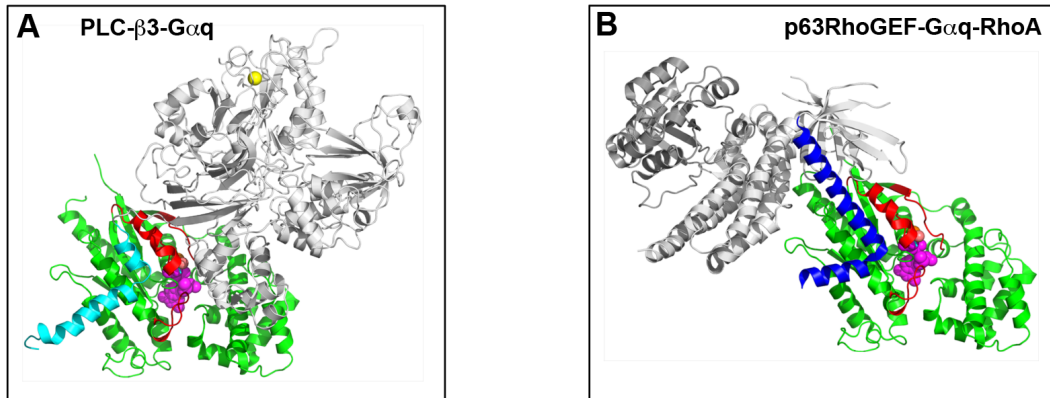
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Figure 4.2. **Three-dimensional crystal structure of PLC-β3 ΔCT in an AlF⁻₄-dependent complex with Gα_q.** (**A**) PLC-β isozymes contain an N-terminal PH domain (blue), four series of EF hands (yellow), a catalytic TIM barrel (red), a C2 domain (cyan), and a long C-terminal extension of ~400 amino acids (white). For generating the crystal structure, PLC-β3 was truncated to remove the entire C-terminal extension (residue 886-1234) shown in the box. (**B**) Gα_q interacts with PLC-β3 at three distinct interfaces, a 20 amino acid region following the C2 domain, a region before the C2 domain, and a short loop within the EF hands. The colors used in the domain diagram are the same as in the structure. Gα_q is shown in green, and the three switch regions (SwI, SwII, SwIII) are highlighted in red. GDP (magenta), Mg^{2+} (orange), and the catalytic water (salmon) are depicted as spheres and AlF⁻₄ is presented as sticks. The calcium ion in the active site of PLC-β3 is highlighted in yellow.

A PLC-β3-Gαq

B p63RhoGEF-Gαq-RhoA

C Overlay of PLC-β3 and p63RhoGEF

<u>Residues of Gα_q binding surface</u>

```
p63RhoGEF  QRDFLNALQSPIEYQSPIE  486
PLC-β3      HQDYAEALINPIKHVSLMD  870
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D

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     human PLC-β1  DYVPDTYADVIEALSNPIRYVNLMEQRAKQLAALTLEDEEE   837
    bovine PLC-β1  DYVPDTYADVIEALSNPIRYVNLMEQRAKQLAALTLEDEEE   837
       rat PLC-β1  DYVPDTYADVIEALSNPIRYVNLMEQRAKQLAALTLEDEEE   837
     mouse PLC-β1  DYVPDTYADVIEALSNPIRYVNLMEQRAKQLAALTLEDEEE   837
     human PLC-β2  DYIPGAWADLTVALANPIKFFSAHDTKSVKLKEAMGGLPEK   839
       rat PLC-β2  DYVPDTWADLTVALANPIKYFSAHDKKSVKLKEVTGSLPEK   847
     mouse PLC-β2  DYIPDTWADLTVALANPIKYFNAQDKKSVKLKGVTGSLPEK   844
     human PLC-β3  DYIPDDHQDYAEALINPIKHVSLMDQRARQLAALIGESEAQ   886
       rat PLC-β3  DYIPDDHQDYAEALINPIKHVSLMDQRAKQLAALIGESEAQ   885
     mouse PLC-β3  DYIPDDHQDYAEALINPIKHVSLMDQRAKQLAALIGESEAQ   887
      frog PLC-β   DYIPDDHQGYANALTNPIKHISLMDQRAKQLAALMGENEQP   872
     human PLC-β4  TYVPDGFGDIVDALSDPKKFLSITEKRADQMRAMGIETSDI   862
       rat PLC-β4  TYVPDGFGDIVDALSDPKKFLSITEKRADQLRAMGIETSDI   863
     mouse PLC-β4  TYVPDGFGDIVDALSDPKKFLSITEKRADQMRAMGIETSDI   862
 fruit fly PLC-β   IYVPDGFEDFMAMLSDPRGFAGAAKQQNEQMKALGIEE---   844
 c. elegans PLC-β  TYVPDELSGLVDALADPRAFLSEQKKRQEALAHMGVDDSDI  1055
```

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Figure 4.3. **Comparison of the binding interfaces of PLC-β3·Gα$_q$ and p63RhoGEF·Gα$_q$** (**A**) Structure of PLC-β3 in an AlF$_4^-$-dependent complex with Gα$_q$. C2 domain extension of PLC-β3 is highlighted in cyan and engages the α2 and α3 helices of Gα$_q$. The remaining regions of PLC-β3 are shaded in gray, and the calcium ion in the active site of PLC-β3 is depicted as a sphere (yellow). Gα$_q$ is shown in green, and the three switch regions are highlighted in red. GDP (magenta), Mg^{2+} (orange), water (salmon) are depicted has spheres and AlF$_4^-$ (cyan) is shown as sticks. (**B**) Crystal structure of p63RhoGEF in a nucleotide-dependent complex with Gα$_q$ and RhoA (Lutz et al., 2007). The PH domain extension of p63RhoGEF (blue) docks between the α2 and α3 helices of Gα$_q$. The remaining regions of p63RhoGEF and RhoA are shaded in gray. (**C**) Structural overlay of PLC-β3·Gα$_q$ and p63RhoGEF·Gα$_q$ complexes. Also shown is a sequence alignment of the Gα$_q$ binding domain of human p63RhoGEF and human PLC-β3. Residues in p63RhoGEF required for Gα$_q$ binding, Gα$_q$-stimulated exchange activity, and the corresponding residues in PLC-β3 are shown in red. (**D**) Multiple sequence alignment of PLC-β isozymes. Highlighted in red are key residues in the helix-turn-helix domain of PLC-β3 that are required for Gα$_q$-dependent PLC activity and the corresponding residues in other PLC-β isozymes.
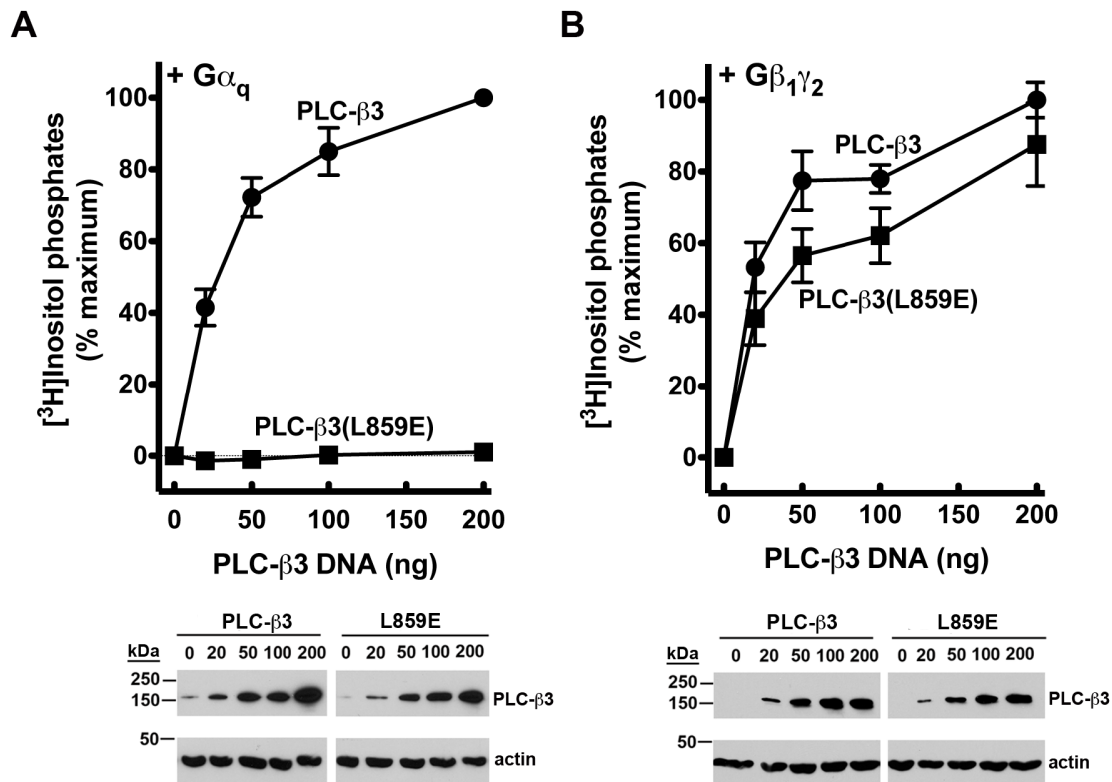
Figure 4.4. **Mutation of L859 abolishes $G\alpha_q$ but not $G\beta\gamma$-dependent activation of PLC-β3**. COS-7 cells were cotransfected with (**A**) $G\alpha_q$ or (**B**) $G\beta_1\gamma_2$ and varying amounts of either wildtype (●) or mutant (■) PLC-β3 DNA. Accumulation of [^{3}H]inositol phosphates was measured 36 h after transfection as described in Materials and Methods. Data (mean ± S.E.) are shown as percent of maximum wildtype PLC-β3 activity and averaged from three individual experiments. Cell lysates from COS-7 cells transfected as described above were subjected to SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with rabbit polyclonal anti-PLC-β3 antibody. Membranes were also probed with mouse monoclonal anti-actin antibody to control for loading.
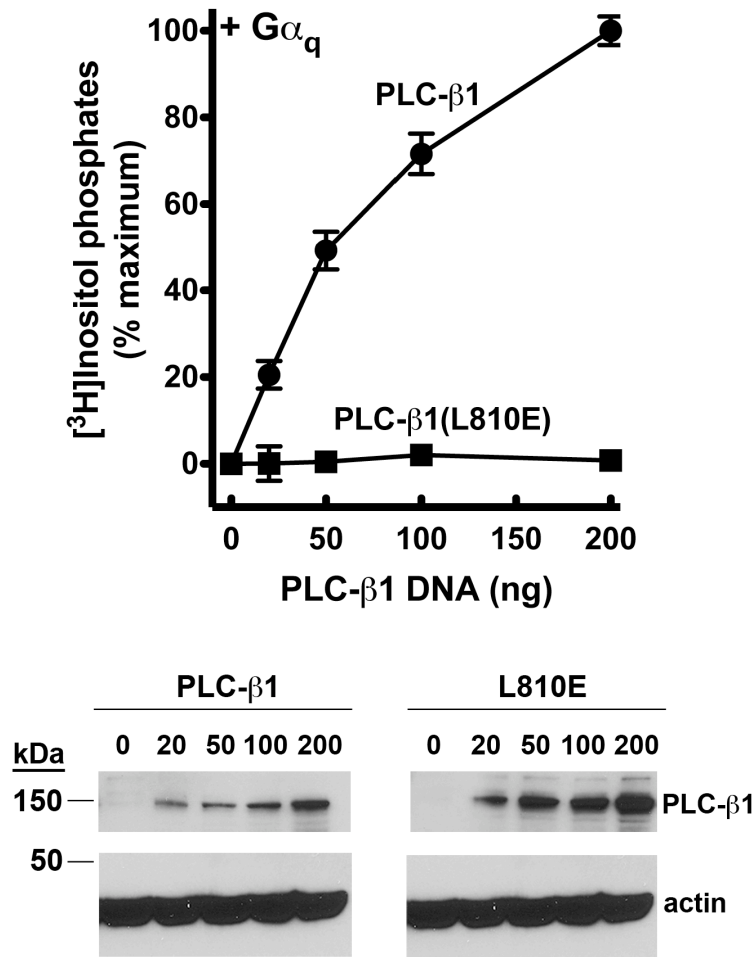
Figure 4.5. **The conserved leucine residue in the PLC-β3·Gα$_q$ binding interface serves the same function in PLC-β1**. COS-7 cells were cotransfected with Gα$_q$ and varying amounts of either wildtype (●) or mutant (■) PLC-β1 DNA. [^{3}H]inositol phosphates were quantified 36 h after transfection as described in Materials and Methods. Data (mean ± S.E.) are shown as percent of maximum wildtype PLC-β1 activity and averaged from three separate experiments. Cell lysates from COS-7 cells transfected as described above were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with rabbit polyclonal anti-PLC-β1 antibody and mouse monoclonal anti-actin antibody as a loading control.
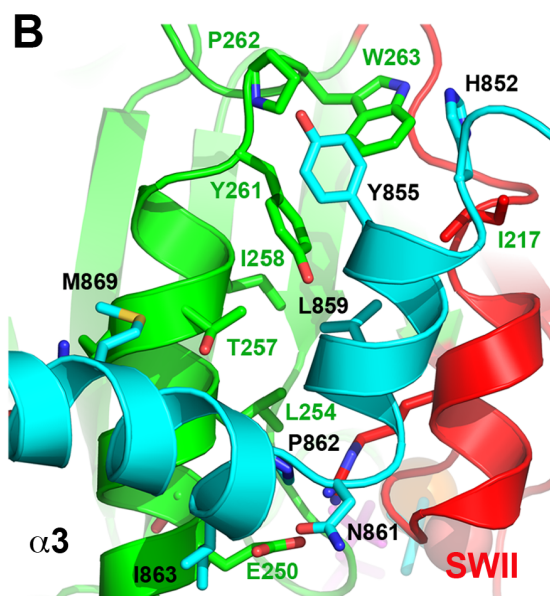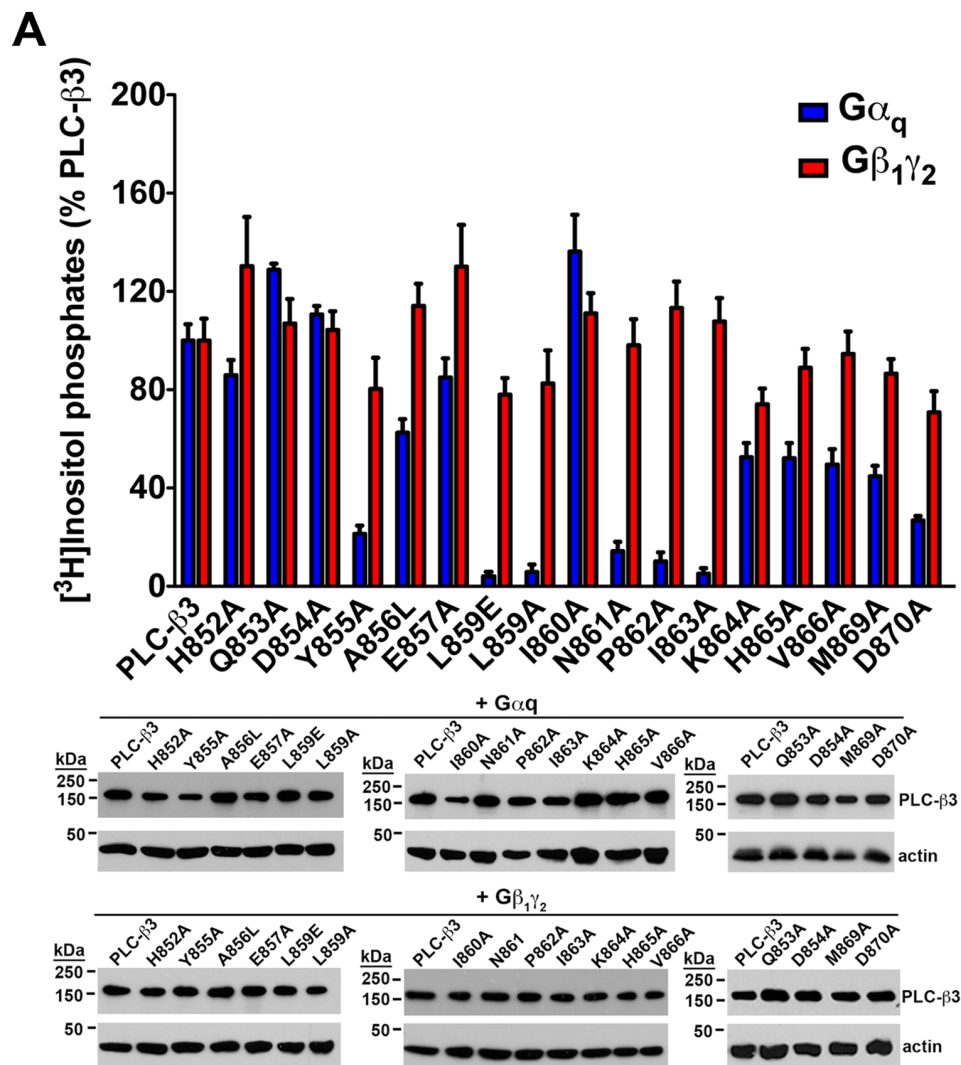
Figure 4.6. **PLC-β3 helix-turn-helix mutants are defective in Gα$_q$ but not Gβγ-stimulated PLC activity**. (**A**) COS-7 cells were cotransfected with either Gα$_q$ (red) or Gβ$_1$γ$_2$ (blue) and 50 ng wildtype or mutant PLC-β3 DNA. Total [^{3}H]inositol phosphates were measured as described in Materials and Methods. Data (mean ± S.E.) shown is averaged from four individual experiments and presented as percent of wildtype PLC-β3 activity. Immunoblot analysis shows equal expression of wildtype PLC-β3 and each PLC-β3 mutant. Membranes were also probed for actin to control for loading of cell lysates. (**B**) Close-up view of the PLC-β3 (cyan) and Gα$_q$ (green; switch regions in red) binding interface shows that the helix-turn-helix region of PLC-β3 docks between switch II (SwII) and the α3 helix of Gα$_q$, forming nonpolar interactions with the hydrophobic cleft of Gα$_q$.
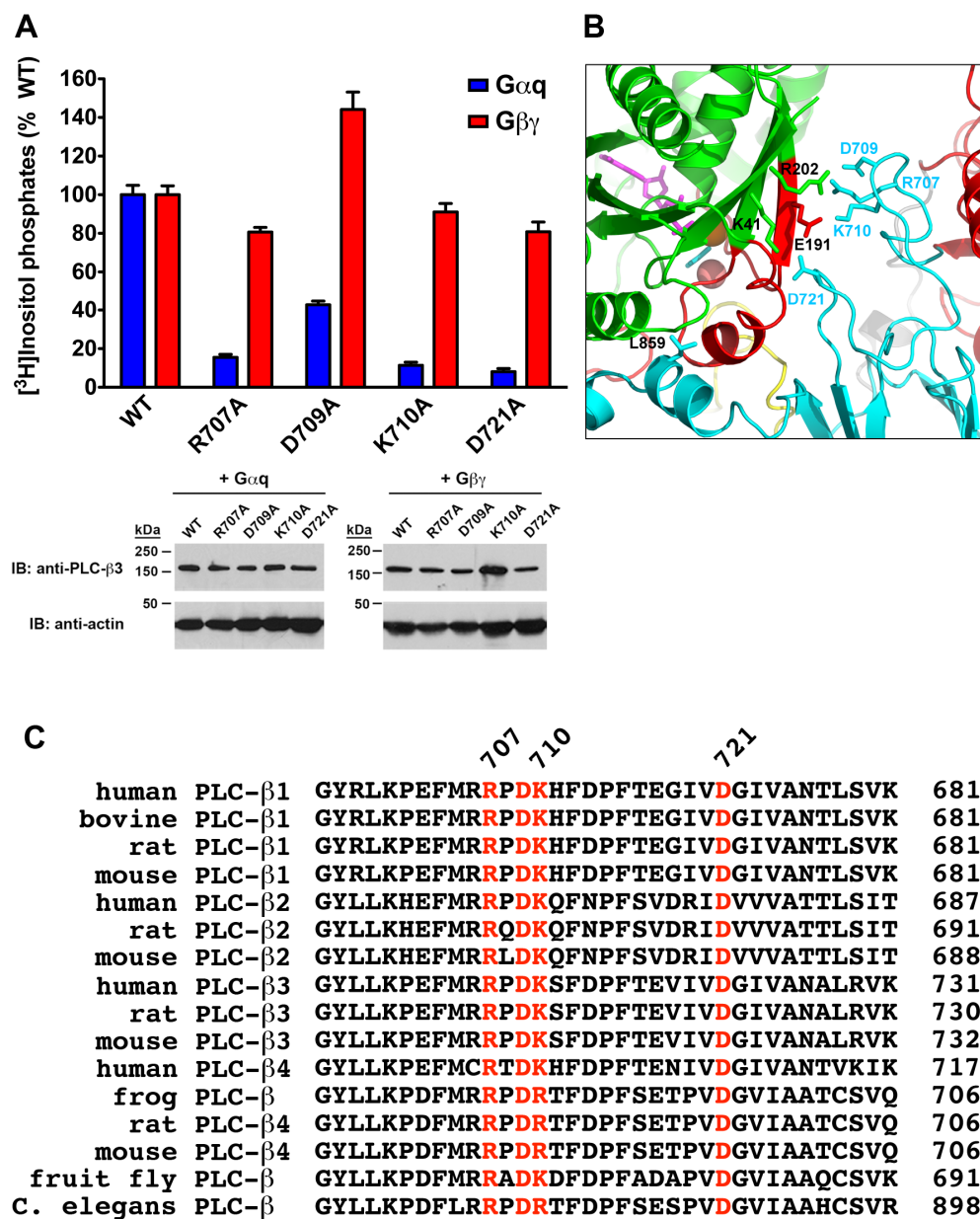
Figure 4.7. **Mutation of charged residues preceding the C2 domain of PLC-β3 attenuates Gα_q but not Gβγ-promoted PLC activity**. (**A**) COS-7 cells were cotransfected with either Gα_q (red) or Gβ_1γ_2 (blue) and 50 ng wildtype or mutant PLC-β3 DNA. Accumulation of [³H]inositol phosphates was measured as described in Materials and Methods. Immunoblots show expression levels of wildtype or mutant PLC-β3 and actin as a loading control. (**B**) Structural detail of contacts between PLC-β3 (cyan) and Gα_q (green). The switch regions of Gα_q are shown in red. (**C**) Multiple sequence alignment of PLC-β isozymes. Highlighted in red are charged residues mutated in PLC-β3 and the corresponding residues in other PLC-β isozymes.
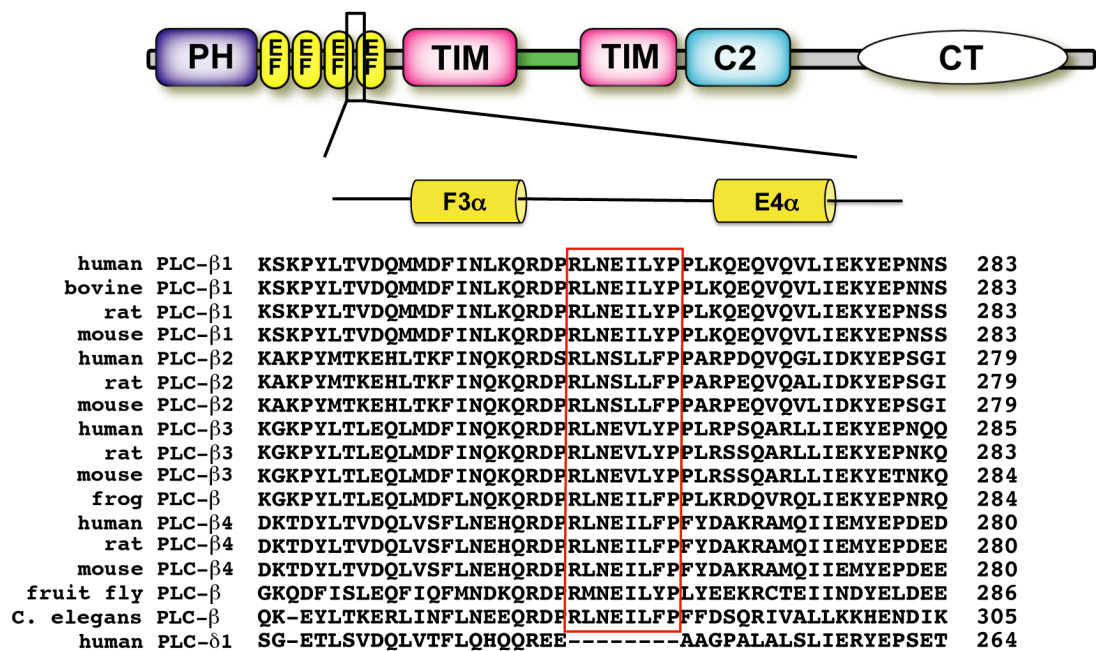
Figure 4.8. **PLC-β3 EF hand insert is conserved in other PLC-β isoforms**. A multiple sequence alignment of PLC-β isozymes and human PLC-δ1 reveals that the EF hand domain contains an eight amino acid insert that is not present in PLC-δ1. The EF hand insert is located between the third and fourth EF hands of PLC-β isozymes shown in the box. Also shown is the secondary structure of portions of the third and fourth EF hands. Unstructured regions are depicted as black lines.
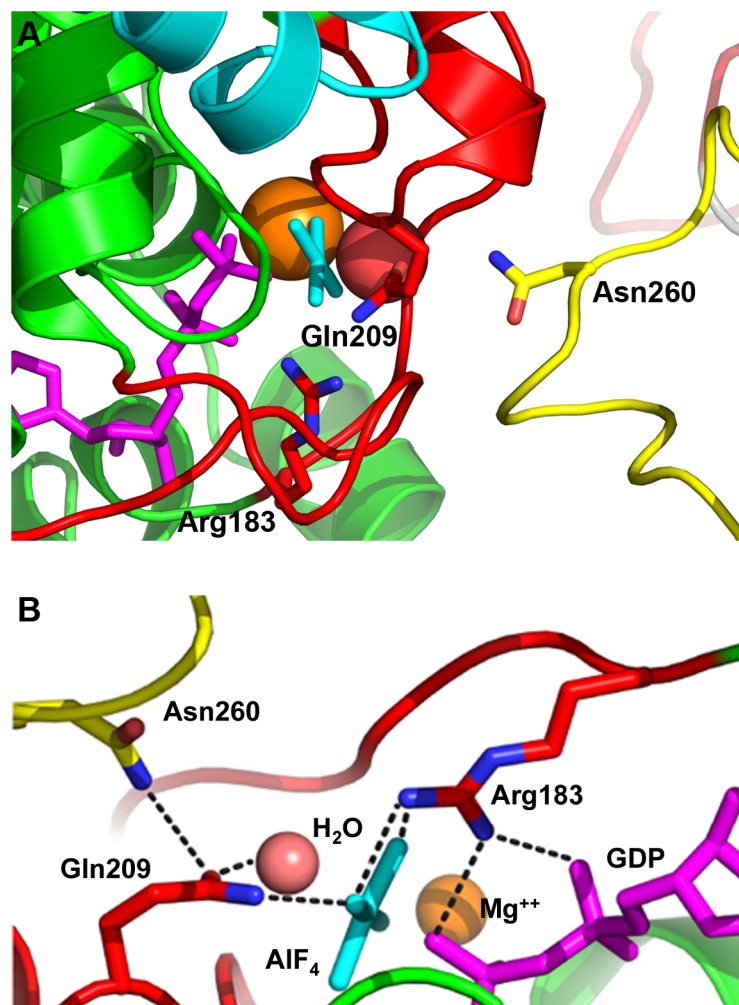
Figure 4.9. **Structural details of the nucleotide binding site of Gα_q in the PLC-β3 ΔCT·Gα_q complex.** (**A**) Residue Asn260 (yellow) of PLC-β3 positions conserved Gln209 (red) of Gα_q, which stabilizes nucleophilic water for GTP hydrolysis. Residue Arg183 of Gα_q stabilizes the γ-phosphate leaving group of GTP and also interacts with the α and β phosphates of GTP. GDP (magenta) and AlF$_4$ (cyan) are shown as sticks, and Mg^{2+} (orange) and catalytic water (salmon) are depicted as spheres. (**B**) The side chain nitrogen of Asn260 forms hydrogen bonds (dashed lines) with the carbonyl oxygen of Gln209, stabilizing the GTP transition state for GTP hydrolysis.
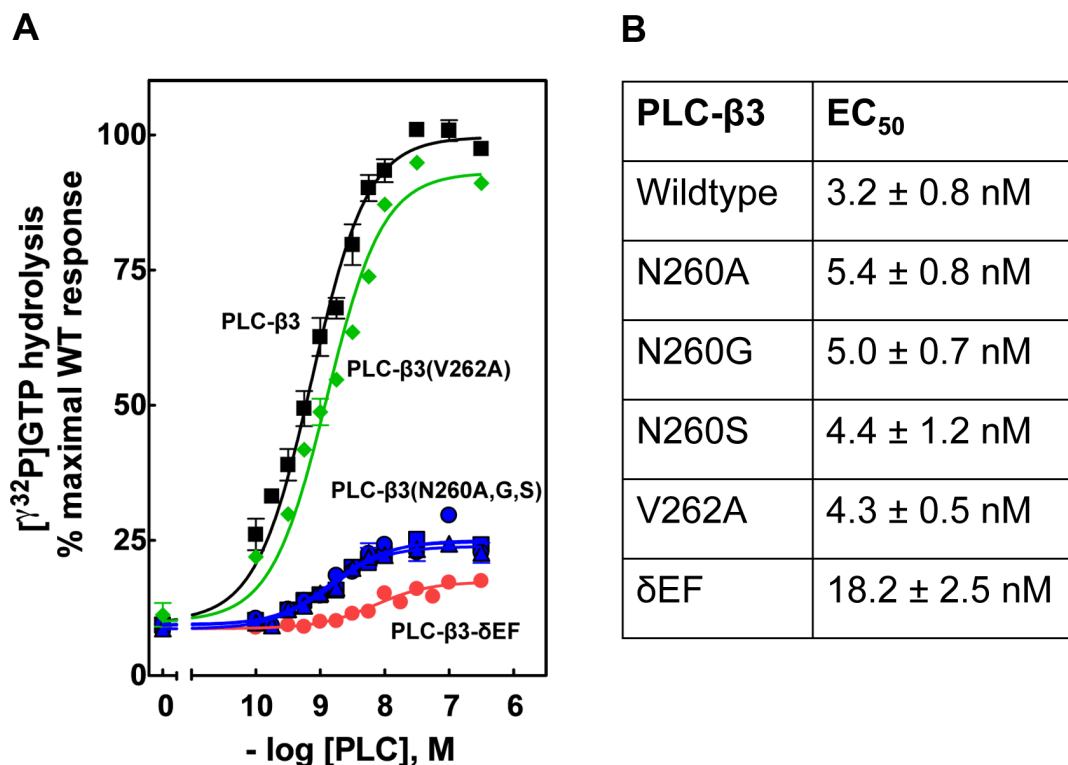
Figure 4.10. **Mutation of the PLC-β3 EF hand loop abrogates PLC-β3-promoted GTP hydrolysis on Gα$_q$.** (**A**) Purified P2Y$_1$-R, Gα$_q$, and Gβ$_1$γ$_2$ were reconstituted into phospholipid vesicles, and steady-state hydrolysis of [γ-^{32}P]GTP was measured in the presence of 0.3 μM 2MeSADP and increasing concentrations of wildtype PLC-β3 (■) or the following PLC-β3 mutants: PLC-β3 δEF (●; red), N260A (■; blue), N260G (●; blue), N260S (▲; blue), and V262A (◆; green). The data (mean ± S.E.) shown are averaged from three individual experiments and presented as percent of maximal PLC-β3 wildtype response. (**B**) Table presented shows the calculated EC$_{50}$ values (mean ± S.E.) for GTP hydrolysis in the presence of wildtype and mutant PLC-β3.
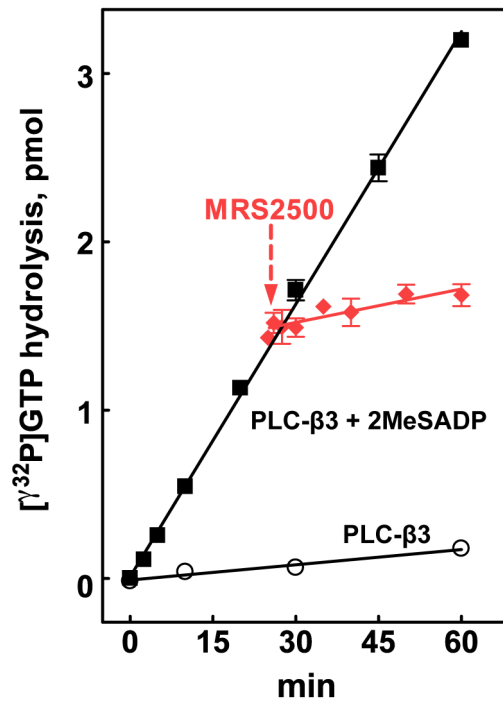
Figure 4.11. **Competitive antagonist of P2Y$_1$-R rapidly terminates P2Y$_1$-R and PLC-β3-stimulated GTP hydrolysis of Gα$_q$**. Purified P2Y$_1$-R, Gα$_q$, and Gβ$_1$γ$_2$ were reconstituted into phospholipid vesicles. Steady-state hydrolysis of γ-[^{32}P]GTP was quantified at 30°C at the times indicated in the presence of 100 nM PLC-β3 alone (○) or both 0.3 μM 2MeSADP and 100 nM PLC-β3(■). P2Y$_1$-R competitive antagonist, MRS2500, (◆; red) was added at 10 μM saturating concentration at the times indicated before quantifying GTP hydrolysis.
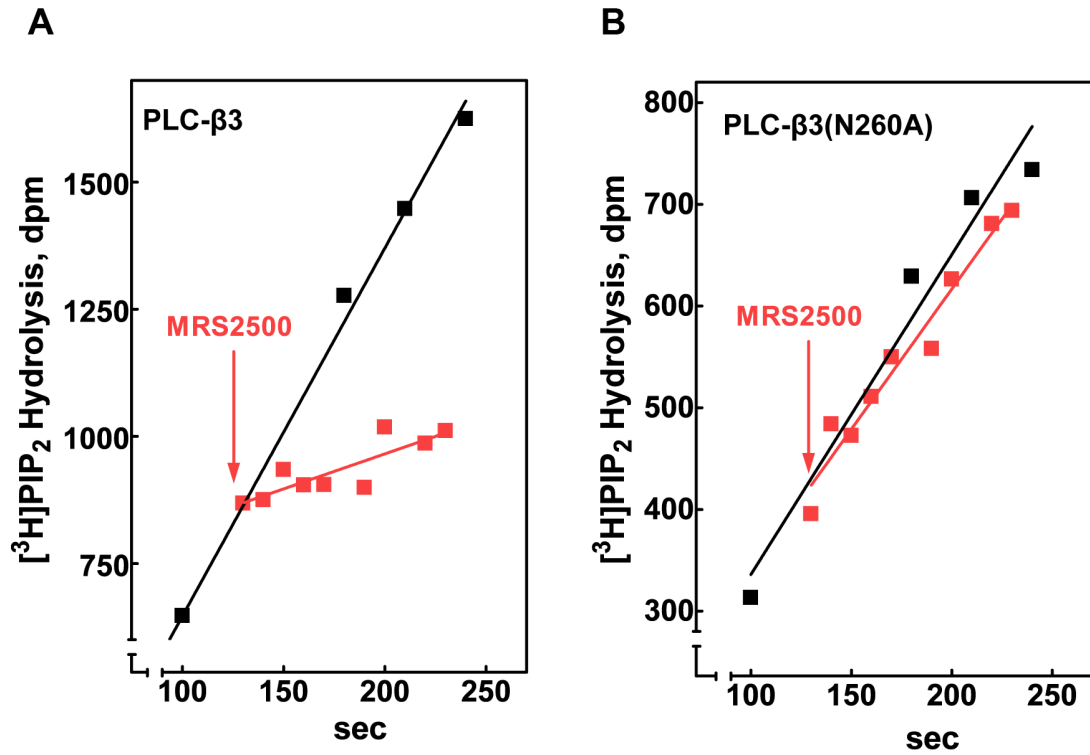
Figure 4.12. **GAP-deficient PLC-β3(N260A) mutant displays a much slower rate of deactivation in the presence of P2Y$_1$-R competitive antagonist**. Purified P2Y$_1$-R, Gα$_q$, and Gβ$_1$γ$_2$ were reconstituted into phospholipid vesicles containing [^{3}H]PtdIns(4,5)P$_2$. 2MeSADP (0.3 μM) was added to activate P2Y$_1$-R and catalyze GTP exchange on Gα$_q$ at 25°C. Proteoliposomes were incubated with 30 nM PLC-β3 wildtype (**A**) or PLC-β3(N260A) (**B**), and steady-state PtdIns(4,5)P$_2$ hydrolysis was measured at the indicated times. P2Y$_1$-R competitive antagonist, MRS2500, (red) was added to proteoliposomes at 10 μM, and total [^{3}H]inositol phosphates were quantified at the times shown.
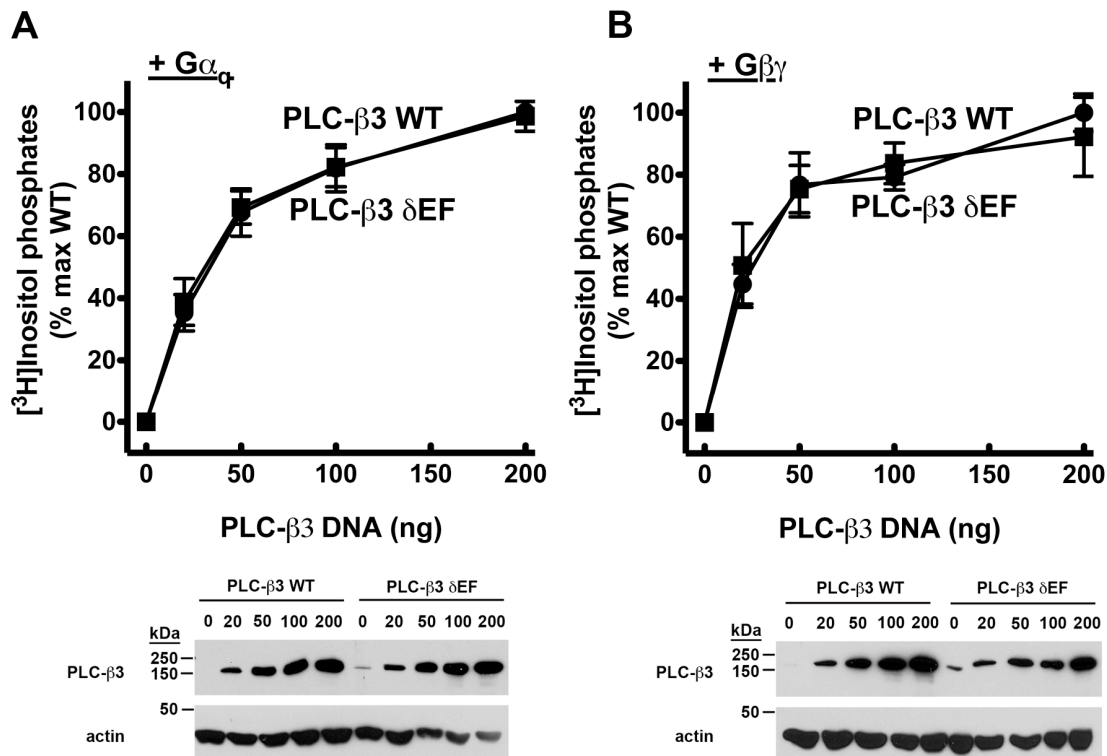
Figure 4.13. **Activation of GAP-deficient PLC-β3 by Gα_q in intact cells**. COS-7 cells were cotransfected with either Gα_q (**A**) or Gβ_1γ_2 (**B**) and a broad range of PLC-β3 wildtype (●) or δEF (■) DNA concentrations. Accumulation of [^{3}H]inositol phosphates was measured as described in Materials and Methods. Data (mean ± S.E.) are shown as percent of maximum wildtype PLC-β3 activity and averaged from three individual experiments. Cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with rabbit polyclonal anti-PLC-β3 antibody and mouse monoclonal anti-actin antibody.
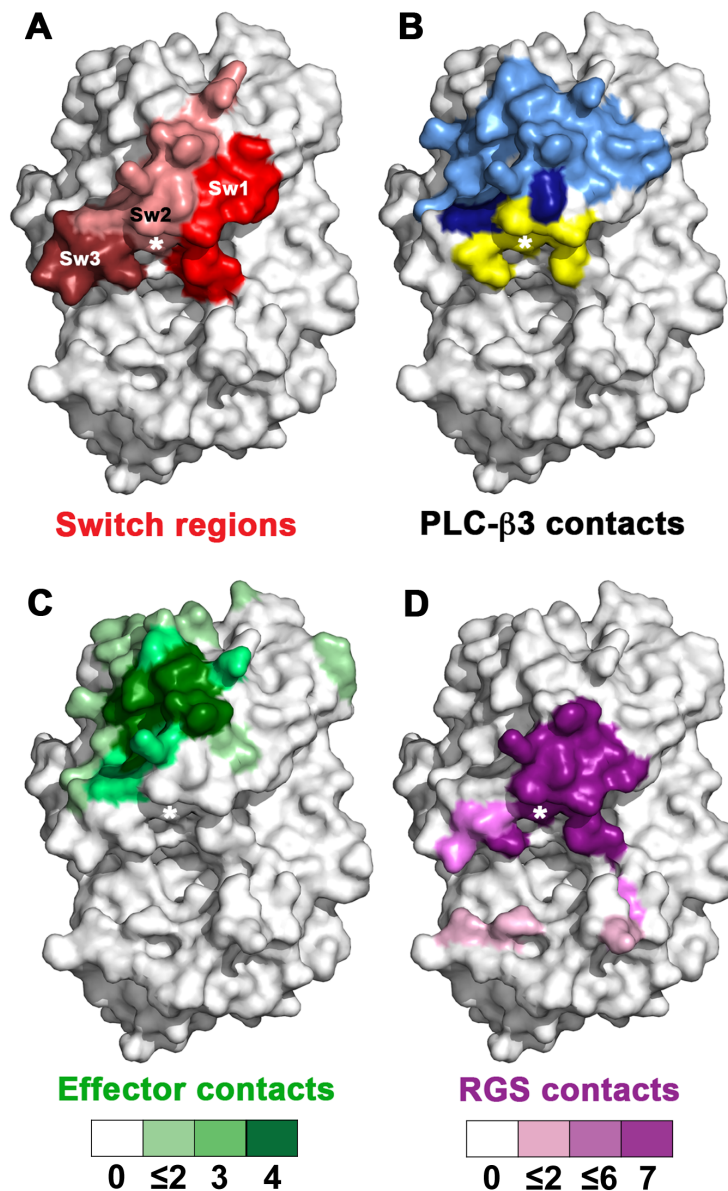
Figure 4.14. **PLC-β3 engages both the conserved effector and RGS binding domains of Gα_q.** (**A**) Switch I (Sw1), switch II (Sw2), and switch III (Sw3) are highlighted on the surface of Gα_q. The white asterisk indicates the position of highly conserved Gln209. (**B**) PLC-β3 (blue) and heterotrimeric G protein effectors (green) share common Gα contact sites. The EF hand loop of PLC-β3 (yellow) engages residues on the surface of Gα_q that are also observed between RGS proteins (purple) and their interacting Gα subunit. The EF hand loop and other regions of PLC-β3 have some overlapping contact residues shown in dark blue. (**C**) The sites of interaction between effectors and their heterotrimeric G proteins were mapped onto the surface of Gα_q. These effector·Gα complexes included Gα_s·adenylyl cyclase, Gα_t·PDEγ,

Gα_q·GRK2, and Gα_q·p63RhoGEF (Tesmer et al., 1997b; Slep et al., 2001; Tesmer et al., 2005; Lutz et al., 2007). Structures of seven RGS·Gα complexes were used to highlight RGS contacts sites and include RGS1·Gα_{i1}, RGS4·Gα_{i1}, RGS8·Gα_{i3}, RGS9·Gα_t, RGS10·Gα_{i3}, RGS16·Gα_{i1}, and RGS16·Gα_o (Tesmer et al., 1997a; Slep et al., 2001; Slep et al., 2008; Soundararajan et al., 2008). Colors in (**C**) and (**D**) were varied to depict low (light green or purple) to high (dark green or purple) conservation of effector and RGS contact sites.
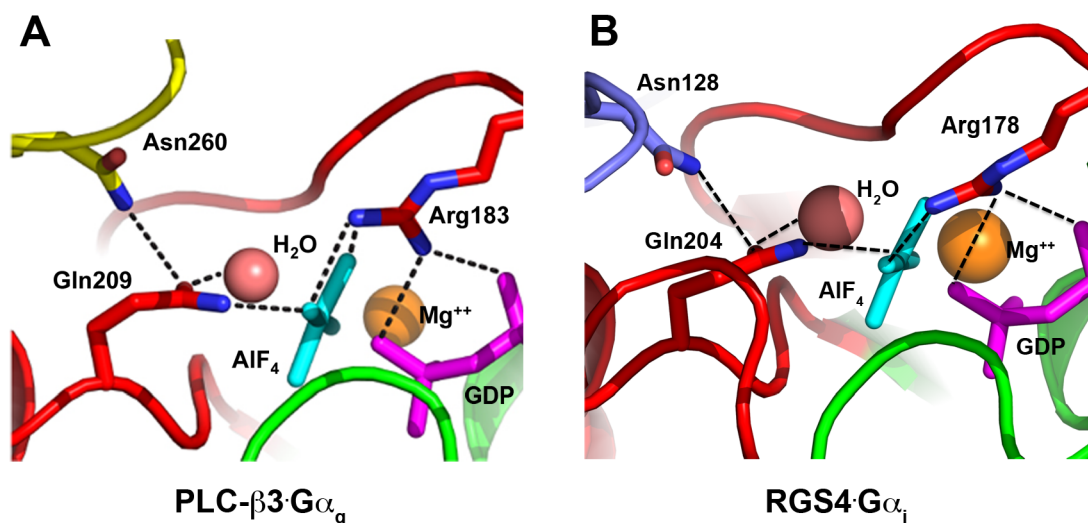
Figure 4.15. **Structural comparison of the nucleotide binding site of Gα in the PLC-β3 ΔCT·Gα$_q$ and RGS4·Gα$_i$ complexes**. (**A**) Residue Asn260 (yellow) of PLC-β3 interacts with highly conserved Gln209 (red) of Gα$_q$ to stabilize the GTP transition state for GTP hydrolysis. (**B**) Residue Asn128 (blue) of RGS4 serves the same function in stabilizing conserved Gln204 of Gα$_i$ for GTP hydrolysis. GDP (magenta) and AlF$_4^-$ (cyan) are presented as sticks, and Mg^{2+} (orange) and catalytic water (salmon) are shown as spheres. Hydrogen bonds between atoms are depicted has dashed lines.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Cells respond to changes in their surrounding environment by using cell surface receptors to detect extracellular cues and relay information to intracellular compartments. Heterotrimeric G protein-coupled receptors (GPCRs) are the largest and most diverse family of cell surface receptors encoded in the mammalian genome and respond to various extracellular stimuli, including hormones, neurotransmitters, peptides, and proteases. In Chapter 2 of this thesis, we examined signal regulation of G protein-coupled protease-activated receptor 2 (PAR2), a cell surface receptor for extracellular proteases. Unlike classic GPCRs, PAR2 is irreversibly activated by proteolytic cleavage, and the mechanisms of signal regulation are likely unique from reversibly activated GPCRs. Most activated GPCRs are rapidly desensitized and internalized following receptor phosphorylation and β-arrestin binding. For PAR2, however, the role of phosphorylation in regulating signaling and trafficking is not clearly understood and is the focus of Chapter 2 in this thesis.

Activated PARs and other GPCRs catalyze GDP/GTP exchange on the α subunit of heterotrimeric G proteins. GTP-bound Gα dissociates from Gβγ, and both Gα and Gβγ dimers bind and activate downstream effectors including adenylyl cyclases, ion channels, RhoGEFs, and PLC-β isozymes. In Chapter 4 of this dissertation, we examined the mechanisms of $G\alpha_q$-dependent activation and deactivation of PLC-β

isozymes. The Harden lab has recently generated a three-dimensional crystal structure of PLC-β3 in an AlF$^-_4$-dependent complex with Gα_q. We have identified three novel contacts within the PLC-β3·Gα_q interface and used structure/function analysis to determine the importance of these interactions in PLC-β3 activation and deactivation (Chapter 4).

5.1 Signal Regulation of Protease-activated Receptor 2

In the present study, we found that phosphorylation of PAR2 differentially regulates desensitization and trafficking. Upon agonist addition, wildtype PAR2 was rapidly and robustly phosphorylated on serine and threonine residues within the cytoplasmic tail (C-tail). A phosphorylation-deficient mutant (PAR2 0P) failed to recruit β-arrestins to the cell surface and was markedly impaired in receptor desensitization. We also showed that PAR2 0P internalizes independent of G protein activation and phosphorylation, suggesting that phosphorylation or a distinct conformation of the receptor C-tail are required for cell surface retention. In addition, we demonstrated that the phosphorylation-deficient PAR2 0P mutant constitutively internalizes and sorts through the endocytic pathway to lysosomes through a dynamin-dependent but clathrin- and β-arrestin-independent pathway. These results suggest that phosphorylation is dispensable for PAR2 endocytic trafficking but essential for controlling receptor desensitization and β-arrestin binding.

Ligand-bound GPCRs are rapidly phosphorylated by G protein-coupled receptor kinases (GRKs) or second messenger kinases. For PAR2, however, the protein kinases responsible for receptor phosphorylation are not currently known. Previous studies found that pharmacological inhibitors of protein kinase C (PKC) enhanced PAR2-stimulated

calcium signaling in KNRK and hBrie 380 cells, suggesting that PKC may be important for PAR2 desensitization (Bohm et al 1996). PAR2 contains several potential PKC sites within its C-tail region that could serve as potential sites of phosphorylation. Currently, there is no direct evidence for PAR2 phosphorylation by PKC.

PKC and other second messenger kinases are known to indiscriminately phosphorylate agonist-occupied receptors as well as unoccupied GPCRs. GRKs, however, strictly recognize ligand-bound receptors. There are seven GRKs (GRK1-7) encoded in the mammalian genome and all share a core domain architecture. GRKs contain a catalytic domain flanked by an N-terminal RGS homology domain and a C-terminal region that functions in membrane association. GRK1 and GRK7 are expressed exclusively in the retina, and GRK4 is primarily found in the testis. The remaining GRKs (GRK2, GRK3, GRK4, GRK5) are ubiquitously expressed in a variety of tissues. GRKs do not appear to recognize particular consensus sequences in GPCRs but specifically bind to the activated form of receptors and phosphorylate serine and threonine residues near negatively charged amino acids. Thus, GRK2, GRK3, GRK5, and/or GRK6 are potential candidates for mediating PAR2 phosphorylation. Future studies are needed to identify the specific GRK(s) and/or second messenger kinases that regulate PAR2 phosphorylation and signaling.

Most GPCRs require phosphorylation by GRKs for β-arrestin binding. Recent studies suggest that phosphorylation of GPCRs at distinct sites by specific GRKs may also regulate β-arrestin function. In studies of V2 vasopressin and AT_{1A} angiotensin receptors, siRNA depletion of GRK2 and GRK3 prevented agonist-induced receptor phosphorylation and internalization but had no effect on arrestin-mediated ERK1/2

activation (Kim et al., 2005; Ren et al., 2005). Alternatively, loss of GRK5 and GRK6 had little effect on receptor phosphorylation and internalization but reduced arrestin-dependent ERK1/2 activation. Similar findings were also shown for the $\beta_2 AR$ and follicle-stimulating hormone (FSH) receptor (Kara et al., 2006; Shenoy et al., 2006). The authors proposed that GRK5 and GRK6 phosphorylate distinct sites on GPCRs that allows β-arrestins to bind and adopt a conformation for mediating ERK1/2 activation whereas phosphorylation by GRK2 and GRK3 fails elicit the same response. Similar to V2 vasopressin and AT_{1A} receptors, PAR2 also stimulates β-arrestin-mediated ERK1/2 signaling that is reported to be independent of heterotrimeric G protein activation (DeFea et al., 2000; Stalheim et al., 2005). The distinct determinants within PAR2 that are critical for the diverse functions of β-arrestins are not clearly understood. Differential phosphorylation by specific GRK subtypes may be a mechanism to confer distinct signaling events of PAR2 and is an important future pursuit to better understand regulation of PAR2 signaling.

PAR2 appears to internalize in a dynamin-dependent but clathrin- and β-arrestin-independent pathway in the absence of receptor phosphorylation. Most activated GPCRs associate with β-arrestins, which couple receptors to clathrin and adaptor protein complex-2, components of the endocytic machinery. β-arrestin binding promotes GPCR internalization through clathrin-coated pits. Some GPCRs, however, use a dynamin-dependent but clathrin-independent pathway for receptor internalization. Clathrin-independent internalization occurs through lipid raft microdomains enriched in glycosphingolipids and cholesterol (Mayor and Pagano, 2007). A subset of lipid rafts, known as caveolae, have a similar lipid composition as other lipid microdomains but also

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contain additional protein components including the structural protein, caveolin. Caveolin proteins oligomerize, generating a cytoplasmic coat around lipid microdomains and facilitate formation of flask-shaped pits at the plasma membrane.

Caveolin proteins are thought to sequester GPCRs and other membrane proteins into caveolae through protein interactions with caveolin binding motifs. Analysis of the PAR2 protein sequence revealed that the receptor contains a putative caveolin binding motif (YYFVSHDF, $\phi X\phi XXXX\phi$) within the C-terminal end of its seven transmembrane domain. Caveolin may interact with the binding motif to promote movement of PAR2 into caveolar membranes and clathrin-independent endocytosis. In MDA-MB-231 cells, PAR2 was found to co-fractionate with caveolin-1 in detergent insoluble microdomains and also shown to co-localize with caveolin-1 in immunofluorescence microscopy studies (Awasthi et al., 2007). Further analysis is needed to determine if PAR2 directly interacts with ubiquitously expressed caveolin-1 or caveolin-2 and whether phosphorylation-deficient PAR2 0P resides in caveolar membranes and internalizes using lipid rafts. We have previously shown successful depletion of caveolin-1 from EA.hy926 endothelial cells using shRNAs (Russo et al., 2009a). In addition, there are several pharmacological methods to alter cholesterol-rich lipid rafts and inhibit clathrin-independent endocytosis (Ivanov, 2008). For example, methyl-β-cyclodextrin has a high affinity for cholesterol and forms soluble lipid complexes, effectively depleting cholesterol from lipid microdomains and altering their structure. Reintroducing cholesterol to cells can reverse these effects. Filipin and nystatin are polyene antibiotics that remove cholesterol from lipid raft microdomains by forming large cholesterol-rich aggregates. Using these

methods, we can determine the role of caveolae and/or lipid rafts in internalization of phosphorylation-deficient PAR2 0P.

Localization of GPCRs in caveolar membranes and lipid rafts is also thought to compartmentalize GPCR signaling (Chini and Parenti, 2004; Patel et al., 2008). Compartmentalization may be particularly important for PAR2 since this receptor is not only activated by soluble extracellular proteases like trypsin but also by proteases that require membrane bound cofactors. The transmembrane protein tissue factor (TF) is required for the activity of coagulant protease factor (F)VIIa and proteolytic cleavage of PAR2. In MDA-MB-231 cells, cholesterol depletion and siRNA knockdown of caveolin-1 severely impaired TF-FVIIa-dependent PAR2 signaling whereas disruption of lipid rafts and caveolae had no effect on signaling induced by the PAR2-specific agonist peptide (Awasthi et al., 2007). A similar result was also observed for PAR1 signaling in endothelial cells (Russo et al., 2009b). When activated by thrombin, PAR1 stimulates Rho activation, endothelial barrier permeability, and proinflammatory responses (Coughlin, 2005). Alternatively, activation of PAR1 by activated protein C (APC) promotes Rac1 activation, endothelial barrier protection, and anti-inflammatory effects (Feistritzer and Riewald, 2005; Russo et al., 2009b). APC requires the transmembrane cofactor, endothelial protein C receptor (EPCR) and compartmentalization into caveolar membranes for activating PAR1 signaling pathways (Riewald et al., 2002; Bae et al., 2007a; Bae et al., 2007b; Russo et al., 2009b). Collectively, these results suggest that localization of PARs in caveolar membranes may be a general mechanism for protease-selective signaling. Future studies are needed to investigate whether compartmentalization of the membrane bound TF-FVIIa complex and PAR2 activates

PAR2 signaling pathways that are distinct from cellular responses stimulated by trypsin or agonist peptide.

Our studies further suggest that PAR2 is sorted from endosomes to lysosomes and degraded in the absence of receptor activation and phosphorylation. Studies of the β_2AR and CXCR4 indicate that ubiquitination is essential for lysosomal sorting and degradation of these receptors (Marchese and Benovic, 2001; Shenoy et al., 2001; Marchese et al., 2003). Ubiquitin-deficient β_2AR and CXCR4 mutants fail to degrade in the presence of agonists. Ubiquitinated cargo interacts with the ubiquitin binding motif of hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) (Marchese et al., 2003). HRS also associates with tumor suppressor gene product 101 (Tsg101) of the ESCRT complex, which is needed for multivesicular body formation, lysosomal fusion, and degradation of proteins and lipids. Post-endocytic sorting of PAR2 requires receptor ubiquitination and binding of HRS, but the role of Tsg101 and other members of the ESCRT machinery is unknown (Jacob et al., 2005b; Hasdemir et al., 2007). In contrast to wildtype PAR2, δ-opioid receptor and PAR1 are degraded independent of receptor ubiquitination (Whistler et al., 2002; Wolfe et al., 2007). Degradation of δ-opioid receptors is regulated by GPCR-associated sorting proteins (GASP), which target some GPCRs to lysosomal pathways (Whistler et al., 2002). Proper sorting of PAR1 to lysosomes requires association with SNX1, a protein that functions in vesicular trafficking (Gullapalli et al., 2006). Whether phosphorylation-deficient PAR2 0P mutant is ubiquitinated and sorts to lysosomes through an ESCRT-dependent or independent pathway remains to be determined.

5.2 Mechanisms of Gα_q-dependent Activation and Deactivation of Phospholipase C-β

In the present study, we examined the mechanisms of PLC-β activation by Gα_q. Inspection of the PLC-β3·Gα_q crystal structure revealed three novel interactions within the PLC-β3·Gα_q binding interface. The primary region of contact occurs between the hydrophobic effector binding pocket of Gα_q and a 20 amino acid extension following the C2 domain of PLC-β3. The C2 domain extension adopts a helix-turn-helix topology and docks between the α2 (Switch II) and α3 helices of Gα_q. Mutation of key residues (Y855, L859, N861, P862, I863) within the helix-turn-helix of PLC-β3 severely impaired Gα_q-stimulated PLC activity whereas activation by Gβγ remained intact. PLC-β3 also makes electrostatic interactions with Gα_q using several charged residues (R707, D709, K710, D721) in a region preceding the C2 domain. Alanine substitution at each of these amino acids inhibited PLC-β3 activation by Gα_q but not Gβγ. The third region of contact occurs between the nucleotide binding site of Gα_q and a small loop connecting the third and fourth EF hands of PLC-β3. The location of the EF hand loop suggested that this region might be important for the GTPase accelerating activity of PLC-β3. An asparagine residue (N260) within the EF hand loop stabilizes Q209 in Gα_q. Q209 is a highly conserved amino acid in the nucleotide binding pocket of Gα_q and is essential for positioning the nucleophilic water molecule for GTP hydrolysis. Deletion of the entire EF hand loop or single point mutations at N260 abrogated PLC-β3-stimulated GTP hydrolysis on Gα_q. Furthermore, a GAP-deficient PLC-β3 mutant displayed a strikingly slower rate of deactivation since Gα_q remained GTP-bound. Consequently, GAP-

deficient PLC-β3 activity was largely unchanged when agonist was removed compared to the rapid shutoff of wildtype PLC-β3.

PLC-β isozymes are unique from other PLC family members in that they have a long C-terminal extension following the C2 domain. Previous studies of PLC-β isozymes suggested that the C-terminal region was required for $G\alpha_q$ binding and stimulation of PLC-β. The Harden lab, however, found that a truncated mutant of PLC-β3 (PLC-β3 ΔCT), lacking ~350 amino acids of the C-terminal region, still forms a high affinity complex with $G\alpha_q$. In fact, both full-length PLC-β3 and PLC-β3 ΔCT have virtually the same affinity for $G\alpha_q$ by surface plasmon resonance analysis, suggesting that the C-terminal region is actually dispensable for PLC-β binding to $G\alpha_q$. Instead, the primary region of $G\alpha_q$ binding is a small helix-turn-helix region directly after the C2 domain. Based on these observations, the role of the long C-terminal extension of PLC-β isozymes in regulating enzyme activity is not clearly understood.

Mutational analysis of the PLC-β1 C-terminal region suggested that this domain is also important for membrane association. PLC-β1 isozymes lacking residues 903-1030 of the C-terminal region were found in soluble fractions of COS-7 cell lysates whereas full-length PLC-β1 was present in particulate fractions (Wu et al., 1993). Consequently, loss of membrane association completely abrogated $G\alpha_q$-stimulated PLC activity. Similarly, expression of $G\alpha_q$ in COS-7 cells failed to activate PLC-β3 ΔCT whereas $G\alpha_q$ robustly stimulated full-length PLC-β3 (data not shown). It remains to be determined if PLC-β3 ΔCT is sufficiently targeted to membranes for activation by $G\alpha_q$. A future direction of this work is to determine the importance of the C-terminal extension in membrane association versus $G\alpha_q$ binding. Membrane localization sequences are often

used to target cytosolic proteins to the cell surface. A common membrane localization sequence used for prenylation of target proteins is the C-terminal CaaX box (where C is cysteine, a is usually an aliphatic residue, and X is the last residue of the protein) of N-, H-, and K-Ras GTPases (Stickney et al., 2001). A polybasic region or palmitoylation signal is also added to the N-terminal end of the CaaX box motif to enhance membrane association. Addition of a membrane localization sequence to PLC-β3 ΔCT would determine if simply anchoring PLC-β isozymes to the plasma membrane is sufficient for activation by Gα$_q$ proteins and would show that the C-terminal region primarily functions in attaching PLC-β isozymes to lipid bilayers rather than in binding to Gα$_q$.

PLC-β isozymes are both effectors and GAPs of the Gα$_q$ family of heterotrimeric G proteins. As a result, Gα$_q$ proteins directly stimulate PLC activity, and PLC-β isozymes accelerate the intrinsic GTP hydrolysis of Gα$_q$ to deactivate phospholipid signaling. As effector GAP proteins, PLC-β isozymes must maintain phospholipid signaling at steady-state and also rapidly terminate signaling when agonist is removed. The mechanism by which PLC-β isozymes act as both activators and deactivators of phospholipid signaling is not clearly understood. We have identified a small loop between the third and fourth EF hands of PLC-β3 that mediates the GTPase accelerating activity of PLC-β3. We have demonstrated that removing the entire loop or introducing single point mutations in PLC-β3 inhibits PLC GAP activity without affecting the apparent affinity of PLC-β3 for Gα$_q$.

Identifying the GAP domain of PLC-β isozymes and generating GAP-deficient mutants have provided the tools to examine the effects of PLC-β GAP activity on phospholipid signaling. We found that a GAP-deficient PLC-β3 mutant has a much

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slower rate of deactivation when agonist is removed. Consequently, the rate of PtdIns(4,5)P$_2$ hydrolysis was largely unchanged. Interestingly, analysis of GAP-deficient PLC-β3 in intact cell assays showed that Gα_q stimulated the GAP-deficient mutant to the same extent as wildtype PLC-β3 despite the loss of deactivating GAP activity. In these experiments, COS-7 cells are co-transfected with wildtype or mutant PLC-β3 and Gα_q, and hydrolysis of [^{3}H]PtdIns(4,5)P$_2$ is quantified after incubation with LiCl for 1 h. Under these experimental conditions, only the accumulation of inositol phosphates is measured, suggesting that GAP activity of PLC-β isozymes has no effect on the overall extent of phospholipid signaling. It is also possible that a more sensitive technique, like measuring intracellular calcium levels, will better demonstrate differences in wildtype and GAP-deficient PLC-β3 activity. In future studies, we will examine how loss of GAP activity affects the rate of PLC-β3-promoted release of intracellular calcium.

The intact cell assays are performed in the absence of receptor stimulation, which may be needed to observe any differences in wildtype and GAP-deficient PLC-β3 activity. We have stimulated several recombinant and endogenous Gα_q-coupled receptors and measured accumulation of [^{3}H]inositol phosphates in COS-7, HEK293, and HeLa cells. Unfortunately, robust activation of endogenously expressed PLC-β isozymes masked any effects of over-expressed wildtype or GAP-deficient PLC-β3. Thus, our new strategy is to deplete cells of endogenous PLC-β isozymes using shRNAs and reintroduce siRNA-resistant forms of either wildtype or GAP-deficient PLC-β3 in HEK293 cells. Using these cell lines, we will examine the effects of GAP activity on receptor-promoted PtdIns(4,5) hydrolysis and calcium mobilization and measure the rates of signal initiation and termination as well as quantify changes in signal magnitude and duration.

PLC-β isozymes are activated by all members of the Gα$_q$ family of heterotrimeric G proteins as well as Gβγ dimers released from abundantly expressed Gα$_i$ subtypes. PLC-β2 and PLC-β3 are the most responsive to Gβγ dimers whereas PLC-β1 shows little to no activation (Park et al., 1993a; Smrcka and Sternweis, 1993). Currently, the differential effects of PLC-β activation by Gα$_q$ proteins versus Gβγ dimers is not clearly understood. The crystal structure of the PLC-β3 ΔCT·Gα$_q$ complex has revealed specific contacts between Gα$_q$ and PLC-β isozymes that are distinct from the proposed regions of Gβγ binding (Kuang et al., 1996; Sankaran et al., 1998; Wang et al., 1999b; Wang et al., 2000). In fact, single point mutations in the helix-turn-helix domain of PLC-β3 completely ablate Gα$_q$-stimulated PLC activity with no effect on activation by Gβγ dimers. It is possible that the cellular consequences of Gβγ activation of PLC-β isozymes are unique from that of Gα$_q$ proteins. Activation and deactivation of Gα$_q$ proteins are regulated by PLC-β isozymes due to the GTPase accelerating activity of PLC-β (Berstein et al., 1992; Biddlecome et al., 1996; Mukhopadhyay and Ross, 1999). In contrast, signal termination of Gβγ signaling would require Gα$_i$-specific RGS proteins and reassociation of Gβγ and GDP-bound Gα$_i$. Consequently, the rates of signal initiation and termination as well as signal magnitude and duration may be significantly different from Gα$_q$-promoted PLC activity. Using PLC-β2 or PLC-β3 mutants that disrupt Gα$_q$ binding, we can specifically examine the effects of Gβγ-promoted PLC-β activity in cells and *in vivo* in the absence of Gα$_q$ signaling.

The mechanism of Gβγ-promoted activation of PLC-β isozymes is currently unknown and an important future pursuit to fully understand heterotrimeric G protein activation of PLCs. A future direction of these studies is to generate a high-resolution

crystal structure of a PLC-β isozyme in complex with Gβγ. Gβγ has a higher affinity for PLC-β2 than PLC-β3, suggesting that PLC-β2 may be a better crystallography target for this complex (Park et al., 1993a; Smrcka and Sternweis, 1993). A fragment of PLC-β2 containing the N-terminal PH domain, four EF hands, catalytic TIM barrel, and C2 domain was used to generate two crystal structures and a similar fragment of PLC-β3 was used to generate the PLC-β3·Gα$_q$ structure (Jezyk et al., 2006; Hicks et al., 2008). Based on these observations and previous studies suggesting that Gβγ binds to the PH domain and Y box of PLC-β2, this fragment of PLC-β2 is a good initial target for crystallizing the PLC-β2·Gβγ complex (Kuang et al., 1996; Sankaran et al., 1998; Wang et al., 1999b; Wang et al., 2000).

Structural information from PLC-β2 in complex with Rac1 and the PLC-β2 holoenzyme provide a possible mechanism for Gβγ-stimulated activation of PLC-β isozymes. Comparison of these two crystal structures revealed that PLC-β2 does not undergo a conformational change upon Rac1 binding. In addition, an ordered portion of the X/Y linker blocks the active site, forming hydrogen bonds with several residues on the catalytic TIM barrel (Jezyk et al., 2006; Hicks et al., 2008). Removing this ordered portion or the entire X/Y linker of PLC-β2 increased basal PLC activity by 5-20-fold in intact cell assays. These results were confirmed with purified proteins in reconstitution experiments (Hicks et al., 2008). Removing the X/Y linker of PLC-β1 or divergent PLC isozymes PLC-δ1 and PLC-ε also caused a robust increase in basal PLC activity. The X/Y linkers of PLC isozymes vary in length and amino acid sequence, but they all contain a large number of negatively charged residues. Hicks et al. proposed that Rac1 recruits PLC-β isozymes to lipid bilayers and negatively charged membranes repel the

negatively charged X/Y linker, releasing autoinhibition of PLC-β (Hicks et al., 2008). The authors also proposed that this is a possible mechanism for Gβγ-dependent activation of PLC-β isozymes. Comparison of the PLC-β2 holoenzyme to a structure of Gβγ in complex with PLC-β2 will provide evidence for either Gβγ-dependent recruitment of PLC-β2 to the membrane or a conformational change to confer activation.

G protein-coupled signaling transmits information from extracellular stimuli to intracellular compartments and controls a host of physiological responses including cardiac function, vision, olfaction, and neurotransmission. Cell surface receptors, like PAR2 and other GPCRs, are essential for transmitting these signals across the plasma membrane by coupling to heterotrimeric G proteins and promoting activation of intracellular protein effectors like PLC-β. Each of these components of the G protein-linked signaling pathway is tightly regulated to maintain the fidelity and temporal control of transmembrane signaling. The work presented here and the proposed future directions will further our knowledge of PAR2 signal regulation at the cell surface and PLC-β activation and deactivation within intracellular compartments.

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