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Scaffolding of the Cofilin Pathway by Beta-arrestin-1

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biochemistry and Molecular Biology

by

Alice Lin

December 2012

Dissertation Committee:
Dr. Kathryn A. DeFea, Chairperson
Dr. Jolinda A. Traugh
Dr. Leonard J. Mueller

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The Dissertation of Alice Lin is approved:

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Committee Chairperson

University of California, Riverside

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It was a risky decision to come to University of California, Riverside right after I finished my undergraduate degree in Taiwan. I will never become independent and brave as I am right now if I did not choose to come here alone and challenge myself. Although there were some difficulties along the journey, my family and friends are always supportive.

Special thanks and love to my parents, who may not really understand what I am studying, but they always listen to me and believe in me.

"Life will find a way." This wonderful quote was in my statement of purpose for applying the PhD program here. I am really glad that I finally overcome all the doubts and obstacles and find my own way in research and in life here in United State.

ABSTRACT OF THE DISSERTATION

Scaffolding of the Cofilin Pathway by Beta-arrestin-1

by

Alice Lin

Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology

University of California, Riverside, December 2012

Dr. Kathryn A. DeFea, Chairperson

β-arrestins are well-known mediators involved in both activation and termination of G-protein-coupled receptor (GPCR) signaling pathways. β-arrestins transduce signals by binding to, and either activating or inhibiting, various downstream signaling proteins, leading to a variety of cellular responses, which can occur independent of the heterotrimeric G-protein coupling. Since ~40% of currently used therapeutics target GPCRs, understanding the functions of β-arrestins is an important biological question. We established a model that shows β-arrestins mediate the actin cytoskeletal reorganization downstream of protease-activated receptor-2 (PAR-2) by temporally and spatially controlling cofilin activation. Cofilin severs actin filaments and is controlled by opposing actions of LIM kinase (LIMK) (which inactivates it by phosphorylation on Ser3) and cofilin-specific phosphatase, chronophin (CIN) that activates it. My study elucidated

how β-arrestin-1 (β1) can interact and regulate LIMK and cofilin in PAR-2-enhanced actin dynamics. First, β1 bound and inhibited LIMK activity directly. At least two specific binding sites on β1 to LIMK1 or to cofilin were identified within residues 1 to 99 and 183-418 by using multiple recombinant β1 truncations and a deletion mutant (residues 146 to 182 were deleted) in sandwich immunoassays. The deletion mutant showed high binding affinity and direct inhibition of LIMK1, which suggested that if β1 is induced into the appropriate conformation for exposing the amino and carboxyl binding regions, β1 can directly bind and inhibit LIMK1 activity. β1 may protect cofilin from phosphorylation and inactivation, too. Results from spot peptide arrays confirmed the amino region of β1 was the specific binding site for LIMK1. Co-immunoprecipitation and bioluminescence resonance energy transfer assay further proved the interaction of β1 and LIMK1 in cells. The absence of β-arrestins disrupted the cofilin activation and caused abnormal development of growth cones and mature dendritic spines in mouse hippocampus. This dissertation demonstrated that the specific regions in β1 scaffold and regulate the kinase activity of LIMK1. Thus, β1 contributes to the spatial regulation of cofilin activation by sequestering cofilin with CIN while inhibiting LIMK at cell protrusions.

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CHAPTER ONE

Introduction

Chapter 1 Introduction

Chemotaxis, the directed cell migration of cells toward agonists, is critical in immunity, angiogenesis, wound healing, embryogenesis, and neuronal patterning. Chemotaxis first requires a chemoattractant to interact with a cell-surface receptor, then stimulate the reorganization of actin cytoskeleton and then form a leading edge (Ponti, Machacek et al. 2004). Recently, β-arrestins, well-known mediators of both activation and termination of G-protein-coupled receptor (GPCR) signaling pathways, have been emerged as key regulators of chemotaxis contributing to temporal and spatial actin reorganization. We have shown that β-arrestins scaffold members of cofilin pathway, cofilin, LIM kinase 1 (LIMK1) and chronophin (CIN) downstream of protease-activated receptor-2 (PAR-2). This research project elucidates the molecular mechanism and the details about how β-arrestins specifically interact with these molecules, and further temporally coordinate and spatially control cytoskeleton reorganization (Figure 1.1).

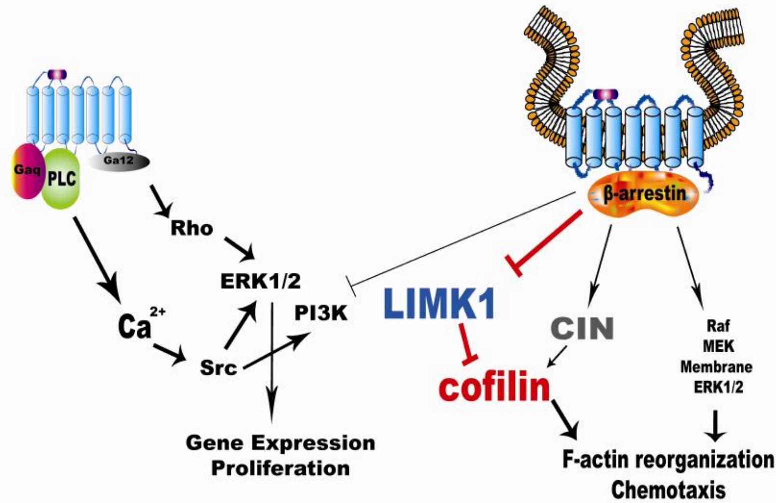


Figure 1.1 Multiple signaling arms downstream of protease-activated receptor-2. Activation of PAR-2 by serine proteases or activating peptides to unveil a tethered ligand is likely to induce a conformational change within the transmembrane helices that expose receptor cytoplasmic surfaces important for interaction with the α subunit of heterotrimeric G proteins at the intercellular loop of the receptor. The activated PAR-2 couples to $G_{\alpha q/11}$ and subsequently leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Presence of cytosolic IP3 induces intracellular influx of calcium (Ca^{2+}), where DAG and Ca^{2+} activate protein kinase C (PKC). Subsequent activation of mitogen-activated kinase proteins (MAPK) by PKC leads to activation and nuclear translocalization of ERK1/2. On the other hand, activated PAR-2 can also elicit signaling independently of G-protein coupling by binding of β-arrestins to agonist-induced PAR-2 triggers the assembly of a MAP kinase activation complex using β-arrestins as scaffold, which subsequent activation of β-arrestin-bound pool of ERK1/2. The function of β-arrestin-bound ERK1/2 is presently unknown, possibly favoring the phosphorylation of plasma membrane, cytosolic or cytoskeletal ERK1/2 substrates. β-arrestins also chemotaxis by scaffolding cofilin signaling pathway members, cofilin, LIMK1 and CIN.

1.1 β-arrestin-dependent signaling pathway

β-arrestins (β-arrestin-1 and β-arrestin-2) are mediators of both GPCR signaling and signal termination. Traditionally, GPCR signaling transduction was thought to be entirely dependent on heterotrimeric G-proteins, but the paradigm has recently shifted as many studies prove that β-arrestins can mediate GPCR signaling without G-proteins. The traditional role of β-arrestins is to uncouple, internalize and desensitize GPCRs by being recruited to membrane and binding to the stimulated GPCRs. However, β-arrestins can also serve as adaptors to pleitropic scaffold proteins and lead to localized activation and inhibition of signaling molecules. β-arrestins not only are capable of bringing key components of a signaling cascade in proximity to each other, but they may also directly affect the enzymatic activities of downstream signaling molecules. This β-arrestin-dependent GPCR signaling adds a new twist to the function of cellular trafficking machinery (Shenoy and Lefkowitz 2003; Ma and Pei 2006; Zoudilova, Kumar et al. 2007).

1.1.1 The characteristics of β-arrestin-1

Studied based on the crystal structures of β-arrestins provide valuable information about the mechanism of scaffolding and regulation by β-arrestins to the downstream signaling molecules (Figure 1.2). There are more than 100 binding partners have been proteomics screen identified, however, only a fraction of them have been demonstrated biochemically to interact with β-arrestins. By adopting multiple conformations that expose different sets of binding regions, β-arrestins can interact with different GPCRs to promote formation of a specific set of complexes (Figure 1.3). The crystal structures of β-arrestin-1 and β-arrestin-2 share similar characteristics with visual arrestin-1. The structure of β-arrestin is consists of two lobes that can rotate on a central hinge region. The sequence between residues 357-382 is the "disordered region", containing the clathrin binding domain, which is exposed upon activation of GPCRs. Binding to GPCR appears to induce a conformation change results in closer apposition of the two lobes and exposure of the clathrin binding domain. Additional region composed of amino acid residues 282-309 is the highly conserved "lariat" loop that has been shown to act as a polar core, which is important for stabilizing the arrestins in their basal conformations.

The residues 46-86 appear to be important for binding to most GPCRs. There are other potential binding sites for receptor including peptide 164-172 or 44-66. However, these peptides do not form a contiguous surface on β-arrestin suggests that β-arrestin may have more than one conformation for receptor bindings. The receptor-bound β-arrestin adopts in a major conformation change that allows C-tail to move away from the original position and exposes potent binding regions to downstream molecules such as ERK1/2 or

Raf. The scaffolding of signaling molecules by β-arrestins will be discussed in the next session (Han, Gurevich et al. 2001; Gurevich and Gurevich 2003; Vishnivetskiy, Hosey et al. 2004).

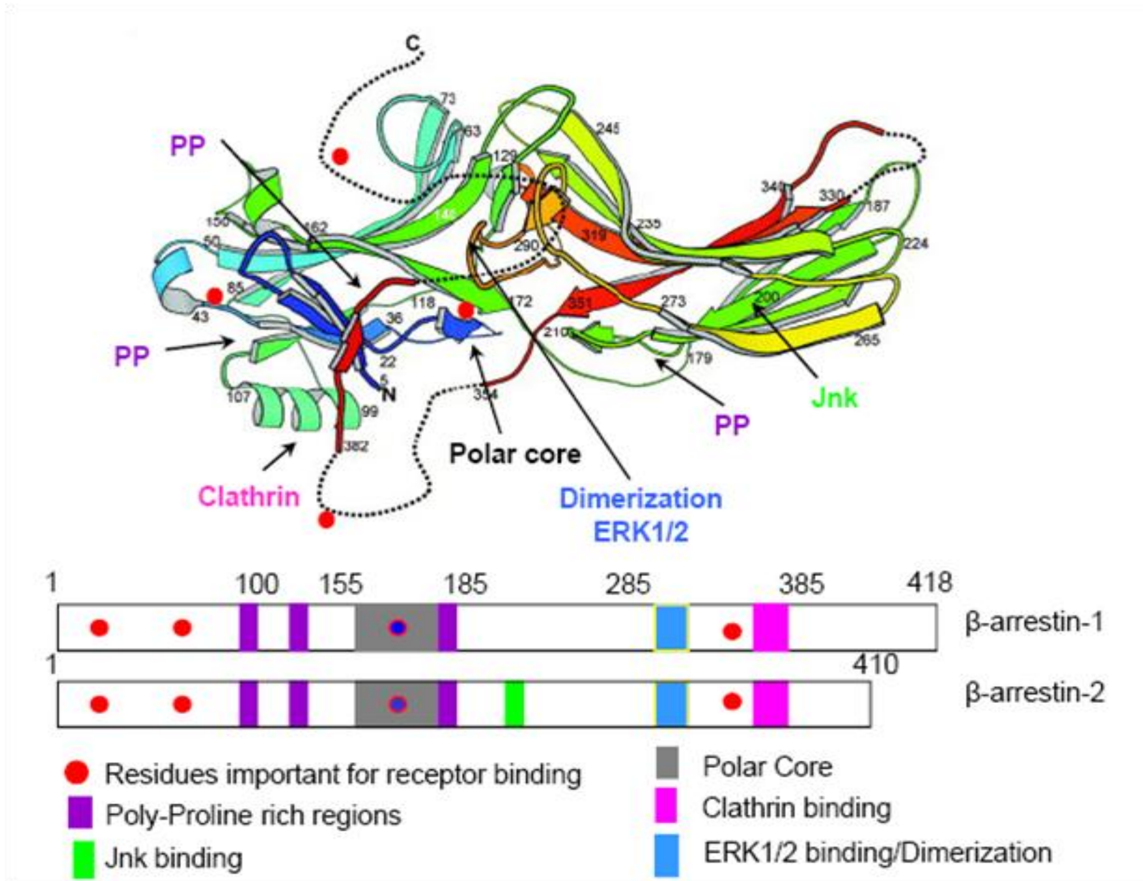


Figure 1.2 Structural predictions for β-arrestin-scaffolding protein interactions.
A ribbon diagram of predicted inactive β-arrestin structure is shown, with interacting domains identified for various signaling proteins indicated by colored arrows and boxes. Red circles indicate residues thought to mediate receptor binding.

(A)

```
P49407      MGDK-GTRVFKKASPNGKLTVYLGKRDFVDHIDLVDPVDGVVLVDPEYLKERRVYVTLTC     59     ARRB1
P32121      MGEKPGTRVFKKSSPNCKLTVYLGKRDFVDHLDKVDPVDGVVLVDPDYLKDRKVFVTLTC     60     ARRB2
            **:* *******:*** **************:* ************:***:*:*:*****

P49407      AFRYGREDLDVLGLTFRKDLFVANVQSFPPAPEDKKPLTRLQERLIKKLGEHAYPFTFEI     119    ARRB1
P32121      AFRYGREDLDVLGLSFRKDLFIATYQAFPPVPNPPRPPTRLQDRLLRKLGQHAHPFFFTI     120    ARRB2
            **************:******:*..*:***.*:..:******:**::***:**:*******

P49407      PPNLPCSVTLQPGPEDTGKACGVDYEVKAFCAENLEEKIHKRNSVRLVIRKVQYAPERPG     179    ARRB1
P32121      PQNLPCSVTLQPGPEDTGKACGVDFEIRAFCAKSLEEKSHKRNSVRLVIRKVQFAPEKPG     180    ARRB2
            ************************:*::****..****.**************:***:**

P49407      PQPTAETTRQFLMSDKPLHLEASLDKEIYYHGEPISVNVHVTNNTNKTVKKIKISVRQYA     239    ARRB1
P32121      PQPSAETTRHFLMSDRSLHLEASLDKELYYHGEPLNVNVHVTNNSTKTVKKIKVSVRQYA     240    ARRB2
            ***:*****:*****:.**********:******:.********:.*******:******

P49407      DICLFNTAQYKCPVAMEEADDTVAPSSTFCKVYTLTPFLANNREKRGLALDGKLKHEDTN     299    ARRB1
P32121      DICLFSTAQYKCPVAQLEQDDQVSPSSTFCKVYTITPLLSDNREKRGLALDGKLKHEDTN     300    ARRB2
            *****.*********.**.**.*.**********:**:*..*******************

P49407      LASSTLLREGANREILGIIVSYKVKVKLVVSRGGLLGDLASSDVAVELPFTLMHPKPKEE     359    ARRB1
P32121      LASSTIVKEGANKEVLGILVSYRVKVKLVVSRGG--------DVSVELPFVLMHPKPHDH     352    ARRB2
            *****:::****:*:***:***:***********        **:*****.******:**

P49407      P----PHREVPENETPVDTNLIELDTN---DDDIVFEDFARQRLKGMKDDKEEEEDGTGS     412    ARRB1
P32121      IPLPRPQSAAPETDVPVDTNLIEFDTNYATDDDIVFEDFARLRLKGMKDDDYDD------     406    ARRB2
            .    *...***.:.************   ***********.********..::

            PQLNNR
            -QLC-
             **:
```

(B)

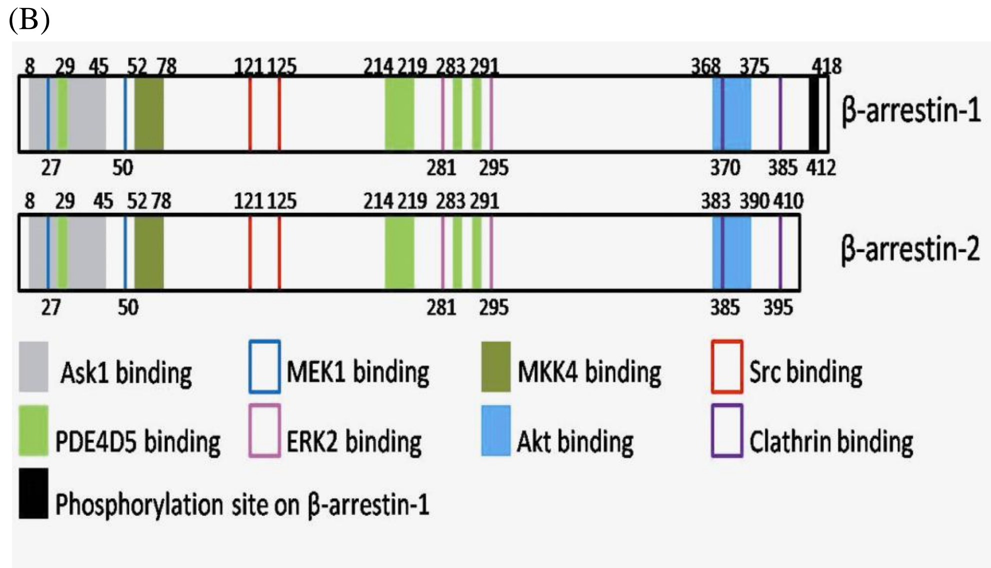


Figure 1.3 Comparison of amino acid sequences of β-arrestin-1 and β-arrestin-2.
(A) Alignment of putative interaction domains on β-arrestin-1 and 2. The sequence of β-arrestin-1 and 2 are aligned and domains that have been shown to mediate interactions with various downstream targets are shown. Gray shading indicates Ask1 binding sites and blue boxes indicate MEK1 binding sites, with red letters indicating specific amino acids that are required for binding to both. Dark green shading indicates MKK4 binding sites. Red shading indicates polyproline stretches important for Src SH3 domain interactions. Green boxes indicate PDE4D5 binding sites. Pink shading indicates ERK2 binding sites with essential lysines highlighted in white. Light blue shading indicates an Akt binding motif. Black box indicates clathrin binding domain. Black shading indicates site for C-terminal serine phosphorylation on β-arrestin-1.
(B) The binding domains of β-arrestin-1 and β-arrestin-2 are aligned and drawn into color boxes for better demonstration.

1.1.2 The scaffolding role of β-arrestins

Recently, there have been increasing numbers of studies on the scaffolding role of β-arrestins. While β-arrestins serve as adaptor proteins that are recruited to G-protein-coupled receptors (GPCRs) to promote receptor desensitization and internalization, their newly discovered ability to promote G-protein-independent signals, has revealed an even more diverse array of responses than originally thought. Upon GPCR binding, β-arrestins scaffold different proteins into signaling complexes; by now over 100 β-arrestin binding partners have been identified (Lefkowitz and Shenoy 2005; Shukla, Xiao et al. 2011). Out of these numerous β-arrestin scaffolding complexes that have been reported, many of them contain kinases, such as mitogen-activated-protein kinases (MAPKs), Raf, MEK1, Ask1, MKK4, phosphoinositol-3-kinase (PI3K), Akt, AMPK and LIM kinase (LIMK) (DeFea 2011; Shukla, Xiao et al. 2011). In some cases, the effect of β-arrestin association is positive; in others, association with β-arrestins directly inhibits enzymatic activity. In still other cases, such as PI3K and Akt, β-arrestins appear to be able to positively and negatively regulate kinase activity (Defea 2008). The regulation of kinases is a critical step in signal transduction and, despite the known role of β-arrestins in this process; the underlying molecular mechanisms remain poorly understood. The more we understand about the interactions of β-arrestins with variety of kinases, the more we determine the important roles of β-arrestins for different cellular effects.

This dissertation focuses on the scaffolding and regulation of LIM kinase 1 and its substrate, cofilin, by β-arrestins. Data and results will be discussed in chapter 2 and 3.

Moreover, the scaffolding and regulating of different kinases by β-arrestins are reviewed in this chapter, too (See the *session 1.4~1.6, chapter 1*).

1.2 Protease-activated receptor-2 (PAR-2)

The scaffolding functions of β-arrestins are typically associated with G-protein coupled receptor (GPCR) activation, and in fact regulation of LIMK activity by β-arrestins occurs downstream of a specific GPCR, protease-activated-receptor-2 (PAR2) and may occur downstream of other GPCRs as well. About 2000 GPCRs are expressed from approximately 600 genes in human genome have been identified. These GPCRs respond to different extracellular stimuli (including light, odor, peptides, neurotransmitters, lipids, inflammatory molecules and hormones) and have been reported to relate to numerous diseases making them important therapeutic targets (Lappano and Maggiolini ; Lander, Linton et al. 2001; Rana, Shiina et al. 2001; Venter, Adams et al. 2001).

PAR-2 is one of four members in the protease-activated receptor subfamily of GPCRs, all of which are activated by proteolytic cleavage on their extracellular amino termini, which reveals tethered ligand that then binds to PARs for activation(Hollenberg and Compton 2002). While the ligand of PAR-1, PAR-3 and PAR-4 is identified as trypsin, the physiological agonist of PAR-2 is diverse. Many proteases, including pancreatic trypsin, mass cell tryptase, coagulation factor (factor VII/Xa) and a membrane-bound serine protease (MBSPII) perform the N-terminal cleavage and activate PAR-2 (Dery and Bunnett 1999; Camerer, Huang et al. 2000; Coughlin 2000; Takeuchi, Harris et al. 2000; Coughlin and Camerer 2003; Stefansson, Brattsand et al. 2008). PAR-2 is widely expressed numerous tissues including brain, eye, airway, heart, GI tract, pancreas, kidney, liver, prostate, overy, testes, skin and cardiovascular system (Bucci,

Roviezzo et al. 2005) as well as in different cell types: immune cells, osteoblasts, myocytes, fibroblasts, neurons, glial cells, endothelial and epithelial cells. Moreover, expression of PAR-2 has been reported in many tumor cell lines from lung, colon, breast, and prostate. PAR-2 signaling pathway indeed controls variety of cellular responses, such as proliferation, chemotaxis, ion transportation and tumor metastasis (Figure 1.4). Because we have previously demonstrated that PAR2 promotes inhibition of LIMK activity through β-arrestins which then contributes to cofilin activation and actin cytoskeletal reorganization, many of my studies utilize PAR-2 signaling as a means of inducing LIMK/β-arrestin interactions.

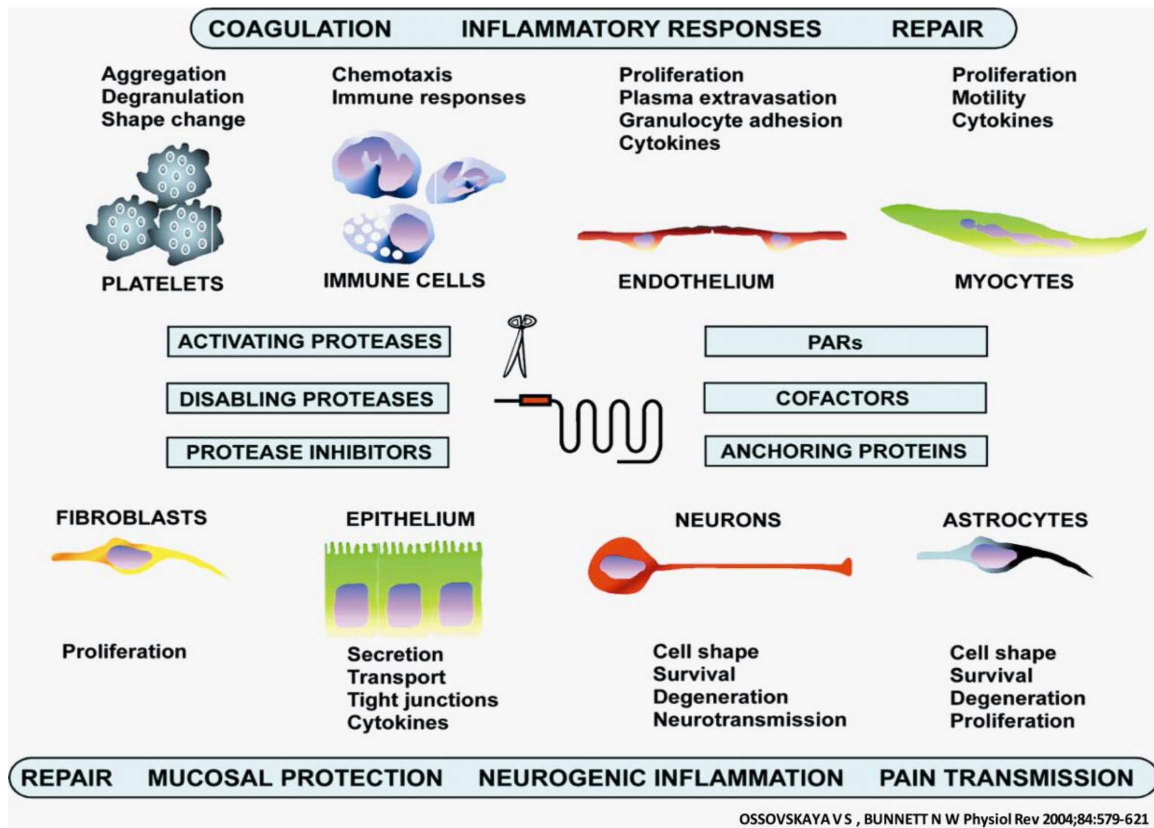


Figure 1.4 A reference summary of the potential physiological roles of protease and PARs in different cell types.
Signaling depends on the availability of proteases, which can activate or disable PARs, protease inhibitors, PARs, and protease cofactors and anchoring proteins. PARs signal a wide variety of cells to regulate critically important biological processes, with implication for physiological regulation and disease. (Figure is cited from Ossovskaya, V. S. and N. W. Bunnett (2004). "Protease-activated receptors: contribution to physiology and disease." Physiol Rev **84**(2): 579-621.)

1.2.1 The activation of PAR-2 by the tether ligand

The extracellular amino terminal cleavage of PAR-2, which is executed by trypsin and many other proteases, exposes the tethered ligand that bind to the second extracellular domain of PAR-2, change its conformation, and triggers downstream signaling (Nystedt, Emilsson et al. 1994; Nystedt, Emilsson et al. 1995; Nystedt, Larsson et al. 1995). The proteases cleave PAR-2 at $R^{34}\downarrow S^{35}$LIGKV to reveal the tethered ligand SLIGKV in humans and at $R^{34}\downarrow S^{35}$LIGRL to expose SLIGRL in mouse (Vu, Hung et al. 1991; Hollenberg and Compton 2002). Synthetic peptides that mimic the first six amino acids of the newly formed N-terminus (SLIGRL-amide in mouse and SLIGKV-amide in humans) can activate PAR-2 independent of proteolytic cleavage (Vu, Hung et al. 1991; Vu, Wheaton et al. 1991; Vu, Liu et al. 1997). More recently, synthetic activating peptide with an N-terminal furoyl group modification (*e.g.,* 2-furoyl-LIGRL-ornithine-NH_2 (2fAP)) mimics the tethered ligands, and has been reported to be equally effective to activate PAR-2 and more potent than SLIGRL-amide (Bernatowicz, Klimas et al. 1996; McGuire, Saifeddine et al. 2004; Kanke, Ishiwata et al. 2005). Thus, in these studies, we use this specific ligand, 2fAP, to specifically activate PAR-2 even in the absence of enzymatic cleavage (Ge, Ly et al. 2003).

The activated PAR-2 changes its conformation and couples to $G_{\alpha q/11}$ and subsequently leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Presence of cytosolic IP3 induces intracellular influx of calcium (Ca^{2+}), where DAG and Ca^{2+} activate PKC (Corvera, Dery et al. 1997; Nguyen, Moody et al. 1999). Activated PKC subsequent

activates downstream signaling transductions such as mitogen-activated protein kinase (MAPK) and ERK1/2. However, unlike other PARs, once PAR-2 is activated, the G-protein-dependent signaling pathway is not the only downstream signaling. Via β-arrestins coupling, PAR-2 can also trigger several cellular events without G protein involvement. For example, our laboratory discovered that the β-arrestin-dependent signaling downstream of PAR-2 prolongs ERK activation and sub-cellular localization of the ERK1/2 module to the membrane. The activation of PAR-2 not only regulates the MAPK module, but also stimulates many other downstream β-arrestin-dependent signaling pathways and leads to the activations of PI3K, RhoA, JNK, NFκB and cofilin (Zoudilova, Min et al. ; DeFea, Vaughn et al. 2000; DeFea, Zalevsky et al. 2000; Ge, Ly et al. 2003; Ge, Shenoy et al. 2004; Stalheim, Ding et al. 2005; Wang and DeFea 2006; Wang, Kumar et al. 2007; Zoudilova, Kumar et al. 2007).

PAR-2 is one of the first GPCRs shown to utilize β-arrestin-dependent scaffolding to facilitate chemotaxis. One study demonstrated that PAR-2-mediated motility in breast cancer cell lines requires β-arrestins and secreted trypsin for ERK1/2 activation and cell migration, suggesting that autocrine activation of PAR-2 by secreted proteases may contribute to the migration of metastatic tumor cells through a β-arrestin scaffolding complex (Ge, Ly et al. 2003; Ge, Shenoy et al. 2004). In 2007, we further revealed a novel β-arrestin-dependent mechanism of cofilin activation downstream of PAR-2 activation. We suggested that the cofilin-mediated actin activity is mediated by β-arrestins coupling with cofilin regulators, LIM kinase 1 (LIMK1) and chronophin (CIN) results in increasing actin reorganization and chemotaxis.

1.3 Actin filament reorganization by cofilin

Actin filaments (F-actin) are polar structures with fast-growing barbed end that dominates assembly kinetics and a slow-growing pointed end. Cells must create new barbed ends quickly for the rapid changes necessary for chemotaxis or cell migration. Cofilin severs F-actin filaments to generate more barbed ends that can serve as de novo actin nucleation sites, so the rate of actin polymerization increases (Figure 1.5). Detailed information about cofilin is presented in the chapter 4.

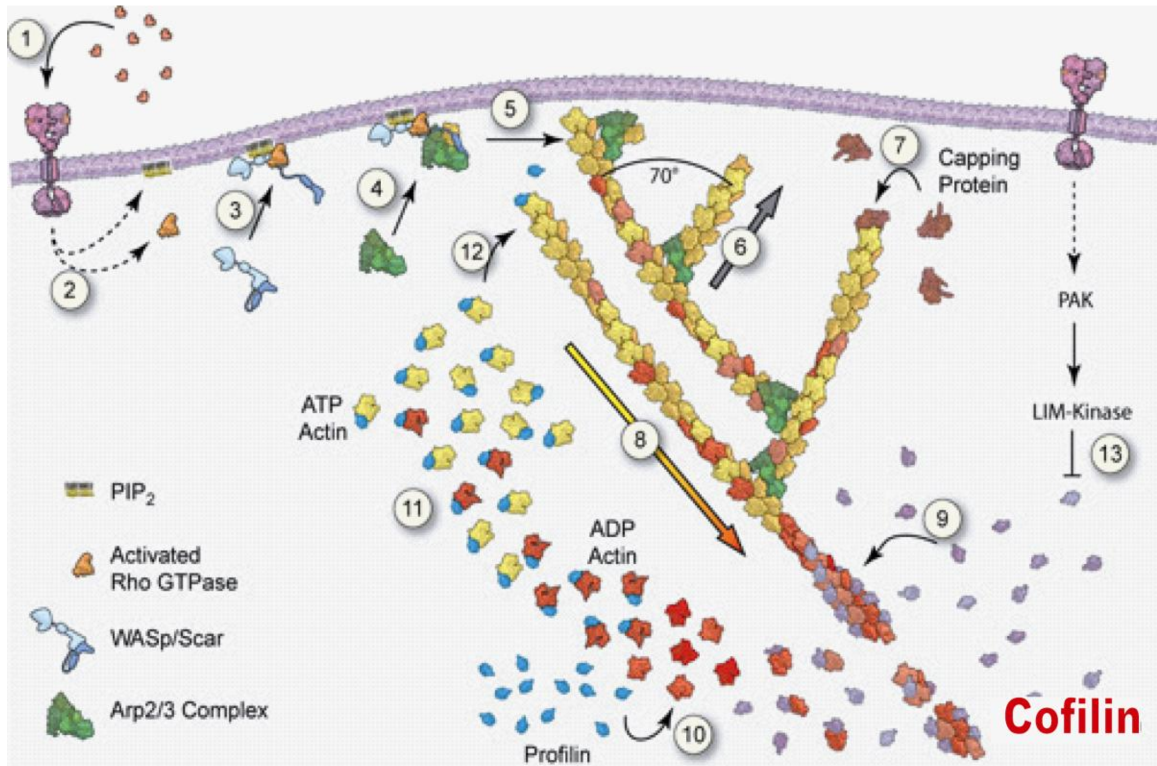


Figure 1.5 A summary cartoon figure to demonstrate the function of cofilin in the mechanism actin filament reorganization.

Cofilin binds to the existing actin filament (F-actin) between adjacent subunits along the two helix. Binding of cofilin induces twisting of actin filaments and introduces strains in the actin filament. This contributes to the increase in the filament dynamics by increasing the rate of actin monomer recycling and actin filament reorganization.

1.3.1 LIM kinase 1 (LIMK1)

LIM kinase 1 is a member of the LIM kinase family (LIMK1 and LIMK2). LIM kinase regulates the actin cytoskeleton by phosphorylating cofilin on serine 3, and inactivating it. LIMK activity can be regulated by several upstream signaling pathways. LIMK activity can be regulated by several upstream signaling pathways that activate Rho GTPases. These Rho GTPases phosphorylate LIMK and increase its activity (Figure 1.6). Immunohistochemical staining demonstrates that LIMK1 is widely expressed in embryo and adult, especially high expression in the brain, kidney, lung, stomach and testis. Defections of LIMK1 result in variety of human diseases. The human genetic multi-syndrome disorder, Williams-Beuroen syndrome (WBS) has been implicated caused by the microdeletion of *limk1* on chromosome 7. Deletion of *limk1* leads to abnormalities in synaptic structure and spine development most likely due to aberrant regulation of actin cytoskeleton, causing deficits similar to those observed in WBS, such as behavioral alterations and cardiovascular diseases. Also, the hyperactivation of LIMK1 is observed in Alzheimer's disease. This evidence indicates the development of normal central nervous system is reliant upon the presence of LIMK1. Furthermore, dys-regulation of LIMK is associated with tumor cell invasiveness and metastasis as well as cardiovascular disorders, since in ithe absence of LIMK1 the balance between phosphorylation and dephosphorylation of cofilin is altered which can cause actin cytoskeleton disorders. Structural studies reveal that LIMK1 has variety possible binding domains (Figure 1.7.). Via different protein-protein interactions, LIMK1 can integrate upstream signaling and then regulate downstream signaling (Tassabehji 2003; Foletta, Moussi et al. 2004; Morris,

Lenhoff et al. 2006; Scott and Olson 2007; Wen, Han et al. 2007; Pober, Johnson et al. 2008; Yildirim, Janssen et al. 2008).

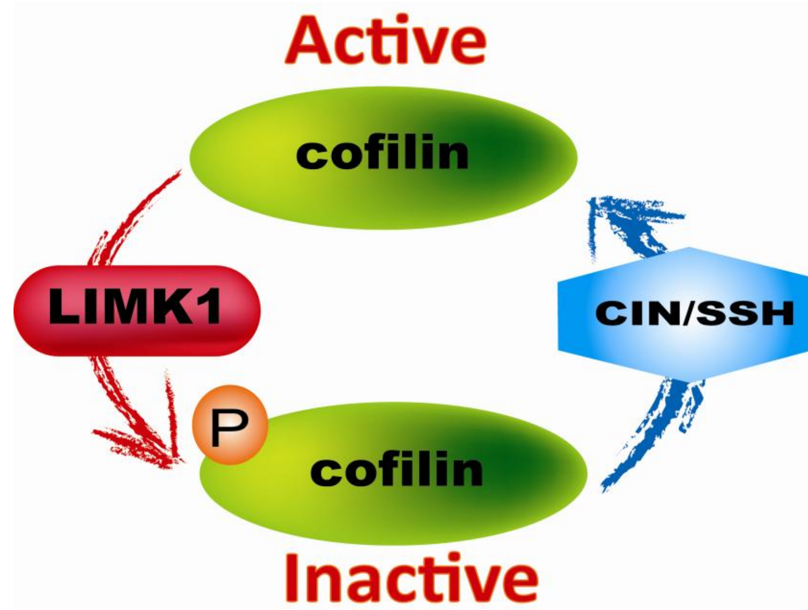


Figure 1.6 Regulation of cofilin activity.
LIMK1 phosphorylates and inactives cofilin, while chronophoin (CIN) or Slingshot (SSH) dephosphorylates cofilin and activates its actin sever ability.

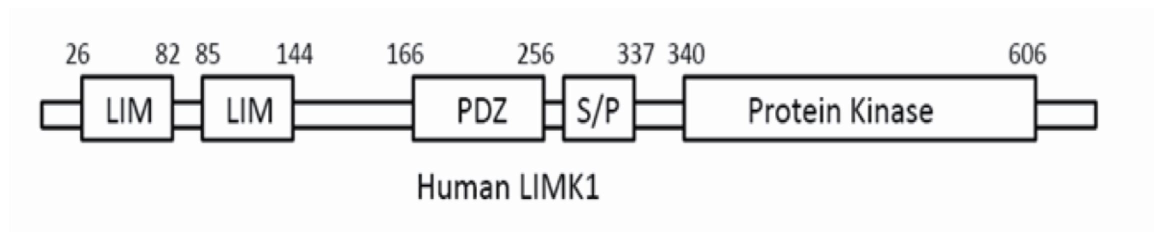


Figure 1.7 Functional domains of LIMK1.
LIMK1 has a unique organization of signaling domains, with two amino-terminal LIM domains, adjacent PDZ and proline/serine (P/S)-rich regions, followed by a carboxyl-terminal kinase domain. LIM and PDZ both have highly possible function of mediating protein-protein interactions.

1.3.2 Cofilin

β-arrestins regulate the cofilin activation by directly regulating the LIMK1 and scaffolding its substrate cofilin. LIMK phosphorylates and inactivates the actin filament severing protein, cofilin in response to various extracellular stimuli. Dephosphorylated cofilin binds to filamentous actin (F-actin), destabilizing it and creating multiple smaller F-actin seeds with free barbed ends to which G-actin monomers rapidly add. LIMK thus acts as an important regulator of actin dynamics as its activation stabilizes F-actin and its inactivation results in dynamic cytoskeletal reorganization. Both processes are crucial to directed cell migration or chemotaxis. Phosphorylation of LIMK at Thr^{508} by Rho-Activated Kinase (Rajagopal, Whalen et al.) and p21-activated kinase-1 (PAK1) within the catalytic loop results in a conformational change that increases its activity. Binding of hsp90 to LIMK promotes homodimerization which then facilitates trans-autophosphorylation within the kinase domain that further stabilizes the active conformation (Li, Soosairajah et al. 2006). Downstream of PAR-2 activation, β-arrestin-1 mediates inhibition of LIMK activity and a concurrent decrease in cofilin phosphorylation, as indicated by the fact that siRNA knockdown of β-arrestins abolishes the PAR-2 stimulated decrease in LIMK activity and cofilin dephosphorylation. β-arrestin-dependent activation of cofilin has also been observed downstream of At1AR although a role for LIMK inhibition in this pathway has not been investigated. The LIMK kinase domain directly interacts with β-arrestin-1 in vitro and LIMK co-localizes with β-arrestin-1 at the plasma membrane upon PAR-2 activation. However, in previous studies, β-arrestin knockdown did not affect LIMK phosphorylation. These data suggest that β-

arrestins might directly inhibit LIMK activity even in the presence of ROCK or PAK-mediated phosphorylation. Likely mechanisms by which β-arrestins inhibit LIMK activity are competition with cofilin for binding to the catalytic domain and/or interference with ATP binding. Because β-arrestins also bind cofilin, one cannot rule out the possibility that they act as a "substrate sponge" sequestering cofilin away from LIMK and protecting it from phosphorylation. Additionally, given that β-arrestins can bind hsp90, another possible mechanism is they sequester hsp90 from LIMK. This latter model does not take into account, the direct interactions of LIMK and β-arrestin-1. Additionally, β-arrestins regulate the downstream target of LIMK, cofilin, by scaffolding it with its upstream phosphatases, slingshot (SSH) and chronophin (CIN).

1.4 Regulation of MAPKs and their upstream kinases by β-arrestins

Mitogen-activated-kinases (MAPKs) were the first β-arrestin signaling targets discovered and elucidation of their regulation paved the way for the identification of numerous other β-arrestin binding partners and signaling complexes(Shenoy and Lefkowitz 2003; Shenoy and Lefkowitz 2005). The typical MAPK "module" is composed of three kinases: MAPKK kinase (MAPKKK) serine phosphorylates the dual specificity kinase, MAPK kinase (MAPKK or MEK). β-arrestins can form complexes with MAPK modules from the extracellular regulated kinase 1 and 2 (ERK1/2) and Jun-kinase (Jnk) subfamilies (Lefkowitz and Shenoy 2005; DeWire, Ahn et al. 2007; DeFea 2011; Shukla, Xiao et al. 2011). In both cases, association with β-arrestins both facilitates activation of the kinase cascade and ensures the proper sub-cellular localization. The mechanisms by which β-arrestins activate these cascades and the composition of individual MAPK module/β-arrestin complexes vary between receptors.

1.4.1 Activation of the ERK 1/2 cascade

β-arrestins scaffold the ERK1/2 module kinases into multi-protein signaling complexes downstream of many receptors (DeWire, Ahn et al. 2007; DeFea 2011), regulating their activation, sub-cellular localization and ultimate cellular responses. In the ERK1/2 pathway, the MAPKKK is the oncoprotein, Raf. Raf phosphorylates and activates the dual specificity kinases, MEK 1 and 2, which in turn phosphorylate and activate ERK1/2. Therefore, Raf is the upstream kinase in the ERK1/2 module. Raf consists of an N-terminal regulatory and a C-terminal kinase domain. In its inactive state, interactions between the N and C-termini render Raf inactive. The most common

mechanism for activation of Raf involves binding to the small G-protein, Ras, which brings it to the membrane and induces a conformational change that leads to exposure of the C-terminal kinase domain (Winkler, Cutler et al. 1998; Luttrell, Ferguson et al. 1999; Terai and Matsuda 2005). Subsequently, phosphorylation of Raf by secondary kinases such as PKCε and PAK, as well as autophosphorylation, can stabilize the active conformation (Basu and Sivaprasad 2007; Zang, Gong et al. 2008). The most common mechanisms for activating the ERK1/2 module downstream of GPCRs involve either G-protein βγ subunits, $G\alpha_q$-dependent Ca^{2+} and PKC or transactivation of receptor tyrosine kinases, all of which can lead to Ras-dependent ERK1/2 activation (Luttrell, Roudabush et al. 2001; Luttrell 2003). However, it is now clear that β-arrestins can activate ERK1/2 independently of these more common pathways. Over a decade ago, studies on the β-2 adrenergic receptor (β_2AR) revealed that there was a β-arrestin-dependent component of ERK1/2 activation, involving scaffolding and activation of the tyrosine kinase, src (Luttrell, Ferguson et al. 1999). Subsequently studies revealed that a number of other receptors require β-arrestins for activation of ERK1/2, but there are receptor-specific differences in the composition of scaffolding complexes, localization of ERK1/2 and ultimate cellular consequence of the signals. For example a number of GPCRs, including PAR-2 and Angiotensin II 1A Receptor (AT1aR), lead to scaffolding of the entire MAPK module (Raf, MEK1 and ERK1/2) with β-arrestins (DeFea, Zalevsky et al. 2000; Luttrell, Roudabush et al. 2001; Tohgo, Pierce et al. 2002; Tohgo, Choy et al. 2003). Both receptors can promote a β-arrestin-dependent/Ras-independent signaling pathway for activation of MAPKs that results in cytoplasmic/membrane sequestration of the activated

kinases. In contrast, neurokinin-1 Receptor (NK1R) and β_2AR promote src and Ras-dependent activation of ERK1/2, resulting nuclear localization and proliferation (Ahn, Maudsley et al. 1999; DeFea, Vaughn et al. 2000; Luttrell, Roudabush et al. 2001). Interestingly, while PAR-2, AT1aR and β_2AR all promote G-protein independent, β-arrestin-dependent ERK1/2 activation, NK1R requires input from both pathways (Defea 2008). Over the past decade, numerous GPCRs have been shown to promote β-arrestin-dependent ERK1/2 activation through mechanisms similar to those identified for the receptors described above, leading to a considerable interest in understanding what determines the composition of each scaffold and how receptor-dependent variations occur(Gesty-Palmer, Chen et al. 2006; Rajagopal, Kim et al. 2010; Shukla, Xiao et al. 2011).

1.4.2 The regulation of Raf by β-arrestins

Because Raf is the most upstream kinase in the ERK1/2 module, it is the most likely target of direct regulation by β-arrestins in the activation cascade. Studies using co-immunoprecipitation and in vitro binding assays have shown that ERK2, MEK1 and Raf-1 can all associate with β-arrestin-1 and β-arrestin-2. Spot peptide arrays and truncated β-arrestin proteins have been used to identify major sites of interaction within each β-arrestin for each MAPK cascade component (Bolger, Baillie et al. 2006; Xu, Baillie et al. 2008; Cheung, Malik et al. 2009; Meng, Lynch et al. 2009; Song, Coffa et al. 2009). However, despite the ability of all three proteins to contact β-arrestin directly, they only stably associate with β-arrestin in cells downstream of certain receptors. Stable formation of a Raf/MEK1/ERK1/2 complex is only observed upon activation of a handful of

receptors, and is associated with Ras-independent ERK1/2 activation (DeFea, Zalevsky et al. 2000; Luttrell, Roudabush et al. 2001; Luttrell 2003; Tohgo, Choy et al. 2003). Current evidence suggests that the interactions of the kinases with β-arrestins may not be enough to trigger the MAPK cascade, unless the β-arrestins hold all the components into a specific conformation so phosphorylation chain can be passed down correctly (Song, Coffa et al. 2009). In another study forced heterodimers were used to demonstrate that membrane localization of β-arrestin-1 was sufficient to trigger ERK1/2 activation, leading to the hypothesis that the ERK1/2 cascade was triggered solely by recruitment to membrane-associated β-arrestins (Terrillon and Bouvier 2004). However, which scaffold was formed and which activation mechanism was triggered was not explored. Additionally, even the theory of "bringing the kinases together at the membrane" does not explain how β-arrestins can trigger Ras-independent Raf activation. A recently published study used alanine-scanning mutagenesis of β-arrestin to identify R307 as a critical residue for binding of β-arrestin-2 to Raf-1(Coffa, Breitman et al. 2011). Furthermore, a mutant β-arrestin-1 in which R307 was changed to A was unable to rescue β_2AR-induced ERK1/2 activation in cells lacking arrestins, confirming that β-arrestin-1 interaction with Raf is required for activation of the cascade. This result is particularly interesting given that β_2AR was not one of the receptors previously demonstrated to induced assembly of the ERK1/2 module onto β-arrestins, although it did promote redistribution of activated Raf to clathrin-coated vesicles(Daaka, Luttrell et al. 1998). Thus, it is possible that other receptors that utilize a different mechanism of β-arrestin-dependent ERK1/2 activation will require different residues within Raf for association

with β-arrestins. Additionally, the analogous mutation in β-arrestin-2 (K308A) did not affect Raf association, suggesting the two β-arrestins may mediate different mechanisms of Raf activation. R307 is located in an exposed surface of the "hinge" region of the non-receptor-bound β-arrestin-1. Structural analysis suggests that R307 of β-arrestin-2 forms a long-ion pair with negatively charged D29 (Coffa, Breitman et al. 2011). Interestingly, D29 was identified in spot peptide residues as a crucial amino acid for binding to MEK1. Thus, if Raf association disrupts this intramolecular interaction, this may serve to facilitate MEK association (Meng, Lynch et al. 2009). Several receptors require both β-arrestins for full activation of ERK1/2 and both β-arrestins have been shown to bind to Raf. An enticing hypothesis regarding the activation of Raf is that either or both β-arrestins can weakly associate with Raf in the absence of receptor binding and that receptor activation serves to bring β-arrestin bound Raf to the membrane, while inducing a conformational change that leads both to a more stable interaction and to exposure of the Raf kinase domain to its downstream substrate, MEK1/2. The fact that both N- and C-terminal truncations of β-arrestins can interact with Raf suggests that there may be a second binding site on the N-terminus that is exposed upon receptor activation and may be important for Raf activation. Additionally, β-arrestins may scaffold Raf with ser/thr kinases that further contribute to its stable activation.

1.4.3 Scaffolding of JNK3 cascade to β-arrestins

The β-arrestin-MAPK module scaffold system is not limited to the ERK module scaffold. It extends to another MAPK pathway, the c-Jun N-terminal kinase 3 (JNK3) signaling pathway. Similar to what was observed with ERK1/2, β-arrestin mediates can scaffold the three key players of the JNK3 kinase cascade together: the apoptosis signal-regulating kinase (ASK1), the dual specificity mitogen-activated protein kinase kinase 4 (MKK4), and c-Jun N-terminal kinase 3 (JNK3) (McDonald, Chow et al. 2000; Miller, McDonald et al. 2001). These interactions were originally observed using yeast-2 hybrid screens and later confirmed with co-immunoprecipitation, proteomics screens and spot peptide arrays. Although all the family members of arrestins (visual arrestin 1 and arrestin 4, non-visual β-arrestin-1 and β-arrestin-2) can interact with the JNK3 module, β-arrestin-2 was the only isoform that facilitated the ultimate phosphorylation and activation of JNK3 (McDonald, Chow et al. 2000; Miller, McDonald et al. 2001; Cheung, Malik et al. 2009). Thus, subtle structural differences between β-arrestins may not affect the association with the binding partners, but slight changes in the conformational arrangements of the proteins within the Jnk scaffold may hinder the downstream signaling.

As was the case for Raf activation, the mechanism by which β-arrestins activate the kinases of the JNk3 cascade remains unclear. The sequence of association may shed some light on the mechanism. One study suggested that while β-arrestin-2 can associate with ASK1, its activation does not require β-arrestins (Miller, McDonald et al. 2001; Seo, Tsakem et al. 2011). However, prior binding to Ask1 strengthened the interaction of β-

arrestin-2 with MKK4 and JNK3 and both MKK4 and Jnk3 phosphorylation are dependent on β-arrestin-2 binding. β-arrestin-2 has a higher affinity for MKK4 than does β-arrestin-1 while both have equal affinities for JNK3. Thus, there is some debate as to how this scaffold facilitates JNK3 activation. Based on cellular studies, one might propose that β-arrestin-2 brings the three kinases in proximity to each other, initiating the cascade by forcing interaction between MKK4 and ASK1. A recent study demonstrated that β-arrestins could directly activate JNK3 by MKK4 and have an optimal concentration at which they facilitate JNk activation. At higher concentrations, β-arrestins did not activate JNK3, although whether they truly inhibit phosphorylation or perhaps oligomerize such that they are unable to serve as scaffolds was not addressed (Seo, Tsakem et al. 2011). Furthermore, the MKK4 used in these in vitro studies was pre-activated; thus, the question of how β-arrestins can trigger the cascade upstream of MKK4 remains unanswered. One possibility is that some other input is required in the cell to activate ASK1 such that it would automatically phosphorylate MKK4 when brought into its proximity. Studies suggest that oligomerization of ASK1 promotes its activation and that the oligomers are rendered inactive by interaction with various membrane proteins. Whether association with β-arrestins disrupts inhibitory interactions or promotes oligomerization has not been addressed but is a possible mechanism by which ASK mediated phosphorylation of MKK4 is achieved.

Many of the specific residues mediating the binding of β-arrestin-2 to the JNK3 module have been determined by using truncations of β-arrestin-2 and spot peptide arrays (Cheung, Malik et al. 2009; Seo, Tsakem et al. 2011). Both the C-terminal chimera

comprising amino acids 182-335 and N-terminal truncation consisting of amino acids 1-181 of β-arrestin-2 could be co-immunoprecipitated with JNK3, leading to the hypothesis that residues in both regions were important for binding(Song, Coffa et al. 2009). A domain, ^{196}RRS^{198} , within the C-terminus of β-arrestin-2 is highly homologous to a known docking motif sequence found in many MAPK binding proteins and proved critical for β-arrestin-2 induced phosphorylation of JNK3(Miller, McDonald et al. 2001). An additional C-terminal site at Val343 of β-arrestin-2 was critical for JNK3 phosphorylation(Seo, Tsakem et al. 2011). A C-terminal region of Ask 1 containing the kinase domain (residues 678-1375) associates with the N-terminal sequence ^{26}DVFD^{29} of β-arrestins, raising the possibility that association increases its activity, even if it does not affect its phosphorylation. It is noteworthy that this same region associates with MEK1, which suggests that both Jnk and ERK1/2 complexes may not be able to form simultaneously. Spot peptide arrays followed by alanine scanning mutagenesis confirmed some of these sites and identified new residues within the N-terminus critical for Jnk3 module interaction with β-arrestins. For example, MKK4 bound to the negative-charged E66/D67/D69 motif of β-arrestin-1. In addition to scaffolding the JNK3 cascade, β-arrestins regulate JNK3 subcellular localization. While the mutations described above are required for interaction with JNK module components, other residues are important for localization. For example, a critical L395 within in the NES signal domain abolished β-arrestin-2 trafficking activity and kept its binding partner, JNK3, in the nucleus (Scott, Le Rouzic et al. 2002).

Besides the two well studied β-arrestin MAPK scaffolds, the other major MAPK cascade group is the p38 MAPK pathway. Although most of environmental stresses and inflammatory cytokines stimuli for triggering JNK3 signal also activate the p38 cascade, whether the β-arrestins play the important roles as signaling transducers in p38 activation or not still need more investigation(Roux and Blenis 2004). Downstream of a well-known chemokine receptor, CXCR4, the expression of β-arrestin-2 increased the p38MAPK activation and led to chemotaxis (Sun, Cheng et al. 2002). Jnk and p38MAPK can be activated by the same upstream components, MKK4/7 and Ask1. Therefore, in some cell types interaction of β-arrestin-2 with Ask1 and MKK4 might also lead to p38MAPK activation.

1.4.4 The activation of MAP kinase cascades by β-arrestins

How β-arrestins serve as signal transducers and what the role of receptor recruitment is in this process? An interesting observation is the fact that there are constitutive interactions between β-arrestins and the MAPK module components. One study revealed that a "receptor-excluded" mutant of β-arrestin-2 with a 7-residue deletion in the central hinge domain was still able to assemble the ASK1-MKK4-JNK3 complex and activate JNK3, although it is locked in the basal conformation and is impaired binding to receptors. Raf, ERK2 and MEK1 as well as Ask1, MKK4 and Jnk3 can all be co-immunoprecipitated with β-arrestin-2 from cells in the absence of receptor activation (Song, Coffa et al. 2009). How then does receptor activation affect kinase activity through these pathways? Receptor engagement increases JNK and ERK1/2 phosphorylation downstream of multiple GPCRs, and may serve to stabilize the

complexes. Studies using a β-arrestin biosensor with an N-terminal luciferase and C-terminal YFP tag suggest that the N and C-termini are brought closer together (consistent with the crystal structure model of active β-arrestin) upon treatment with several GPCR agonists. However, β-arrestin biased agonists, which promoted signaling to MAPK but not G-protein coupling resulted in a decrease in resonance energy transfer between the luciferase and YFP, consistent with a conformational change in which the N and C-terminus move farther apart (Shukla, Violin et al. 2008). Thus, β-arrestins may adopt multiple conformations that expose binding domains for only a subset of their potential binding partners. Specific conformational changes in β-arrestins upon recruitment to certain receptors may alter the activity of the associated MAPK module components, leading to receptor-specific patterns of activation. Additionally, β-arrestins may protect MAPKs from inactivation by their specific phosphatases. β-arrestin-2 specifically associates with the JNK phosphatase, MAP kinase phosphatase 7 (MKP7) and it dissociates upon AT1aR activation, corresponding with the receptor-mediated activation of JNK3 (Willoughby and Collins 2005).

1.4.5 The scaffolding of Src tyrosine kinase family with β-arrestins

As we discussed previously, the β-arrestins can scaffold and activate the entire ERK1/2 module but downstream of some receptors, β-arrestin-dependent ERK1/2 activation is also mediated by Src. β-arrestin-1 can associate with the SH3 domain of Src via poly-proline-rich stretches in its central region (Luttrell, Ferguson et al. 1999; Barlic, Andrews et al. 2000; Miller, Maudsley et al. 2000). Like Raf, Src is autoinhibited in resting cells via intramolecular interactions between a C-terminal phosphotyrosine and its

own SH2 domain, and between a poly-proline rich central region and its SH3 domain. Classically, activation involves dephosphorylation of the C-terminal tyrosine (567) which disrupts intramolecular these interactions and exposes a phospho-acceptor site within the activation loop (Y416) which is then available for phosphorylation by another src molecule or by other cellular tyrosine kinases. Active Src phosphorylates numerous cellular proteins and recruits other tyrosine phosphorylated and proline-rich proteins via its SH2 and SH3 domains, respectively. Some of its cellular substrates are other tyrosine kinases (both receptor and non-receptor), the adaptor protein Shc, and a variety of cellular signaling proteins, including the GTPase dynamin. Downstream of β_2AR, Src-dependent activation of EGFR and recruitment of Grb2-bound SOS (a guanine exchange factor for Ras) are implicated in β-arrestin-dependent activation of ERK1/2 (Wilde, Beattie et al. 1999). Furthermore, Src-dependent phosphorylation of the small GTPase dynamin, induces the internalization of some MAPK signaling complexes(Williams, Weijland et al. 1997; Williams, Wierenga et al. 1998; Ahn, Maudsley et al. 1999; Shi, Resing et al. 2006) and association of β-arrestin-1 with Src induced the dynamin phosphorylation downstream of β_2AR. However, the question of how β-arrestins regulate Src activity has not been completely answered. Since the SH3 domain of Src interacts with its own poly proline region in the inactive state, it is reasonable to postulate that interaction with the β-arrestin polyproline region disrupts this inhibitory interaction. Src activity in vitro in the presence and absence of β-arrestin has not been assayed but could shed light on this possibility, especially since the two proteins have been shown to directly interact. Additionally, src can phosphorylate adaptin and AP2, as well as some

GPCRs; thus β-arrestin-recruited Src might interact via its SH2 domain with phosphotyrosines in the signaling complex which might also contribute to stabilization of the active conformation. Finally, β-arrestins may bring Src in proximity to other tyrosine kinases such as FAK or PYK2, or even other Src molecules, facilitating phosphorylation within its catalytic core.

Besides β-arrestin-Src complexes, several tyrosine kinases have been investigated for their interactions with β-arrestins. The Src family member, Yes, formed a complex with β-arrestin-1, $G\alpha_{q/11}$ and the stimulated ETA receptor (endothelin type A receptor) for GLUT4 (glucose transporter type 4) transportation in the 3T3-L1 adipocytes(Imamura, Huang et al. 2001). Also, in the chemokine receptor CXCR1 reaction, β-arrestin rapidly formed complexes with a src tyrosine family member, Hck. The β-arrestin and Hck showed localization and association in the chemoattractant-mediated granules in the innate immune response by confocal microscopy and co-immunoprecipitation (Barlic, Andrews et al. 2000).

1.5 The scaffolding and regulation of PI3K pathway by β-arrestins

Phosphatidylinositol-3 Kinase (PI3K) is a unique example of kinase regulation by β-arrestins, in that its activity has been shown to be both positively and negatively regulated by β-arrestin association. Class 1A PI3Ks (PI3Kα and PI3Kβ) consist of two subunits: a p85 regulatory domain stabilizes and represses the p110 catalytic kinase domain(Mellor, Furber et al. 2012).Upon association with tyrosine phosphorylated proteins and poly-proline rich proteins via its SH2 and SH3 domains, p85-induced repression is relieved and the catalytic p110 subunit becomes active. Downstream of PAR-2, both β-arrestin-1 and 2 can be co-immunoprecipitated with Class IA PI3Ks and over-expression of β-arrestins results in PAR-2-induced inhibition of PI3K activity. In vitroly, both β-arrestins directly bind the p85α/p110α and p85β/p110β heterodimers. β-arrestin-2 binding directly inhibits both p110α and β activities, while binding to β-arrestin-1 preferentially inhibits p110α. Downstream of other receptors, including receptor tyrosine kinases and GPCRs (protease-activated-receptor-1 (PAR_1) and CCR5), β-arrestin-1 facilitates PI3K activity. Interestingly, in the studies demonstrating the inhibitory effect of β-arrestins on PI3K activity showed low concentrations of recombinant β-arrestin-1 (but not β-arrestin-2) could specifically facilitate the catalytic activity of the p110β catalytic subunit. The studies on CCR5 suggested that β-arrestins could promote PI3K activity by scaffolding it with upstream tyrosine kinases and phosphor-tyrosine containing proteins. Thus, the ability of β-arrestins to act as inhibitors or facilitators of PI3K activity may depend on the nature of their interactions (direct or indirect) as well as receptor-induced conformational changes.

A missing link in the understanding of β-arrestins and how they regulate PI3K activity lies in the identification of sites of interaction and the mechanism by which the catalytic subunit is affected. Like Src, the p85 subunit of Class1A PI3K contains both SH2 and SH3 domains. Thus, direct interactions between PI3K and β-arrestins may be mediated by these domains. However, since all *in vitro* binding studies were done with the p85/p110 holoenzymes (p110 alone is relatively unstable), it is not yet clear whether β-arrestins directly engage one or both subunits. Since β-arrestins are capable of directly inhibiting PI3K, it is likely that the stimulatory interactions may involve scaffolding of PI3K to other cellular proteins, leading to a similar mechanism of activation as is observed downstream of growth factor receptors. The mechanism by which β-arrestin inhibits PI3K activity is less clear. Perhaps, as mentioned above, interaction with the p85 regulatory subunit SH3 and/or SH2 domains results in activation of p110 through induction of a conformational change. Alternatively, β-arrestins may interact directly with the catalytic subunit to relieve the inhibitory action of p85 while stabilizing the enzyme.

A major downstream substrate of PI3K is the ser/thr kinase Akt. Akt, also known as protein kinase B, can interact with the phosphatidyl-inositol-3-phosphate (PIP3), created by activated PI3K. While β-arrestin-dependent Akt activation was shown downstream of the same receptors that utilized β-arrestin to activate PI3K, downstream of the D2-class dopamine receptor (D2R), β-arrestin-2 mediates inhibition of Akt activation. Upon D2R activation, a multi-protein complex containing β-arrestin-2, PP2A (protein phosphatase 2A) and Akt is assembled. By scaffolding Akt to a ser/thr phosphatase, β-

arrestin-2 promotes the dephosphorylation of Akt and inhibition of its activity. This β-arrestin-kinase/phosphatase scaffold controls some of the important DA-associated behaviors and has led to interest in targeting β-arrestin-dependent D2R functions as therapeutics(Del'guidice, Lemasson et al. 2011). The positive effects of β-arrestins on Akt all appear to involve upstream PI3K activity.

1.6 The scaffolding of CAM kinase family by β-arrestins

Two members of the Ca^{2+}/Calmodulin Kinase family (CAMK) have been reported to undergo regulation by β-arrestins, CAMKII and CAMKK2. The CAMKII subfamily locks in an autoinhibitory state in their inactive state due to interaction of a pseudosubstrate region within the autoinhibitory domain with the catalytic domain. A C-terminal self-association domain mediates multimerization of CAMKII molecules. Binding of the Ca2+/calmodulin complex and autophosphorylation relieve autoinhibition resulting in persistent activation. β-arrestins have been shown to directly bind calmodulin and CAMKIIδ(Wu, Hanson et al. 2006; Xiao, McClatchy et al. 2007). Additionally, a recent study revealed that β-arrestins are required for β1AR induced CAMKII activity (Mangmool, Shukla et al. 2010). This study suggested that the effect of β-arrestins was to scaffold CAMKII at the membrane with a cAMP sensitive Guanine exchange factor, ePAC, which activates the GTPase, Rap1. This multimeric complex might hold CAMKII in a conformation allowing phosphorylation by PKC which is activated downstream of Rap. Whether β-arrestin has any direct effect on CAMKII activity was not determined. Interestingly the related β2AR did not promote β-arrestin-dependent CAMKII activation, once again pointing to the fact that each GPCR may induce a unique conformational change in β-arrestins leading to a different set of scaffolds and cellular responses. CAMKII plays an important role in cardiac contractility and further elucidation of this signaling pathway may provide insights for new therapeutic approaches to the treatment of heart failure.

CAMKK2 (also known as CAMKKβ) is another member of the CAMK subfamily, first named for its ability to phosphorylate CAMKII family members and now known to play an important role in the regulation of AMPK (adenosine monophosphate-activated kinase). β-arrestin-2 directly inhibited the phosphorylation of AMPK by CAMKKβ on Thr172 in *in vitro* kinase assay(Wang, Jiang et al. 2010) and could be co-immunoprecipitated with both AMPK and CAMKKβ in cultured fibroblasts and primary mouse fat. Activation of AMPK by protease-activated-receptor-2 (PAR_2) was suppressed by expression of β-arrestin-2. Furthermore, in fat explants from β-arrestin-$2^{-/-}$ mice but not wild type mice, PAR-2 activation decreased AMPK activity. This effect was unique for β-arrestin-2, as PAR-2 did not inhibit AMPK in β-arrestin-1 knockout mice. Like CAMKII, CAMKKβ exists in an autoinhibited conformation in which a region within the N-terminus blocks the activity of the C-terminal kinase domain. Activation of CAMKKβ involves both Ca2+/Calmodulin binding and autophosphorylation. β-arrestin-2 was able to inhibit CAMKKβ-dependent phosphorylation of a non-specific substrate as well as AMPK; therefore, the mechanism of β-arrestin-dependent inhibition is likely to involve a direct effect on activity rather than sequestering AMPK away from CAMKKβ. One possibility is that by binding to calmodulin, β-arrestin-2 prevents activation of CAMKKβ. Further investigation of this pathway may shed light on the role of β-arrestins in metabolic disease. Decreased AMPK activity relates with Type II Diabetes and a number of insulin sensitizing drugs as well as the commonly prescribed anti-cholesterol medication, statins, promote AMPK activity. However, other studies have suggested a positive role for β-arrestins in the maintenance of insulin sensitivity.

CHAPTER TWO

Materials and Methods

Chapter 2 Materials and Methods

Chemicals

All chemicals are from Sigma or Fisher Scientific unless stated otherwise. [γ^{32}P]-ATP *(NEG502A, Perkin Elmer)* was to monitor the kinase activity of LIMK1. Active and unactive LIMK1 *(14-659 and 14-656, Millipore)* were for kinase assay and sandwich immunoassay. All restriction enzymes were from New England Biolabs. Glutathione sepharose 4B *(GE Healthcare)* or Glutathione agarose *(BD Biosciences)* were for the GST-tagged protein purification. PAR2 activating peptide 2fAP (2-furoyl-LIGRL-Ornithine-NH_2) was synthesized by *Tocris Bioscience*. Protein-G agarose and protein-A agarose were from *Upstate*. $PVDF_{fl}$ from *Millipore* is the membrane for western blots.

Antibodies

Antibodies and final dilutions for Western blot (WB), immunoprecipitation (IP) and sandwich immunoassay (SI) were as follows: rabbit anti-phospho (Ser3)-cofilin (*Cell Signaling*, 1:1000 WB), mouse anti-total cofilin (*BD Bioscience*, 1:1000 WB and SI), mouse anti-GFP (*Roche*, 1:500 WB, 2.5μg/ml IP), rabbit anti-GST (*Santa Cruz Biotechnology*, 1:1000 WB), rabbit anti-Flag (*Sigma,* 1:000 WB), mouse anti-β-arrestin-1(*BD Biosciences,* 1:1000 WB), and mouse anti-6xHis (*BD Biosciences*, 1:1000 WB and SI). IR-dye-conjugated secondary antibodies (*Rockland Biosciences)* 1 μg/ml is used in sandwich immunoassay.

Plasmids

The following plasmids were used in transient expression experiments: Flag-tagged β-arrestin-1 plasmids and truncated mutants containing 1 to 163 or 164 to 418

amino acids of β-arrestin-1 were provided from Dr. Robert Lefkowitz (Duke University, Durham, NC). Flag-tagged β-arrestin-1 containing 1 to 99 and 319 to 418 were excised with HindIII and XhoI and subcloned into pcDNA-hygro. For expression in bacteria, GST-tagged WT β-arrestin-1 was from Dr. Robert Lefkowitz (Duke University, Durham, NC). GST-tagged truncated β-arrestin-1 containing 1 to 163 and 164 to 418 amino acids were subcloned into pGEX4T-1.

Cell Culture

HEK 293 Cells were grown in 1X DMEM substituted with 10% FCS. Transient transfections were performed on 70-80% confluent cells using Lipofectamine (*Invitrogen*) or Bio T (*Bioland*) and experiments were performed between 48 and 72 hours after transfection.

Immunoprecipitation

Cleared lysates were prepared as follows: HEK293 Cells (10-cm plates) were maintained overnight in minimal essential medium without serum overnight, incubated with 100 nM 2fAP for 0-5 min at 37℃, and lysed in 0.4 ml lysis buffers (1X HEPES, 1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitors) for LIMK1 or (1X PBS, 1% NP-40 and protease inhibitors) for cofilin. Proteins was analyzed by 10%, 12.5% or 15% SDS-PAGE, transferred to $PVDF_{fl}$ and probed with antibodies at concentrations described above, followed by $Alexa^{680}$ and IR-800-conjugated secondary antibodies. For co-immunoprecipitations, cleared lysates were immunoprecipitated with anti-flag agarose overnight at 4℃ and beads were washed with lysis buffer or were immunoprecipitated with antibody to myc (9E10) or to GFP overnight and pulled down with protein G

agarose; beads were washed and analyzed by 10% or 12.5% SDS-PAGE followed by Western blotting. Blots were imaged and band intensity determined using a LICOR Odyssey Infrared Imaging System (*Li-COR Biosciences*).

Purification of recombinant proteins

GST-tagged WT β-arrestin-1, its truncations (1-163 and 164-418 amino acids of β-arrestin-1), and ERK2 were expressed and purified from BL21 *Escherichia coli* cells using glutathione-sepharose 4B (GE Healthcare). Briefly, *E. coli* BL21 (DE3) cells were transformed with GST-tagged fusion proteins, grown to exponential phase [where OD_{600} (attenuance) is 0.6 and induced with 0.5 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) for 4 hours at 30°C. Bacterial cells were subsequently lysed by sonication (6 bursts of 10 s at 30% power) in PBS supplemented with 10 μg/ml lysozyme, 10 μg/ml DNase 1 and 1% (v/v) Triton X-100. Cleared lysates were incubated with 400 μl of glutathione–Sepharose 4B for 1 hour, washed with 20 volumes of binding buffer supplemented with 1 mM ATP, and the bound protein was eluted using increasing concentrations of free reduced glutathione. Elution fractions containing the fusion protein were dialyzed overnight against PBS and stored in 10% (v/v) glycerol at −80°C until needed. The protein concentration of each elution was determined using the Bradford assay, and the relative purity was determined by SDS/ PAGE (10% gels), followed by staining with Coomassie Brilliant Blue R250. Some breakdown of β-arrestin was observed in each preparation, which was quantified by the Coomassie-stained gels. Removal of the GST moiety from the protein of interest is accomplished through a thrombin cleavage (10U/ml overnight at 4°C) site located between the GST moiety and

the recombinant polypeptide. For solution digestions, GST is easily removed by a second round of chromatography on the glutathione column. Removal of thrombin is facilitated by the use of a benzamidine-agarose column or a gel-filtration step.

Sandwich Immunoassay

Appropriate antibodies against the unactive LIMK or purified cofilin were coated to the bottom of the EIA/RIA 96-well plate to capture LIMK or cofilin. The plate was then blocked with 1%BSA in PBS for 1 hour. Recombinant full length, N (1-163 amino acids) or C (164-418 amino acids)-terminal truncations of β-arrestin-1 tagged by GST were incubated and followed by probing with anti-GST conjugated with IR-800. Wash 3 times by 0.01% Triton X-100 in PBS performed between incubations. Integrated intensity was obtained by the Li-Cor odyssey.

Spot peptide array

Spot peptide array experiments were performed by cooperate laboratory, Dr. G. Baillie, University of Glasgow, Scotland. Peptide libraries were produced by automatic SPOT synthesis and synthesized on continuous cellulose membrane supports on Whatman 50 cellulose membrane using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry with the AutoSpot-RobotASS222 (*Intavis Bioanalytical instruments AG*). The interaction of spotted peptides with GST and GST-fusion proteins was determined by overlaying the cellulose membranes with 10μg/mL recombinant protein. Bound recombinant proteins were detected with specific rabbit antisera and detection was performed with secondary anti-rabbit horseradish-peroxidase-coupled antibody (1:2500 dilution) (*Dianova*) and visualization by ECL.

Data and statistical analysis

All graphs and statistical analyses were performed using Microsoft Excel 2007 or GraphPad Prism 5.0. All experiments were performed a minimum of 3 times. Statistical significance was determined using *t-test* or one-way ANOVA and *Bonferroni* post-hoc tests.

CHAPTER THREE

Elucidation of the mechanism by which β-arrestin-1 regulates LIM kinase 1

Chapter 3 Elucidation of the mechanism by which β-arrestin-1 regulates LIM kinase 1

3.1. Introduction

Shortly after their ability to scaffold signaling molecules was established, a role for β-arrestins in the reorganization of the actin cytoskeleton and cell migration was demonstrated. However, to date only a few β-arrestin binding partners with established roles in actin assembly have been characterized. LIM-Kinase is a β-arrestin binding partner that phosphorylates and inactivates the actin-filament severing protein, cofilin. Stimulation of LIMK will result decreased cofilin-dependent actin filament severing and filament stabilization, while inhibition of LIMK results in increased cofilin activity and dynamic reorganization of the actin cytoskeleton. Downstream of several GPCRs (e.g. PAR2 and AngII) cofilin is dephosphorylated in a β-arrestin-dependent fashion. In the case of PAR2, activation involves both inhibition of LIMK and facilitation of cofilin dephosphorylation by the phosphatase, chronophin (CIN).

To fully understand the function of LIMK and cofilin in cell migration, it is important to review actin assembly. Actin filaments (F-actin) are polar structures with fast-growing barbed end that dominates assembly kinetics and a slow-growing pointed end. Cells must create new barbed ends quickly for the rapid changes necessary for chemotaxis. Active cofilin severs F-actin filaments to generate more barbed ends that can serve as de novo actin nucleation sites, thus the rate of actin polymerization increases. However, this activity must be tightly controlled, both temporally and spatially for

several reasons. First, activation of cofilin uniformly within the cell will reduce cell migration by causing the cell to become unpolarized. Free barbed ends will be created everywhere and no stable filaments will exist to provide a force for forward movement. Uniform inactivation of cofilin will stabilize filaments, essentially paralyzing actin structures and preventing forward movement. Successful migration, then, requires that cofilin be activated within 1-2 microns of the cell membrane, providing free barbed ends for polymerization which allows the membrane to be pushed forward. Simultaneously, towards the back of the leading edge, cofilin activity must be inhibited, such that stable filaments are formed, allowing for ARP2/3- induced filament branching and providing a structure against which the cell can contract. This model for actin remodeling at the leading edge is often referred to as the "dendritic model of nucleation" (Figure 3.1). β-arrestin can contribute to spatial regulation of cofilin activity by sequestering it with its upstream phosphatase at the leading edge while inhibiting LIMK activity in this region.

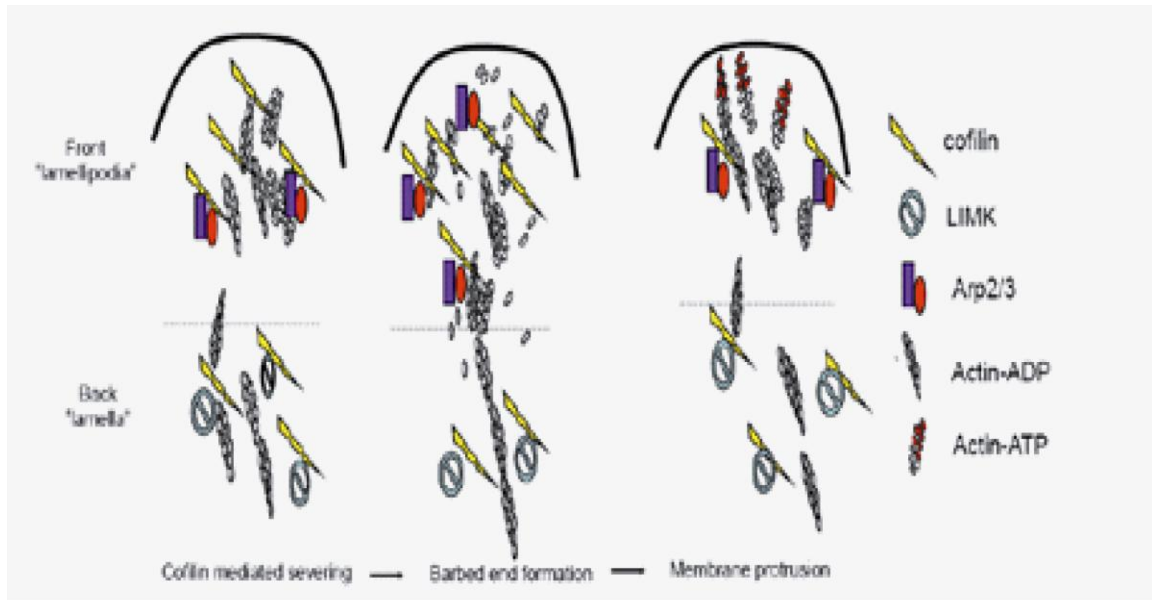


Figure 3.1 Restriction of cofilin activity within the leading edge.
Model for cofilin activity near the protruding edge. Filament severing provides free barbed ends for monomer addition to the front of "lamellipodia". β-arrestins are required for initial cofilin dephosphorylation and physically associates with cofilin upon PAR-2 stimulation at the "back" of lamellipodia. β-arrestins are important for maintaining a pool of dephosphorylated cofilin and colocalizes with cofilin at the tips of membrane protrusions, the lamellipodia, after prolonged PAR-2 activation.

Previously, we demonstrated that PAR2 promotes cofilin dephosphorylation and activation in a human breast cancer cell line (MDA MB468) (Figure 3.2). That cofilin dephosphorylation was accompanied by increased actin filament severing was demonstrated using a modified pyrene actin assembly assay, in which F-actin was formed and extracts from PAR2 activated cells were added. siRNA knockdown of both β-arrestins completely blocked both cofilin dephosphorylation and actin filament severing. Similar observations were made in mouse embryonic fibroblasts (MEFs) from wild type and β-arrestin-1 and 2 knockout mice (Figure 3.3) (Zoudilova, Kumar et al. 2007). These studies also suggested that both β-arrestins were necessary for full cofilin dephosphorylation but that they might differ temporally in this requirement. Finally, these studies demonstrated for the first time, that PAR2 could signal to the cofilin pathway independent of G-protein signaling, as inhibition of various components of the $G_{\alpha}q$, $G_{\alpha}i$ and $G_{\alpha}12$ pathways resulted in even more robust cofilin dephosphorylation.

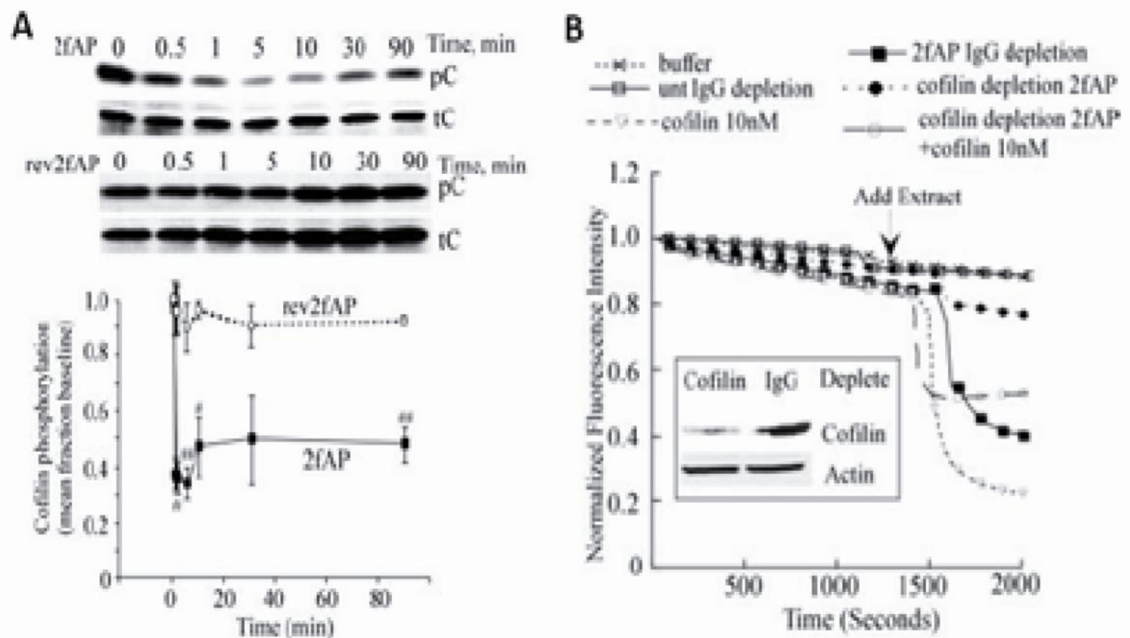


Figure 3.2 PAR-2 promotes cofilin dephosphorylation and severing activity.
A. time course (0-90 min) of cofilin activation in MDA-MB-468 cells after 100nM 2fAP or reverse 2fAP (*rev*2fAP) treatment. *Upper panel*, graph of normalized phosphor-cofilin levels over time presented as a mean fraction of base line ± S.E. (n=10) is shown. Statistically significant differences in cofilin phosphorylation compared with base line are indicated by # ($p<0.05$) and ## ($p<0.005$). **B.** cofilin-dependent actin filament severing activity. Fluorescence-labeled pyrene-actin was used to confirm that PAR-2 actually promoted cofilin-dependent actin filament severing. A decrease in fluorescence intensity in 2fAP IgG depletion represented the increasing of filament severing activity. Fluorescence was monitored for 3000 seconds.

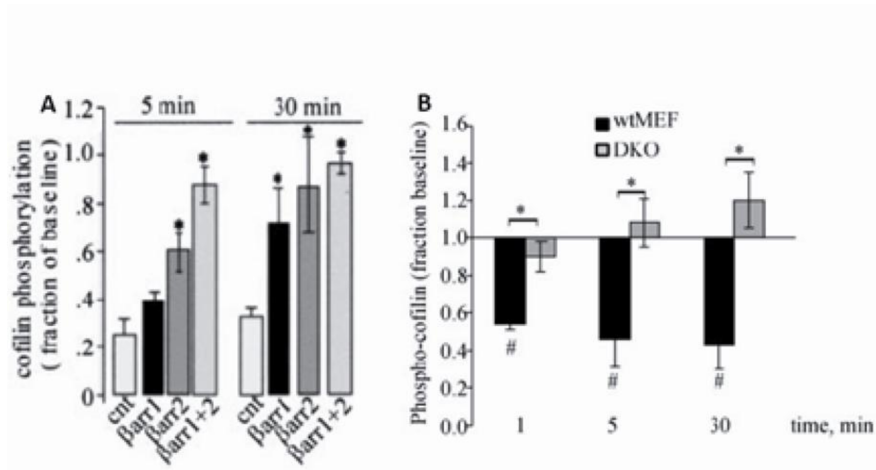


Figure 3.3 siRNA knockdown of β-arrestins inhibits PAR-2-stimulated cofilin dephosphorylation. A. siRNA knockdown in MDA-MB-468 cells. B. siRNA knockdown in MEFs. PAR-2 signaling in MDA-MB-468 cells severs as an excellent model system for studying PAR-2-induced cofilin activation because it has been well characterized. On the other hand, MEFs from β-arrestin-knockout mice provide the best model to address the role of β-arrestins in signaling events, but they express less PAR-2, so PAR-2 responses are less robust.

Further mechanistic exploration into the PAR2-induced cofilin activation pathway revealed that β-arrestins co-immunoprecipitated with cofilin and both of its upstream regulators, LIMK and CIN, although purification of these scaffolding complexes suggests that separate β-arrestin/LIMK and β-arrestin/CIN/cofilin complexes exist. Dominant negative CIN abolishes PAR2 stimulated cofilin dephosphorylation and actin filament severing suggesting facilitation of cofilin dephosphorylation by β-arrestins is involved in cofilin regulation (Zoudilova, Min et al. 2010). However, siRNA knockdown of β-arrestin-1 results in a PAR2 stimulated increase in LIMK activity, suggesting that a second mechanism by which PAR2 promotes cofilin dephosphorylation is through inhibition of LIMK (Figure 3.4).

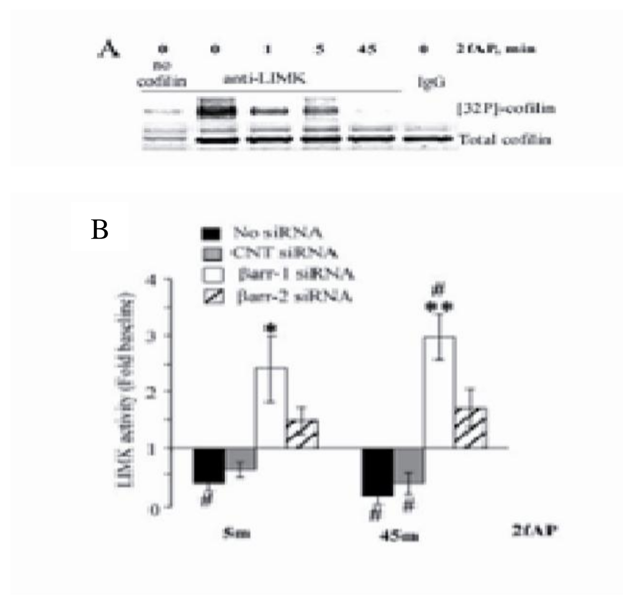


Figure 3.4 β-arrestins inhibit LIMK1 activation downstream of PAR-2. A. LIMK assay: phosphorylation of recombinant cofilin by LIMK immunoprecipitation from MDA-MB-468 cells, after treatment with 2fAP for 0, 1, 5 and 45 min. Upper panel, representative autoradiograph showing radio-labeled cofilin. Lower panel, coomassie-stained gel showing total cofilin. B. LIMK assay after siRNA knockdown of β-arrestins: bar graphs depicting PAR-2 stimulated LIMK activity (mean-fold base line ± S.E.) with and without β-arrestin knockdown. Significant difference between β-arrestin and control siRNA values are indicated by * ($p < 0.01$) and ** ($p < 0.001$), and significant decreases in LIMK activity at each time point with respect to untreated. Cells are indicated by # ($p < 0.01$). m, min; β-arr: β-arrestin.

Recombinant GST-tagged β-arrestin-1 was pulled down together with cobalt-sepharose-bound His-tagged LIMK1 (amino acids 285-639), whereas GST alone and GST-tagged β-arrestin-2 were not. Moreover, no β-arrestin-1 bound to cobalt sepharose alone (Figure 3.5B). Surprisingly, when β-arrestins were expressed in mammalian cells instead of bacterial cells, recombinant β-arrestin-2 also bound to LIMK1 (Figure 3.5C). This result indicates that a mammalian cell-dependent modification of β-arrestin-2 is required for binding to LIMK or that another protein associates with β-arrestin-2 in mammalian cells to facilitate this interaction. This dissertation focuses on the molecular details of the interactions between β-arrestin-1 and LIMK1 in the cofilin activation.

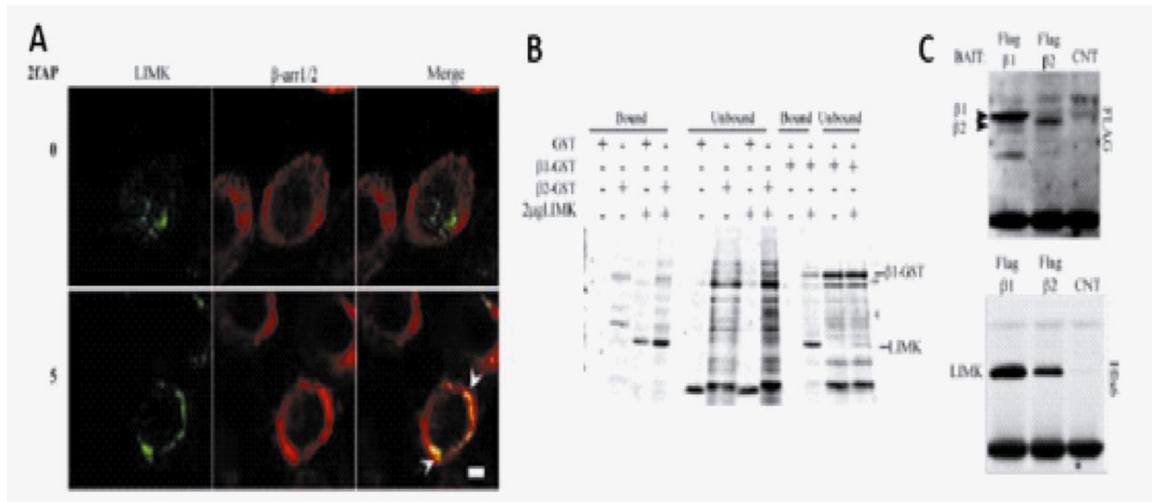


Figure 3.5 β-arrestins bind to and colocalize with LIMK1.
A. Confocal microscopic images of PAR-2 induced β-arrestins to colocalize with LIMK1. MDA-MB-468 cells co-stained with anti-LIMK (*green*) or anti- β-arrestins (*red*). Arrows indicates regions of colocalization after 5 min of 2fAP treatment. *Scale bar = 10µm*. **B.** *In vitro* binding assay of GST-tagged β-arrestins with proteins was analyzed by SDS-PAGE followed by coomassie staining**. C.** *In vivo* binding assay: overexpressed FLAG-tagged β-arrestins were purified with ani-FLAG-agarose in NIH3T3 cells. After incubating with recombinant His6-tagged LIMK1, proteins were eluted from beads. Western blots were probed with anti-FLAG (*upper panel*) or anti-His6 (*lower panel*) to determine interactions. CNT: control; β or β-arr:β-arrestin.

We propose a model in which PAR-2 mediates chemotaxis by coupling with β-arrestins, independent of G-protein signaling to promote the formation of several scaffolding complexes involved in regulation of actin. In this chapter, my studies will further characterize the interaction between β-arrestin-1 and LIMK1, which is a new possible mechanism to control spatial cell migration. While our previous studies did show a role for both facilitation of CIN/cofilin association and inhibition of LIMK activity as contributing to the overall PAR2-induced cofilin dephosphorylation, other studies have indicated that there are cell type differences in the manner in which cofilin is regulated. For example, in neurons, LIMK regulation is critical for controlling cofilin activity in dendritic spines. In contrast, in many tumor cells, CIN and Slingshot (the two cofilin phosphatases) are the primary mediators of cofilin dephosphorylation. In many cells, the relative contributions of these two pathways have not been examined. However, a considerable number of common signaling pathways (e.g. RhoA GTPases) activate LIMK; thus understanding how its activity is controlled is an area in need of more investigation. Since there are so many human diseases related to cytoskeleton dysregulation, understanding the interaction between LIMK1 and β-arrestins, may lead to novel therapeutic targets.

3.2. Results and Discussion

Direct interaction between GST-β-arrestin-1 and LIMK1 was confirmed using a 96 well sandwich immunoassay (see *Material and Method in chapter 1.2.*). Using this assay, we demonstrated the binding between the two proteins, and calculated the apparent EC50 for binding β-arrestin-1 and various truncations of β-arrestin-1 to LIMK1. To assess whether various truncations of β-arrestin-1 were able to bind to LIMK, increasing amounts of either GST-β-arrestin-1 or GST alone were added to LIMK-coated plates and the integrated intensities (proportional to the amount of GST-tagged protein bound) were graphed as a function of moles of GST-tagged protein added. A best-fit binding curve was calculated and drawn using Graphpad Prism software based upon the one-site total binding curve with minor adjustments according to the experimental differences.

Y=Bmax*X/(Kd+X)+NS*X+Background

GST alone is always included in each assay as the control set and gave very low background integrated intensity. The best-fit curve can also determine Bmax and EC50. Bmax is the maximum specific binding. EC50 is a constant that is defined as the half of the maximal effective concentration. EC50 in sandwich immunoassays represent the concentration of the GST-fusion protein needed to achieve the half of the integrated intensity at the protein binding saturation. The higher relative affinity is, the lower EC50 is. NS is the slop of non-specific binding which can be calculated from the GST alone control set. Background is constrained to zero in these tests because the background is already subtracted when the integrated intensity is obtained from the infrared scanning by the Li-Cor Odyssey.

β-arrestin-1 directly bound to LIMK1 and the best-fit curves showed the high relative affinity compared with the GST alone control (Figure 3.6). The amino terminal region (residues 1~163) and the carboxyl terminal region (residues 164~418) of β-arrestin-1 also both bound to LIMK1 (Figure 3.7). These results suggested that β-arrestin-1 contains more than one binding domain for LIMK1 or that a single domain encompassing the C-terminus of the 1-163 and the N-terminus of the 164-418 truncation.

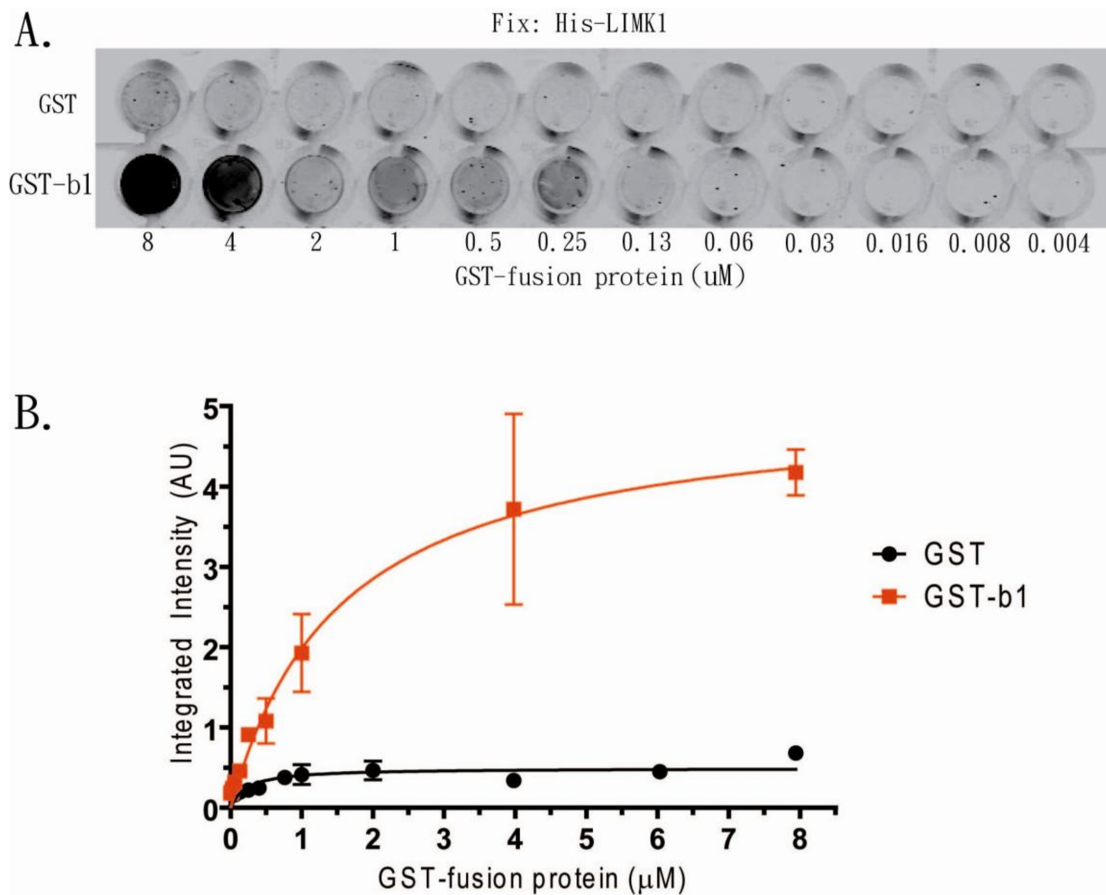


Figure 3.6 β-arrestin-1 directly binds to LIMK1.

A. representative image of the sandwich immunoassay showing the binding of GST-β1 to fixed LIMK1 in the 96-well EIA/RIA plate. **B.** the integrated intensity was obtained from each well. The higher integrated intensity indicated the more GST or GST-β1 bound to LIMK1. The best-fit curves were analyzed and graphed by *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1 or b1: β-arrestin-1.

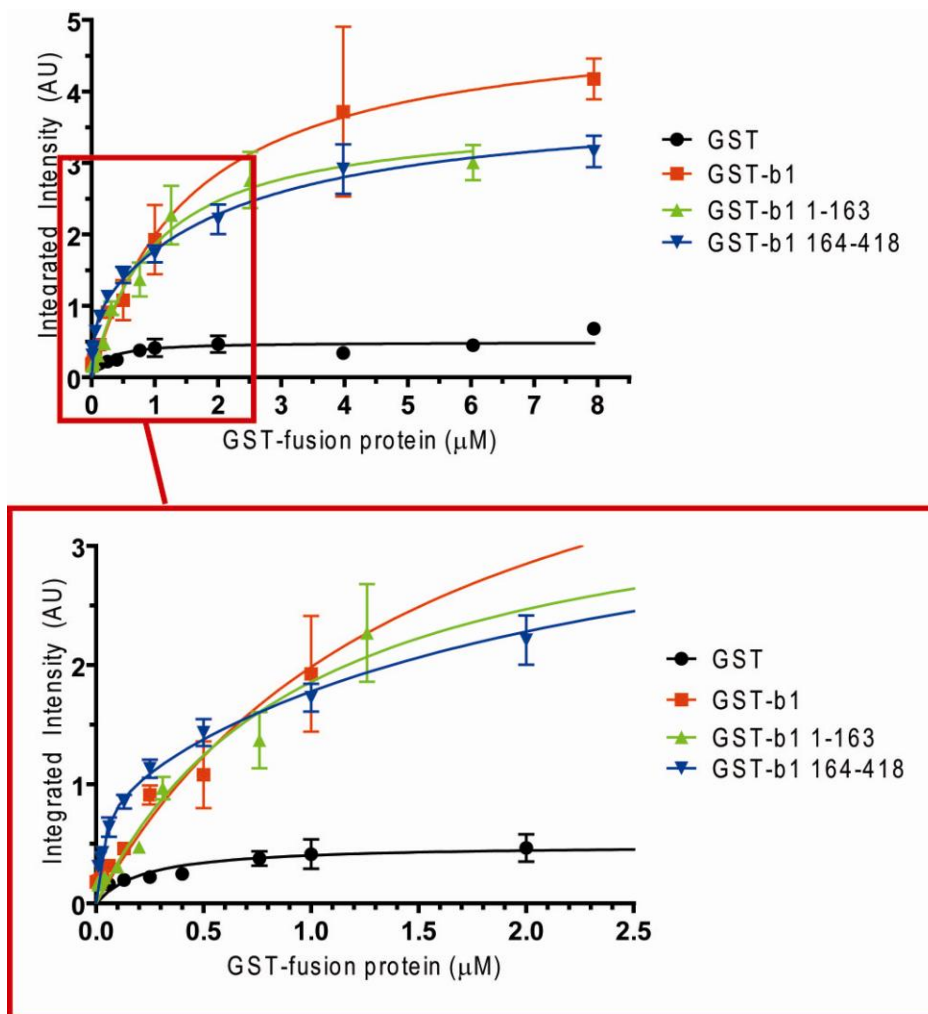


Figure 3.7 GST-β1 amino terminal and carboxyl terminal regions both bind to LIMK1.

The best-fit binding curves according to the sandwich immunoassays showed the directly bindings of GST- β1, 1-163 and 164-418 reached saturation intensities. Zoom-in graph indicated the differences of the EC50s, which were calculated from the curves. GST- β1 amino terminus contains amino acid residues 1 to 163 and GST- β1 carboxyl terminus contains amino acid residues 164 to 418. These integrated intensities were analyzed and graphed with *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1: β-arrestin-1.

We then designed smaller truncations to figure out the specific region of the specific binding of β-arrestin-1 and LIMK1. Truncations of β-arrestin-1 including residues 1-99, 1-145, 183-418, 319-418 were engineered into pGEX4T1 vector and purified after over-expressing in the BL21 *E.coli*. The significant high Bmax and the high EC50 of the small amino acid terminal region (residues 1-99) indicated that this region must played an important role in the directly association with LIMK1 (Figure 3.8). On the other hand, two more small truncations of β-arrestin-1 with residues 1-145 or 183-418 were also purified successfully. The domain including the smaller carboxyl domain of β-arrestin-1 (residues 183 to 418) did not bind to LIMK1. On the other hand, the smaller amino terminal region, residues 1 to 145, could bind to LIMK1 with lower relatively affinities compared to the full-length β-arrestin-1 and the amino terminal region of β-arrestin-1 (Figure 3.9). The comparison of the different β-arrestin-1 truncations indicated that there is a specific binding region, which is most likely located within residues 1 to 99, mediating the directly binding of β-arrestin-1 to LIMK1. However, the binding curves implied that the small regions from residues 146-182 might affect the conformation of the truncated β-arrestin-1 proteins and disrupt their bindings to LIMK1.

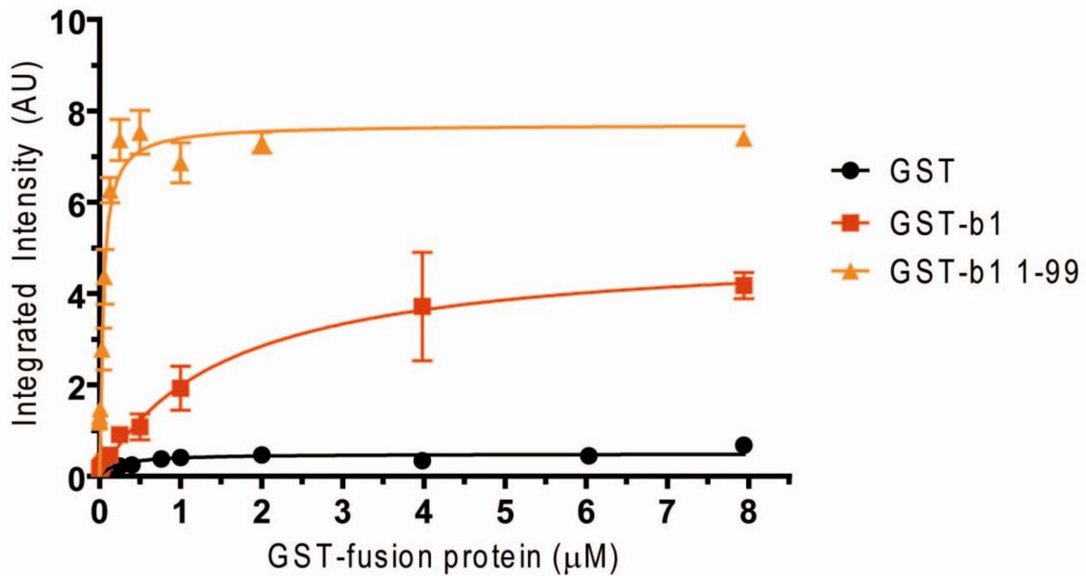


Figure 3.8 GST-β1 small amino terminal including residues 1 to 99 binds to LIMK1. The best-fit binding curves according to the sandwich immunoassays showed the directly bindings of GST-β1 full length and GST-β1 1-99 reached saturation intensities. GST-β1 1-99 contained the amino acid residues 1 to 99 of β-arrestin-1. These integrated intensities were analyzed and graphed with *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1: β-arrestin-1.

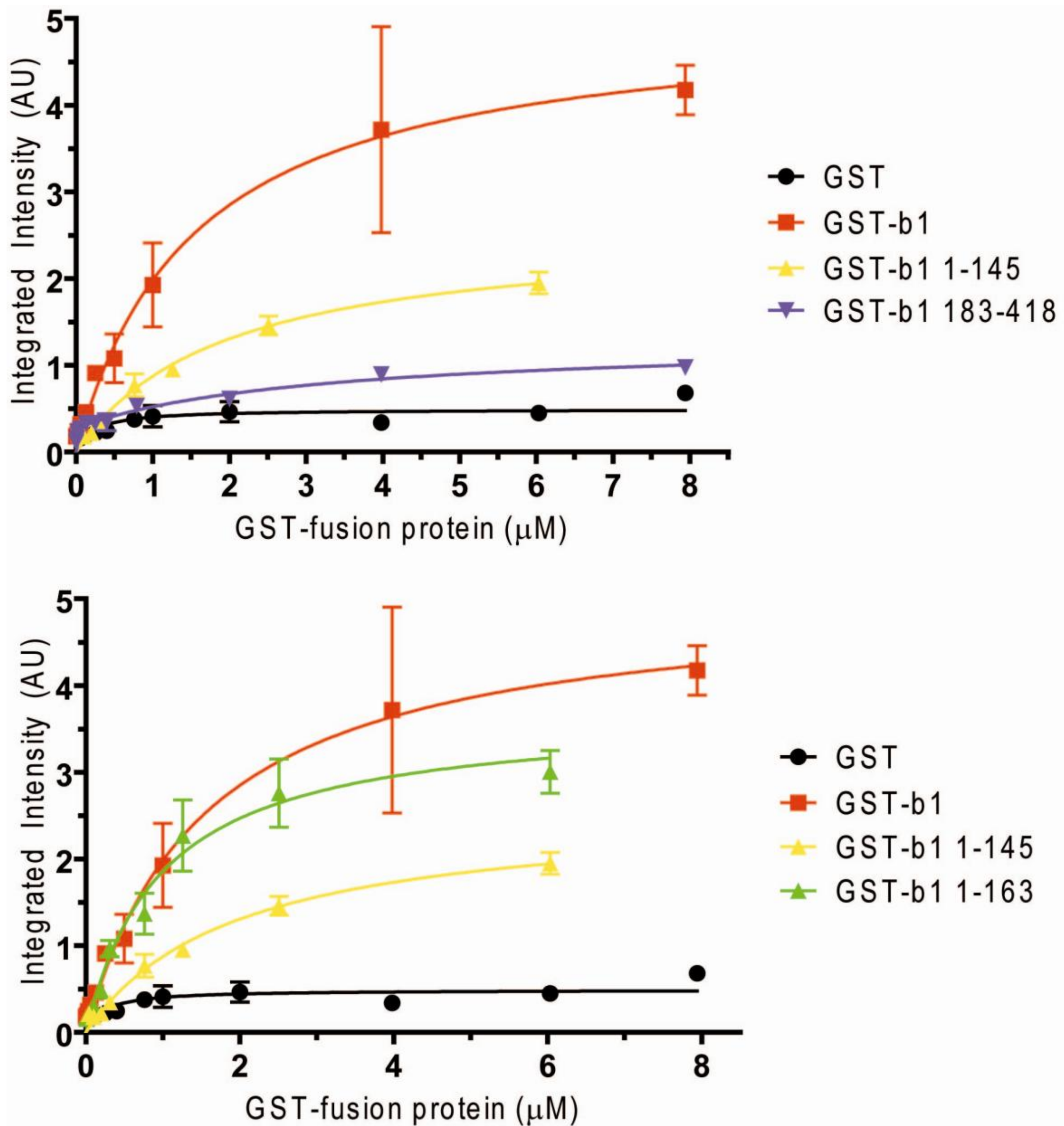


Figure 3.9 GST-β1 1-145 binds to LIMK1 but GST-β1 183-418 does not.
Upper panel, the best-fit curves of GST-β1 full-length, GST-β1 1-145, GST-β1 183-418; *Lower panel*, the best-fit curves of GST-β1 full-length, GST-β1 1-163, and GST-β1 1-145 showing the important binding region in the amino terminus of β-arrestin-1. These integrated intensities were analyzed and graphed with *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1: β-arrestin-1.

The β-arrestin-1 deletion mutant included only residues 1-145 and 183-418 of β-arrestin-1 with a small linker region. The fitting curve demonstrated this deletion mutant regained its binding to LIMK1 with even better relative binding affinity than the full-length β-arrestin-1 (Figure 3.10). The deleted region in this β-arrestin-1 mutant contains the linker region that connects the amino and carboxyl lobes of β-arrestin-1. The deletion mutant surprisingly showed better binding to LIMK1. The specific binding regions of β-arrestin-1 to LIMK1 may be within the amino terminal 1 to 99 and another carboxyl terminal domain between residues 183-418 of β-arrestin-1. The middle linker region of β-arrestin-1 did not affect the directly binding, but it did mediate the conformation of β-arrestin-1 and expose the binding pockets for downstream signaling molecule, in this case, LIMK1.

Spot peptide array data was obtained in collaboration with the laboratory of Dr. George S. Baillie (*University of Glasgow*). Several positive spots were observed, including spots 10-14 (residues 46-90) which is also found within the 1-99 truncation mutant that showed increased affinity for LIMK (Figure 3.11). Thus we propose that a major binding site for b-arrestin-1/LIMK interactions is comprised of amino acids 46-70. Additional sites between amino acids 141-165 (spot 29) and 161-185 (spot 33) were also observed and are consistent with the hypothesis that an additional site in a more central portion of β-arrestin-1 might contribute to the overall binding.

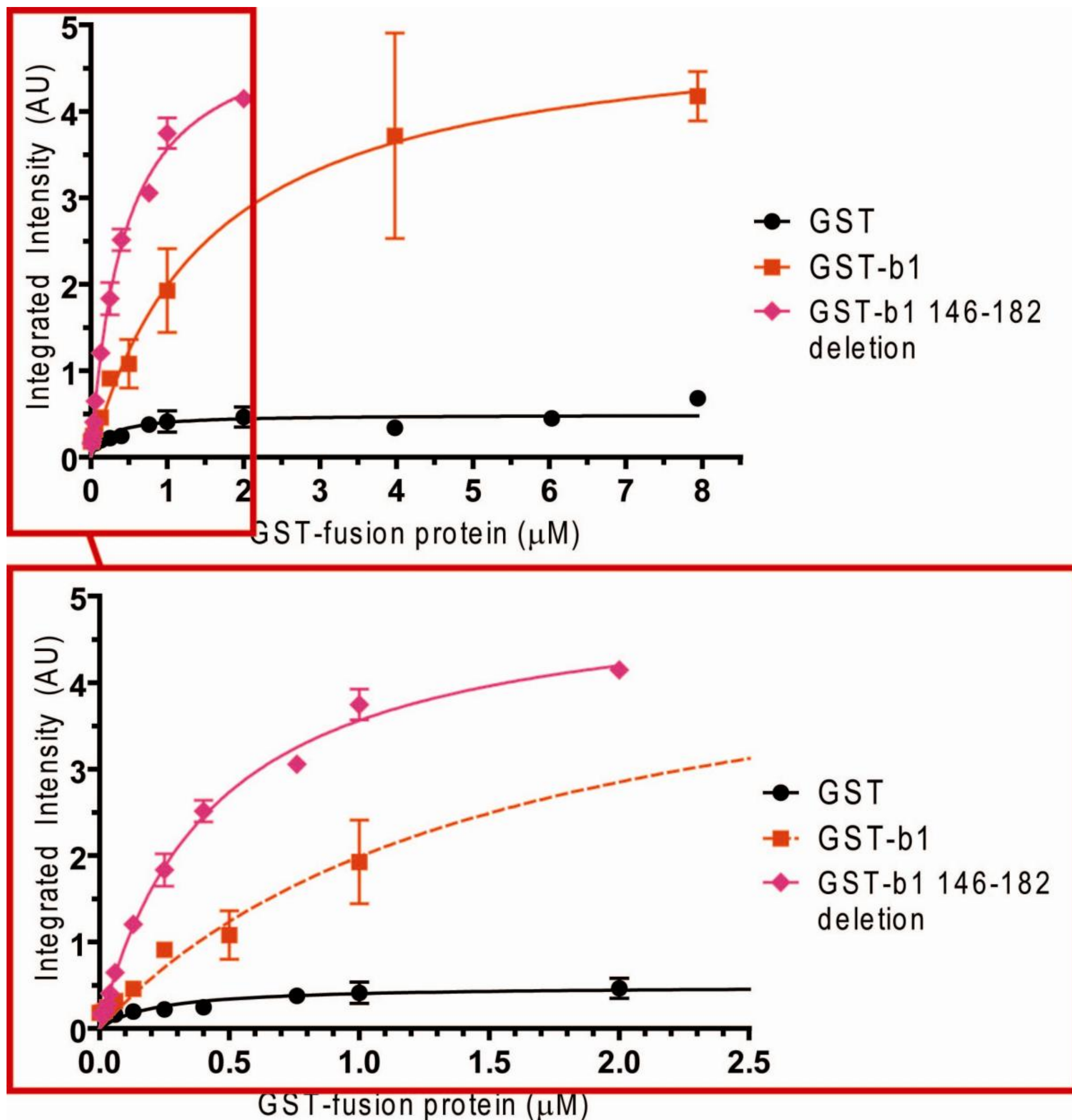
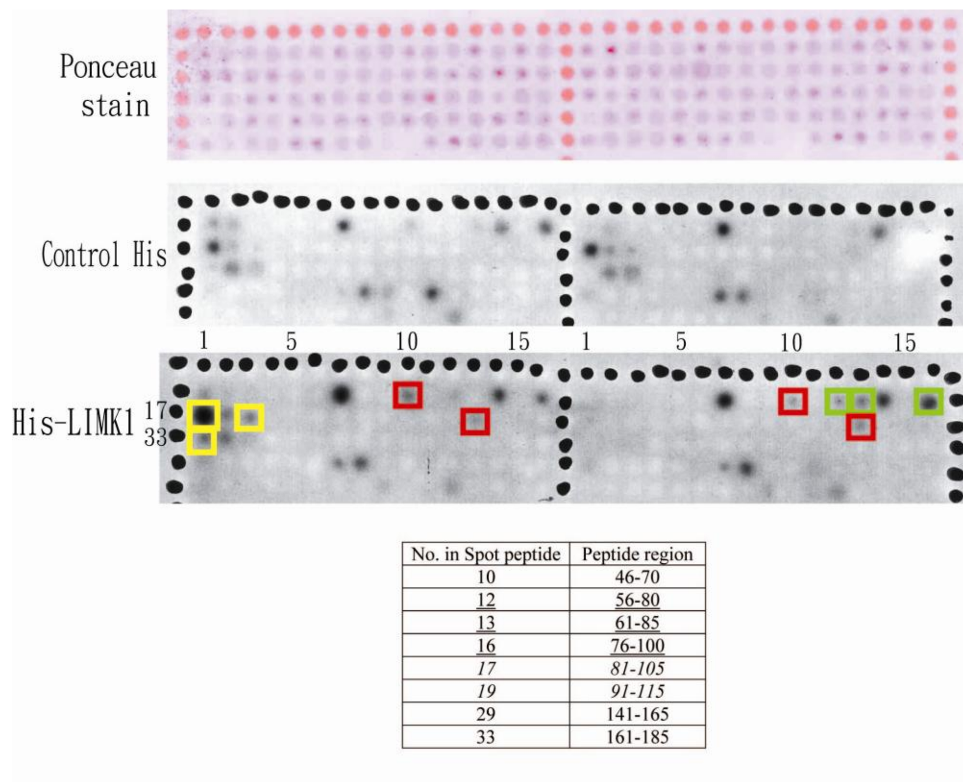


Figure 3.10 The GST-β1 deletion mutant has better binding ability to LIMK1.
The region from amino acid residue 146 to 182 of β-arrestin-1 was deleted in this mutant. The graph included both the curves of GST-β1 full-length and GST-β1 deletion mutant. Zoom-in graph showed the significant differences in these two GST-β1 proteins. These integrated intensities were analyzed and graphed with *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1: β-arrestin-1.



No. in Spot peptide	Peptide region
10	46-70
12	56-80
13	61-85
16	76-100
17	*81-105*
19	*91-115*
29	141-165
33	161-185

Figure 3.11 Spot peptide array shows the possible small binding regions of β-arrestin-1 to LIMK1.

Each spot represents a peptide with 25 amino acid residues synthesized according to the β-arrestin-1 amino acid sequence with a 5 amino acids gap. Duplicate tests were performed on one membrane. Red rectangle: the positive spots that appeared on both tests; yellow rectangle: the positive spots that appeared in test 1 (left part of the array); green rectangle: the positive spots that appeared in test 2 (right part of the array). The corresponding peptide regions of positive spots listed in the table.

We previously showed that β-arrestin-1 decreases LIMK1 activity downstream of PAR-2 activation, but the mechanism has remained vague. We examined the ability of β-arrestin-1 and various truncation mutants to directly inhibit LIMK activity in vitro, using a radioactive kinase assays. By incubating active LIMK1 with general substrate myelin basic protein (MBP) or a specific substrate (recombinant cofilin) and adding increasing amounts of either GST alone or GST-β-arrestin-1, we demonstrated that β-arrestin-1 directly inhibits LIMK activity. MBP contains multiple phosphorylation sites so it can sensitively reflect the activity of general kinase activity. β-arrestin-1 reduced LIMK1 phosphorylation of MBP by 40% while GST alone had no effect (Figure 3.12). The 1-99 β-arrestin-1 truncation, that demonstrated enhanced apparent affinity, was sufficient to directly inhibit the activity of LIMK1 (Figure 3.13). Some truncations of β-arrestin-1, which bound to LIMK1 in sandwich immunoassays, did not directly inhibit LIMK1 activity significantly (Figure 3.14). β-arrestin-1 may have some regions mediating binding and other regions blocking the kinase activity.

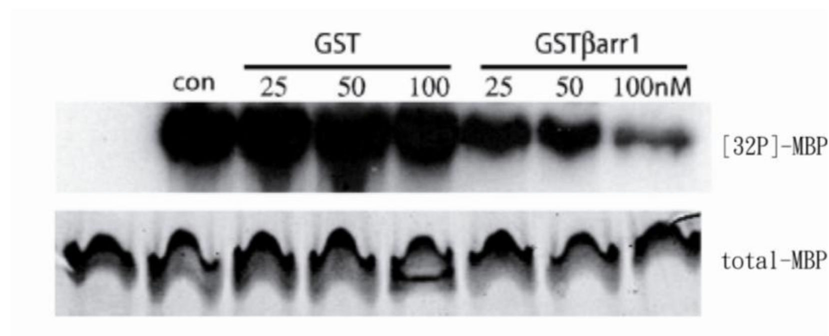


Figure 3.12 β-arrestin-1 directly inhibits LIMK activity *in vitro*.
LIMK assay: myelin basic protein (MBP) was incubated with active LIMK1 with or without different concentrations of GST or GST-β-arrestin-1 with the presence of radioactive ATP. *Upper panel*: representative autoradiograph showing the radio-labeled phosphorylated MBP. *Lower panel*: coomassie-stained gel showing the equally loading of MBP. GST-β1: β-arrestin-1; [32P]-MBP: radioactive ATP labeled MBP.

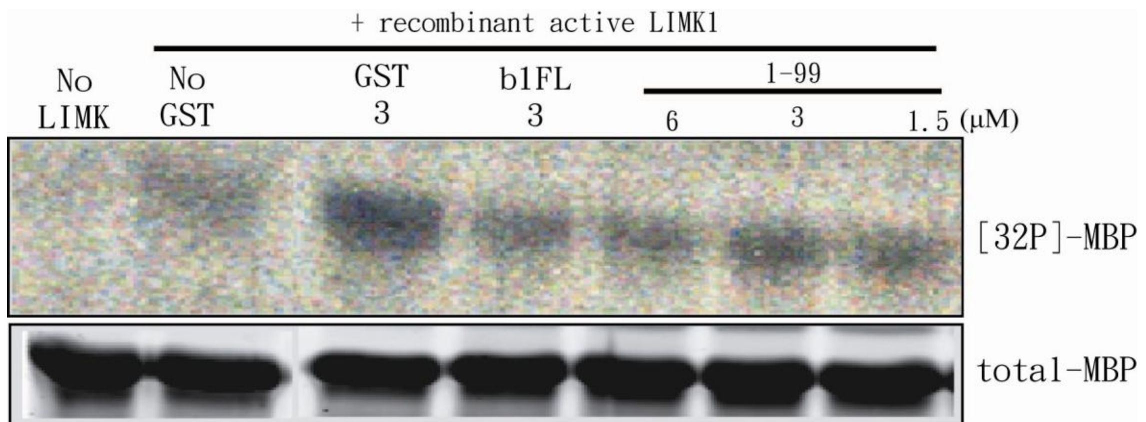


Figure 3.13 β-arrestin-1 small truncation including residues 1 to 99 directly inhibits LIMK activity *in vitro*.
Experiments performed as in figure 2.11. *Upper panel*: representative autoradiograph showing the radio-labeled phosphorylated MBP. *Lower panel*: coomassie-stained gel showing the equally loading of MBP. GST-β1: β-arrestin-1; [32P]-MBP: radioactive ATP labeled MBP.

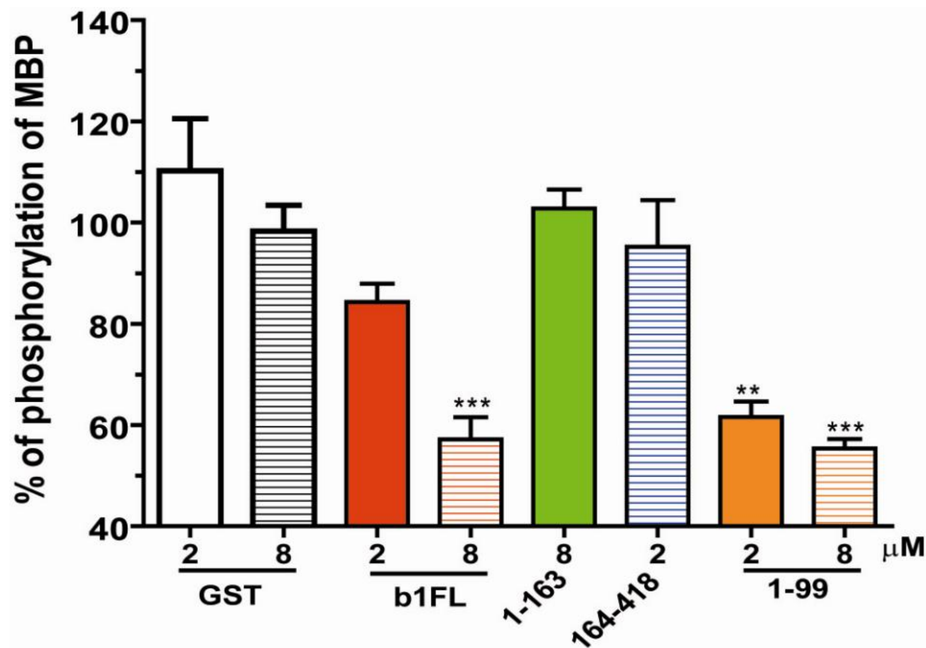


Figure 3.14 Bar graphs depicting the inhibition of LIMK1 by β-arrestin-1 full-length or truncations (mean-% of phosphorylation of MBP ± S.E., n ≥3).

Significant differences of β-arrestin with the control GST only are based on one-way ANOVA and indicated by ** ($p<0.01$) and *** ($p<0.001$). b1FL: GST-tagged β-arrestin-1 full-length; 1-163, 164-418 or 1-99: different GST-tagged β-arrestin-1 truncations which include amino acid residues 1-163, 164-418 or 1-99.

The *in vitro* sandwich immunoassay and kinase assay answered the questions about how β-arrestin-1 directly bind and inhibit LIMK1 activity. The following results further support the mechanism established by *in vitro* experiments. First, PAR-2 induced the association of β-arrestin-1 with LIMK1 in HEK293 cells (Figure 3.15). The highly sensitive BRET assay demonstrated that β-arrestin-1 directly interacts with LIMK1 in response to PAR-2 activation. Once the 2fAP added, the BRET ratio (eYFP/Rlu) increased rapidly (Figure 3.16). Interestingly, the β-arrestin-1 deletion mutant that showed a higher affinity for LIMK than full length β-arrestin-1 in vitro, demonstrated a lower max BRET upon PAR2 activation (Figure 3.17). Although some studies suggested that the 146-182 domain of β-arrestin-1 contains the receptor docking site, both Rlu-β-arrestin-1 and Rlu-β-arrestin-1 deletion mutant bound to PAR2-YFP once PAR2 was stimulated in the BRET assay (Figure 3.18). Thus, the deletion mutant is not deficient in recruitment to PAR2. β-arrestin-1 may have a specific receptor docking site for PAR-2 as well as for other GPCRs. Thus, the deletion of region 146-182 of β-arrestin-1 did not abolish its ability to bind to PAR-2. However, the deletion mutant did not demonstrate 2fAP-induced binding to LIMK1. The direct binding of β-arrestin-1 to LIMK1 may not only depend on the specific binding regions but also the conformational change of β-arrestin-1 downstream of PAR-2 activation. The *in vitro* kinase assays of LIMK1 and the sandwich immunoassays of LIMK1 with β-arrestin-1 elucidated that there were several regions in β-arrestin-1 for important for regulation of LIMK. Some regions of β-arrestin-1 may be responsible to form appropriate binding pockets for its downstream signaling molecules, such as LIMK1 in this chapter. Some regions block the kinase activity.

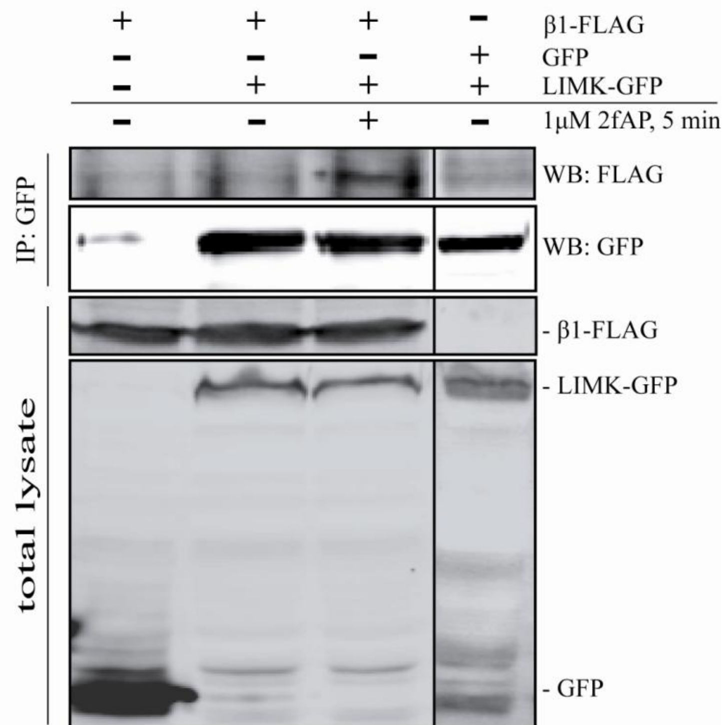


Figure 3.15 β-arrestin-1 associates with LIMK1 downstream of PAR-2 activation. Co-immunoprecipitation assay was performed by transfecting both FLAG-tagged β-arrestin-1 and GFP-tagged LIMK1 in HEK 293 cells. 1μM 2fAP was treated for 5 min. Next, LIMK1 was pulled down by anti-GFP with protein G agarose from the cell lysate. Western blots were probed with anti-FLAG and anti-GFP. Controls with only β-arrestin-1 or LIMK-GFP expression were included in consideration. *Upper panel*: showing the pull-down proteins, FLAG-tagged β-arrestin-1 and GFP-tagged LIMK1. *Lower panel*: total lysate showing the expression of each transfected protein. β1:β-arrestin-1.

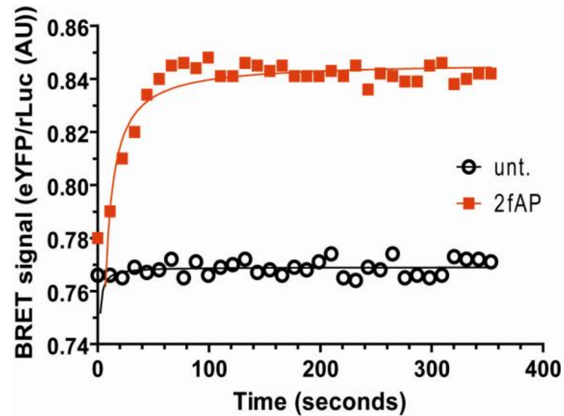


Figure 3.16 β-arrestin-1 directly binds to LIMK1 upon PAR-2 activation.
BRET assay: *Renilla* luciferase-tagged β-arrestin-1 (Rlu- β1) and yellow florescence protein-tagged LIMK1 (YFP-LIMK1) co-transfect in HEK 293 cells. The real-time interactions between the co-expression proteins are monitored by adding the luciferase substrate, coelenterazine *h*, and the released bioluminescence can transfer to the donor, YFP, only if two proteins directly bind to each other. The bioluminescence from luciferase (Rlu) and the emitted fluorescence from YFP (eYFP) are recorded for at least 10 min. The BRET ratio equals to eYFP/Rlu. The increasing BRET ratio with 2fAP treatment showed the directly binding of β-arrestin-1 to LIMK1.

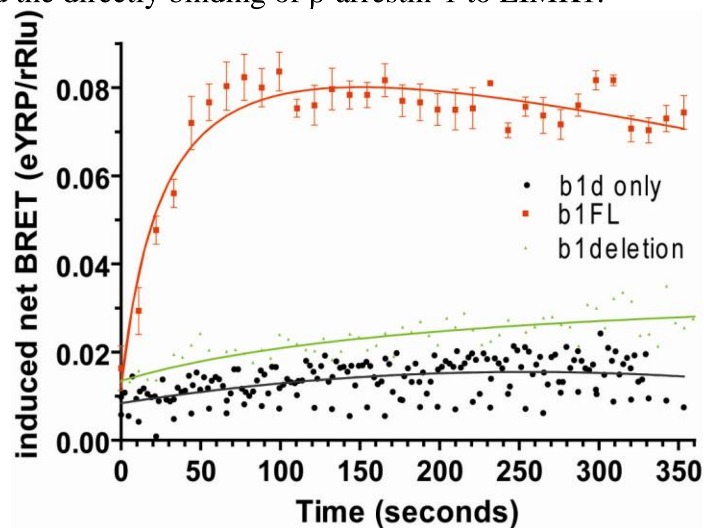


Figure 3.17 The directly interaction of β-arrestin-1 to LIMK1 downstream of PAR-2 activation is disrupted by the deletion including residues 146-182 of β-arrestin-1.
In order to show the rapid BRET ratio increasing upon PAR-2 activation, the induced net BRET was calculated by subtracting the BRET ratio of the paired untreated well in each BRET assay.

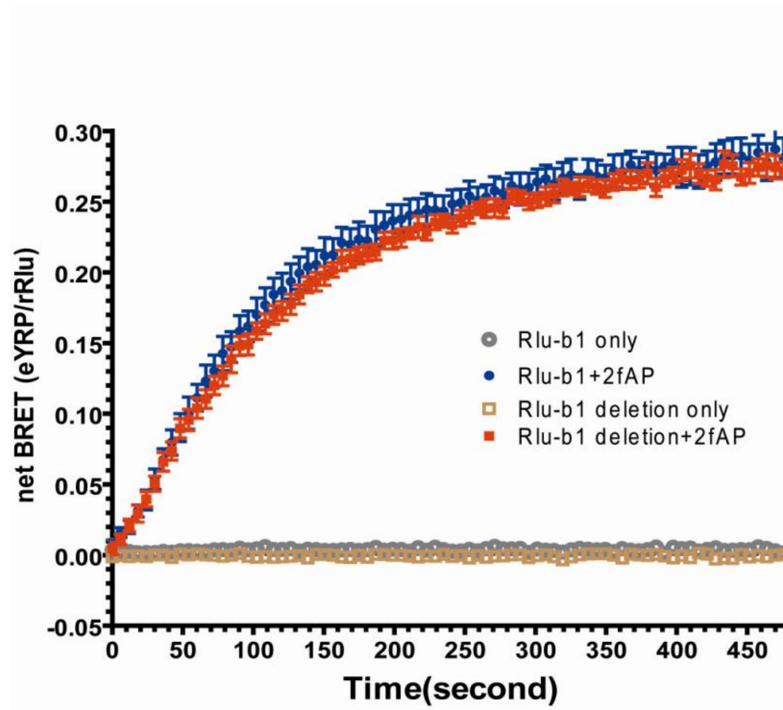


Figure 3.18 The stable and directly binding of β-arrestin-1 full-length or β-arrestin-1 deletion mutant to PAR-2.
BRET ratio curves showed the binding between β-arrestin-1 to PAR-2 did not affect by the deletion of amino acid residues 146-182 of β-arrestin-1.

CHAPTER FOUR

Identification of the mechanism by which β-arrestin-1 regulates cofilin

Chapter 4 Identification of the mechanism by which β-arrestin-1 regulates cofilin

4.1. Introduction

We proposed the molecular model that PAR-2 mediates chemotaxis by coupling with β-arrestins, independent of G-protein signaling, to promote the formation of several scaffolding complexes involved in regulation of actin temporally and spatially. The mechanism of β-arrestin-1 regulating LIMK1 was investigated and discussed in chapter two. In this chapter, we focus on the directly regulation of cofilin by β-arrestin-1.

There are three known regulators for mediating actin dynamics by severing actin filaments: cofilin-1 (as "cofilin" in this dissertation), cofilin-2 and actin depolymerization factor (ADF). Cofilin and ADF mainly express in non-muscle tissue and cofilin-2 distributes in muscle tissue. Cofilin binds to existing actin filaments on the $actin^{ADP}$ sites and introduces twists. The balance of cofilin and actin need to be delicately controlled for proper actin reorganization. During the actin filament treadmilling, $actin^{ATP}$ monomers are adding at the barbed end of actin filaments, hydrolyzing, and releasing its γ phosphate (*Pi*). Cofilin can sever the existing filaments and create many barbed ends as $actin^{ATP}$ adding sites. At the same time, the releasing of *Pi* after the additions of $actin^{ATP}$ increases the binding of cofilin to actin filaments because the depolymerization activity of cofilin increases much more in high pH. *In vitro* studies suggest that when the ratio (cofilin/actin monomer) is low (<1/100 of the K_D), cofilin persists to sever actin filaments. On the other hand, cofilin nucleates with actin and forms bundles at a high ratio of cofilin to actin subunit (Bernard 2007; Van Troys, Huyck et al. 2008). Because cofilin is

sensitive to the pH and the balance with other molecules in microenvironments, the temporally and spatially scaffolding abilities of β-arrestins make them become suitable adaptors and mediators for the activation of cofilin.

LIMK phosphorylates and inhibits cofilin activity by phosphorylating Ser3 of cofilin. The phosphorylation on Ser3 of cofilin inhibits its binding to actin monomers (F-actins), and to actin filaments (G-actins), too. NMR studies reveal that the Ser3 phosphorylated cofilin forms a salt bridge with Lys126 and Lys127. Lys126 and Lys127 are the key residues of cofilin for the association with actin (Pope, Zierler-Gould et al. 2004).

Recently, many other functions of cofilin are proposed. For example, cofilin is reported to sensor the pH and PtdIn(4,5)$P_{2.}$ It also can chaperon actins to nucleus for chromatin remodeling. The regulation of cofilin activation via β-arrestins may coordinate not only the actin dynamic reorganization and chemotaxis, but also other perturbations of cell physiology.

PAR-2 promotes rapid cofilin dephosphorylation via β-arrestins by several possible mechanisms. First, β-arrestins are able to scaffold CIN and cofilin into a complex to increase the activation of cofilin. Second, β-arrestins directly bind and inhibit LIMK1 to decrease the phosphorylation and inactivation of cofilin. We demonstrated that there are specific binding domains between residue 1 to 99 and residue 183-418 for the specific binding of β-arrestin-1 to LIMK1 while the directly inhibition may be mediated by the one of the specific binding regions. Finally yet importantly, we are going to

discuss that β-arrestins may bind and sequester the active and inactive cofilin in appropriate micro-domains at the cell protrusion.

4.2 Results and Discussion

Although the direct binding between β-arrestin-1 and cofilin was published from our laboratory in 2010 (Zoudilova, Min et al. 2010), the specific domains of β-arrestins that bind to cofilin have not been identified. Using sandwich immunoassays, we examined bindings of each set of proteins. First, recombinant β-arrestin-1 bound cofilin reaching saturation integrated intensity (Figure 4.1). The amino terminal domain (residues 1 to 163) of β-arrestin-1 and the carboxyl terminal domain (residues 164 to 418) of β-arrestin-1 both bound to cofilin while the amino terminal domain of β-arrestin-1 showed higher saturation maximum (Bmax) (Figure 4.2). The small amino terminal truncation including β-arrestin-1 amino acid residues from 1 to 99 bound to cofilin with much better relative binding affinity and bigger maximum binding value comparing with the β-arrestin-1 full-length (Figure 4.3). Besides, β-arrestin-1 truncation including amino acid residues 1-145 bound to LIMK1specifically while the region of 183-418 did not bind to LIMK1 (Figure 4.4). Because these two truncations of β-arrestin-1 had lower or no directly binding to LIMK1, we created the β-arrestin-1 mutant without the amino acid residues 146 to 182. However, this β-arrestin-1 deletion mutant maintained its ability to bind to cofilin as well as to LIMK1 (see *Chapter 2*) (Figure 4.5). The middle region of β-arrestin-1 did not responsible for the specific binding of β-arrestin-1 to LIMK1 either cofilin. This region of β-arrestin-1 may affect the conformations of β-arrestin-1 to expose the binding pockets in the amino terminal and carboxyl terminal regions of β-arrestin-1 for LIMK1 and cofilin.

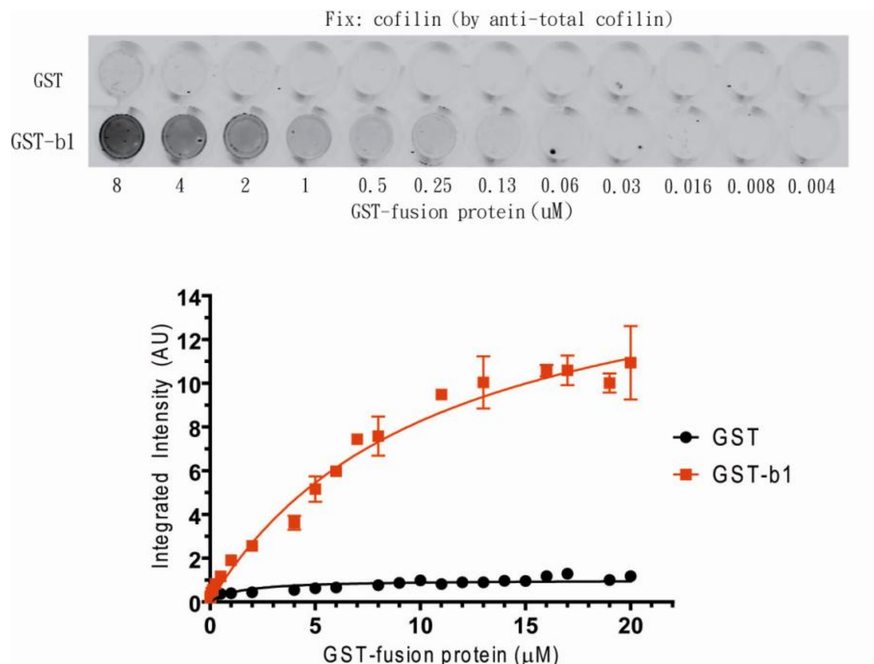


Figure 4.1 GST-β1 directly binds to cofilin.
A representative image of the sandwich immunoassay showed the binding of GST-β1 to the antibody-immobilized cofilin in the 96-well EIA/RIA plate. The integrated intensity was obtained from each well. The higher integrated intensity indicates the higher relative binding affinity to cofilin in the designated GST or GST-β1 concentration. The best-fit binding curve suggested that GST- β1 directly binds to cofilin. These integrated intensities were analyzed and graphed with *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1: β-arrestin-1.

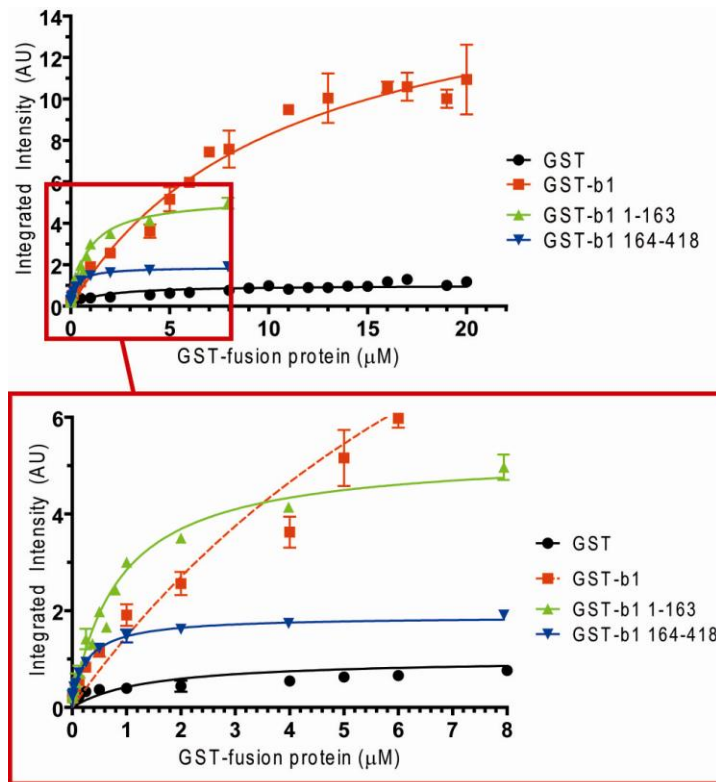


Figure 4.2 The amino terminal and carboxyl terminal regions of GST- β1 both bind to cofilin.

The best-fit curves were graphed according to the results from the sandwich immunoassays (n ≥3). GST- β1 amino terminus contains amino acid residues 1 to 163 and GST- β1 carboxyl terminus contains amino acid residues 164 to 418. These integrated intensities were analyzed and graphed with *GraphPad Prism*. β1: β-arrestin-1.

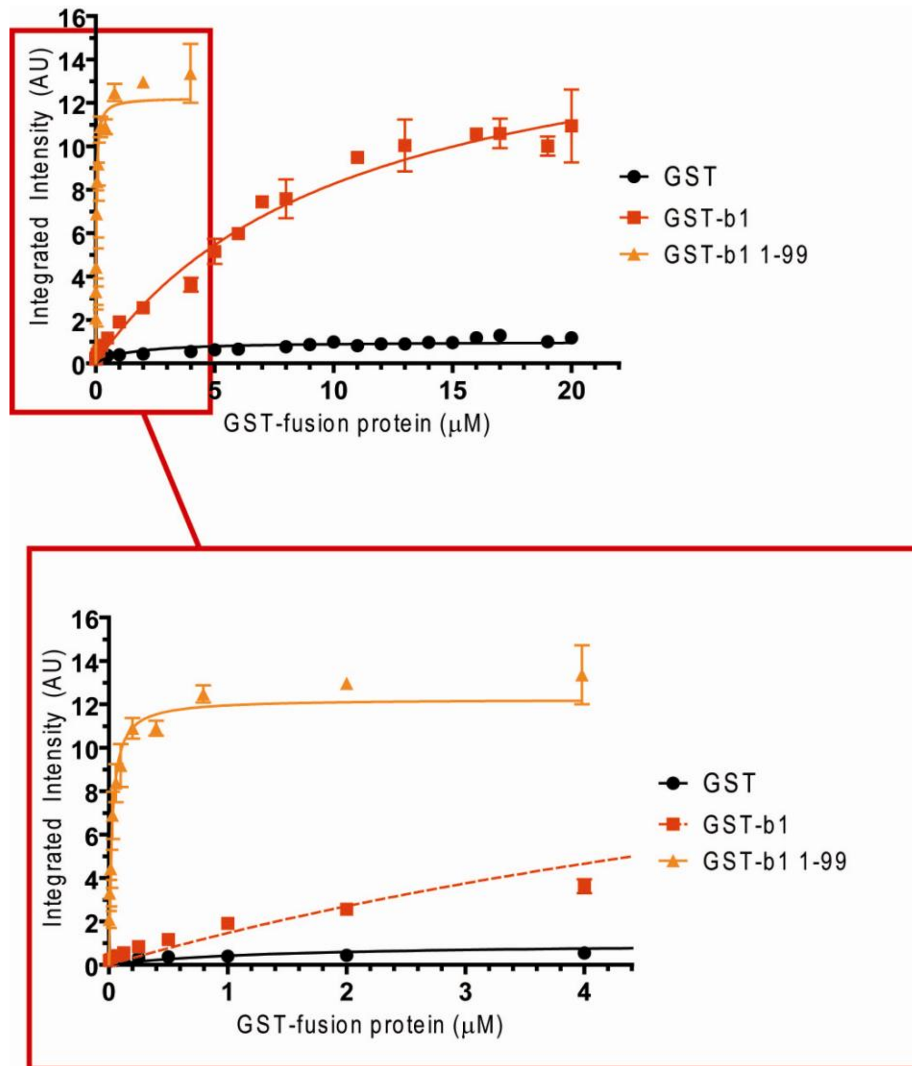


Figure 4.3 GST- β1 1-99 binds to cofilin.

The small truncation GST- β1 1-99 includes amino acid residues 1 to 99 and it demonstrated a significantly greater best-fit curve than the full-length β1. These integrated intensities were analyzed and graphed with *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1: β-arrestin-1.

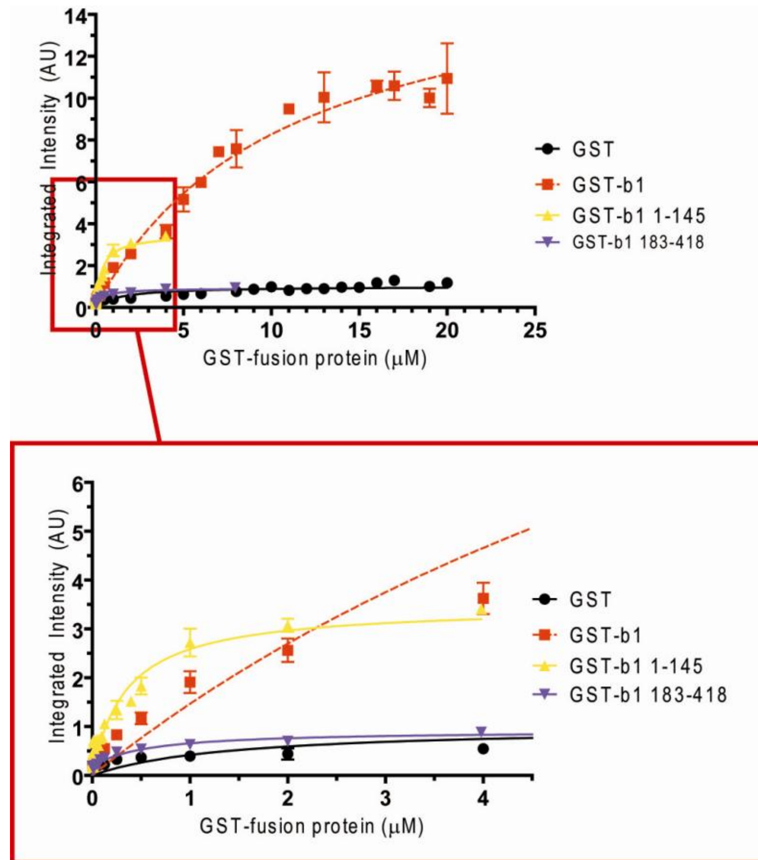


Figure 4.4 GST- β1 1-145 binds to cofilin but GST- β1 183-418 does not.
Two GST- β1 small truncations contain amino acid residues 1 to 145 and residues 183 to 418. The residues 183-418 of β1 truncation did not bind specifically to cofilin, but the residues 1-145 interacted with cofilin with lower relative binding affinity than the GST-β1 full-length to cofilin. These integrated intensities were analyzed and graphed with *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1: β-arrestin-1.

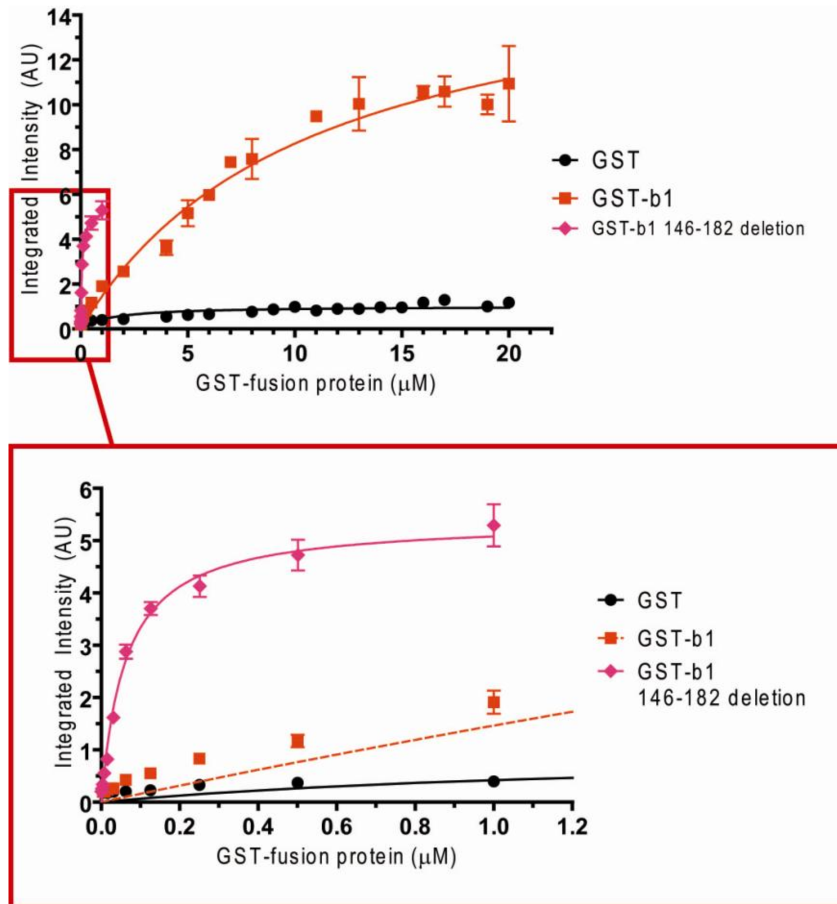


Figure 4.5 GST- β1 deletion mutant binds to cofilin.
The amino acid residues 146 to 182 of β-arrestin-1 were removed from the full-length β-arrestin-1 sequence. This special β-arrestin-1-deleted protein remained the binding to cofilin. These integrated intensities were analyzed and graphed with *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1: β-arrestin-1.

The direct inhibition of β-arrestin-1 to LIMK1 activity was proven by the significant decreases of the phosphorylation of MBP in the presence of β-arrestin-1 full-length or residues 1-99 truncation. However, when we performed the LIMK kinase assays using the specific substrate of LIMK1, cofilin, the inhibitory mechanism of β-arrestin-1 became complicated because of the versatility of β-arrestin-1. First, the full-length β-arrestin-1 still inhibited LIMK1 activity (Figure 4.6 and Figure 4.7). Moreover, the high concentrations of the carboxyl domain of β-arrestin-1 (residues 164-418) and the small amino terminal truncation including residues 1 to 99 showed inhibitions of cofilin phosphorylation (Figure 4.6). Furthermore, the inhibition of cofilin phosphorylation by β-arrestin-1 deletion mutant was better than the full-length β-arrestin-1 (Figure 4.7). The significant differences between truncations and deletion mutant suggested that β-arrestin-1 perform its dual functions: binding and inhibition in the *in vitro* LIMK-cofilin kinase assay (Figure 4.8).

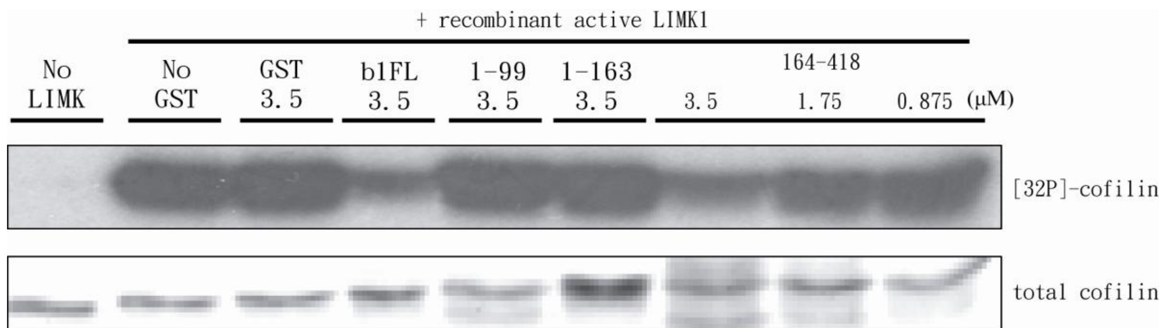


Figure 4.6 Representative autoradiograph shows that β-arrestin-1 directly inhibits LIMK1 to phosphorylate cofilin *in vitro*.
LIMK assay with cofilin as LIMK1 specific substrate: cofilin was incubated with active LIMK1 with or without different concentrations of GST, GST-β-arrestin-1 full-length or truncations with the presence of radioactive ATP. *Upper panel*: representative autoradiograph showing the radio-labeled phosphorylated cofilin. *Lower panel*: coomassie-stained gel showing the equally loading of cofilin. GST-β1: β-arrestin-1; [32P]-cofilin: radioactive ATP labeled cofilin.

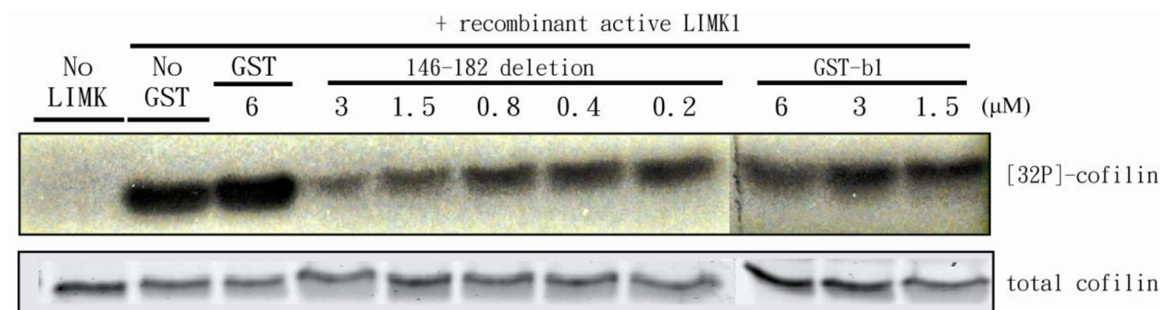
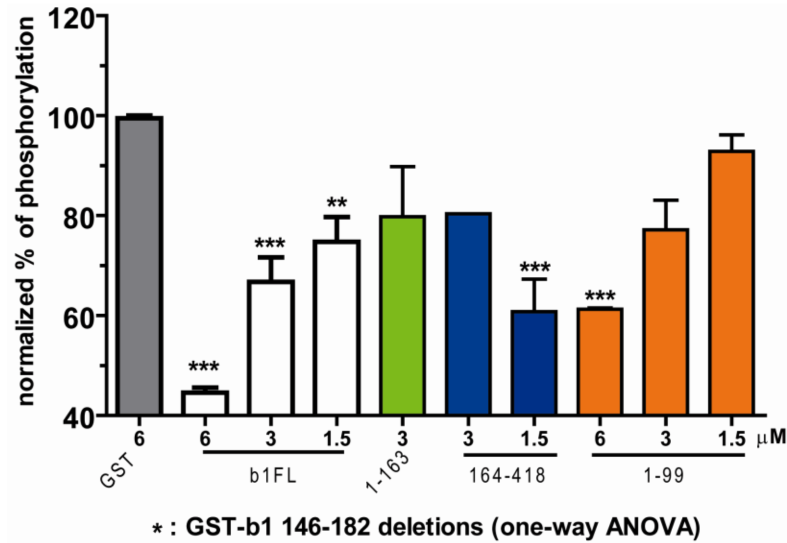


Figure 4.7 GST- β1 deletion mutant inhibits the phosphorylation of cofilin.
A representative autoradiograph of β-arrestin-1 deletion mutant (residues 146-182 were removed). Experiment process was performed as in the figure 4.6.



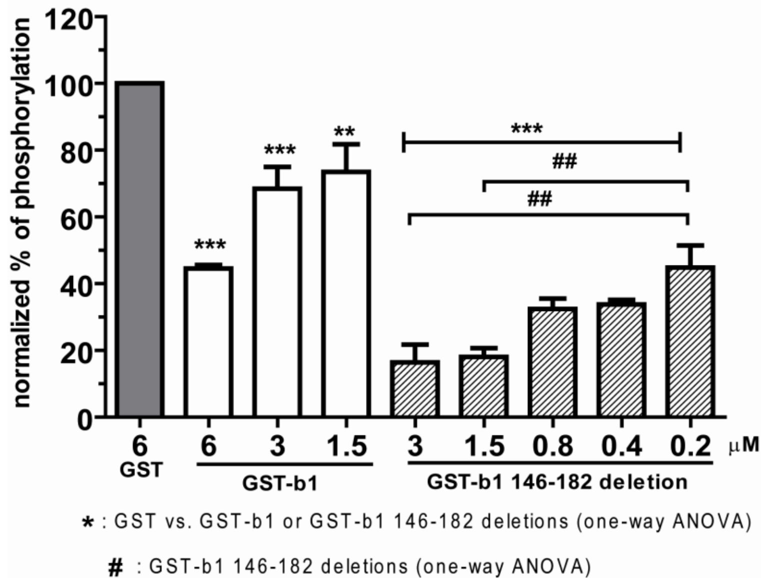


Figure 4.8 Bar graphs depicting the inhibition of LIMK1 by β-arrestin-1 full-length or truncations (mean-% of phosphorylation of cofilin ± S.E., n ≥3).

Significant differences between β-arrestin truncations and the control GST alone were based on one-way ANOVA and indicated by ** ($p<0.01$) and *** ($p<0.001$). The significant differences at different concentrations of β-arrestin-1 deletion mutant were analyzed by *t-test* and indicated by ## ($p< 0.01$). b1FL: GST-tagged β-arrestin-1 full-length; 1-163, 164-418 or 1-99: different GST-tagged β-arrestin-1 truncations which include amino acid residues 1-163, 164-418 or 1-99.

In the mechanism of β-arrestin-1 regulating the activation of cofilin, β-arrestin-1 plays multiple roles with LIMK and cofilin. *In vitro* sandwich immunoassays demonstrate the similarities and differences of β-arrestin-1 to LIMK1 and to cofilin (Figure 4.9).

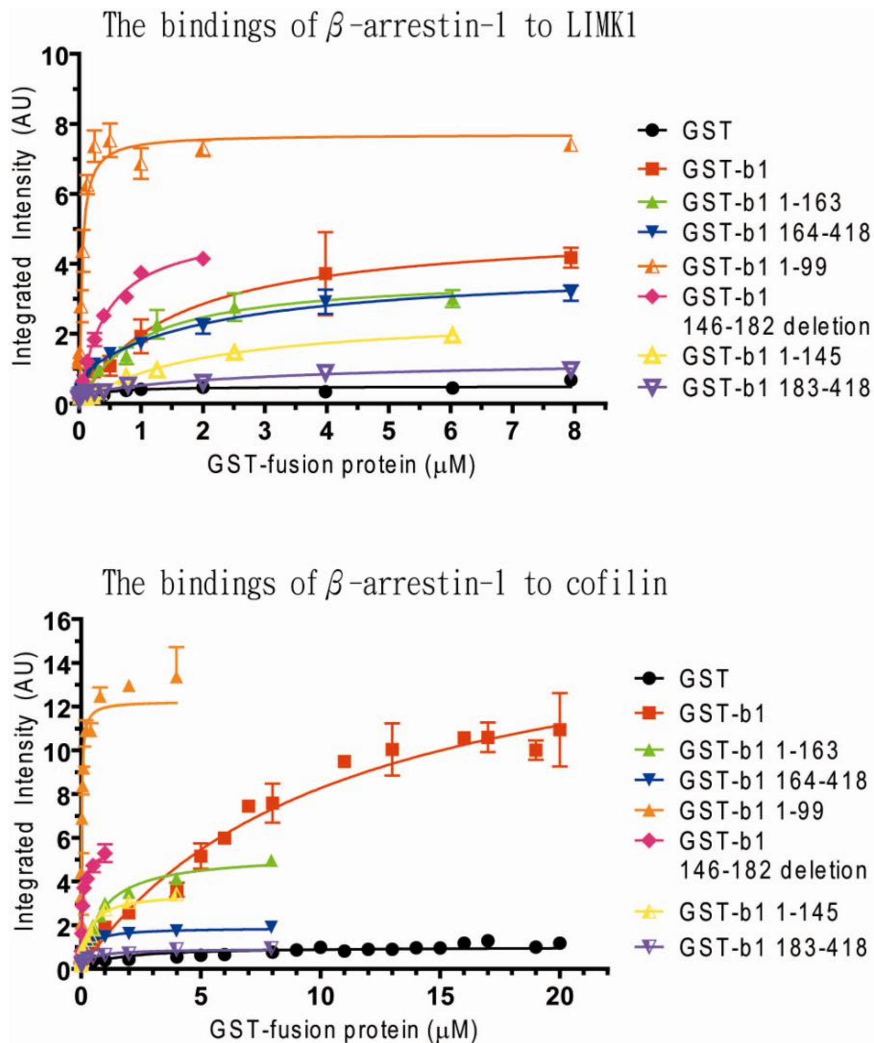


Figure 4.9 The summary of the sandwich immunoassays.
Upper panel: showing the best-fit binding curves of the full-length, truncations and deletion mutant of GST-β-arrestin-1 to LIMK1. *Lower panel*: the best-fit binding curves of the full-length, truncations and deletion mutant of GST-β1 to cofilin. These integrated intensities were analyzed and graphed with *GraphPad Prism*. At least 3 times experiments were performed in each binding conditions. β1: β-arrestin-1.

My project reveals the molecular details about the regulation of LIMK1 and cofilin by β-arrestin-1. First, β-arrestin-1 binds to LIMK1 and direct inhibits the kinase activity. The β-arrestin-1 1-99 binds to LIMK1 with high affinity, suggesting that there is at least one specific binding site for the binding of β-arrestin-1 to LIMK1. β-arrestin-1 1-145, and 1-163 confirm this conclusion by showing binding to LIMK1. When β-arrestin-1 binds to LIMK1, it also directly inhibits the kinase activity. Thus, when the region 1-99 of β-arrestin-1 binds to LIMK1, it may also block the ATP binding to LIMK1 and abolish the phosphorylation process. However, this binding pocket in region 1-99 of β-arrestin-1 is also important for the binding to cofilin. When the kinase assay performed in the presence of cofilin, this small region 1-99 of β-arrestin-1 only showed inhibition at high concentration. β-arrestin-1 needs to play the two roles at the same time: (1) binding and inhibiting LIMK1 will decrease the phosphorylation activity; (2) binding to cofilin may protect it from phosphorylation by LIMK1. The results from the GST-β-arrestin-1 1-99 sandwich immunoassays and kinase assays suggested that LIMK1 and cofilin competed the GST-β-arrestin-1 1-99 *in vitro*. Most of the GST-β-arrestin-1 1-99 molecules bound to cofilin while only small portions of GST-β-arrestin-1 1-99 bound and inhibited LIMK1 activity. Thus, the direct inhibition of LIMK1 by β-arrestin-1 could only observe if extra GST-β-arrestin-1 1-99 molecules are available to LIMK1. The different binding affinities and inhibitions of kinase activity by GST-β-arrestin-1 truncations indicate that there are different specific domains in β-arrestin-1 for mediating different functions. When we consider the results of the carboxyl region 164-418 of β-arrestin-1, the two roles of β-arrestin-1 both involve in the process again but perform with

different manners. The region 164-418 bound to both LIMK1 and cofilin, but this region did not show significantly direct inhibition of LIMK1. The phosphorylation of cofilin decreased in the kinase assay illustrated that the GST- β-arrestin-1 164-418 bound to cofilin and LIMK1 and sequestered them into two separate pools. The phosphorylation of cofilin decreased because the GST-β-arrestin-1 164-418 prevented the interaction between LIMK1 and cofilin. The characteristics of different regions of β-arrestin-1 elucidated more when the GST- β-arrestin-1 deletion mutant was utilized in the experiments. The amino terminal region of β-arrestin-1 may be responsible for the directly inhibition with high binding affinities to LIMK1 and cofilin. The carboxyl terminal region of β-arrestin-1 may control the binding abilities and specificities. The GST-β-arrestin-1 deletion mutant contains both the amino terminal region (residues 1-145) and carboxyl terminal region (residue 183-418). When comparing with the full-length β-arrestin-1, this β-arrestin-1 deletion mutant showed better binding affinities to LIMK1 and to cofilin, too. The results from the kinase assay in the presence of cofilin suggested that β-arrestin-1 deletion mutant not only directly bound and inhibited LIMK1 activity, but also prevented cofilin from phosphorylation by LIMK1 more efficiently than the full-length β-arrestin-1. The deletion of the middle region of β-arrestin-1 may affect the conformation of β-arrestin-1 a lot. While cofilin and LIMK1 compete for the binding sites on β-arrestin-1, the specificities of β-arrestin-1 to LIMK1 or cofilin may also rely on the orientations between the amino terminal lobe and the carboxyl terminal lobe of β-arrestin-1. The deleted region of the β-arrestin-1 mutant, 146-182, contains the linker

region (residue 174-181 in β-arrestin-1), which has been believed for controlling the orientation of the two lobes of β-arrestin-1.

CHAPTER FIVE

Conclusion and Perspectives

Chapter 5 Conclusion and Perspectives

5.1 Conclusion

PAR-2 mediates chemotaxis by using β-arrestins to promote the formation of several complexes for temporal and spatial activation of cofilin. Our previous studies elucidated how β-arrestins regulate CIN and cofilin in this novel mechanism of cofilin activation; however, another signal pathway for cofilin activation is through the β-arrestin-mediated LIMK1 inhibition, which is the major focus in this study.

According to the results and discussion in chapter 2 and chapter 3, we propose two possible mechanisms for the regulation of cofilin by β-arrestin-1 in PAR-2-induced chemotaxis. First, the activated PAR-2 recruits β-arrestin-1 and adapts β-arrestin-1 into different conformations. Thus, some of β-arrestin-1 molecules specifically bind to LIMK and inhibit LIMK1 activity while the other β-arrestin-1 molecules sequencer cofilin, or facilitate the scaffolding with CIN and cofilin together (Figure 5.1). β-arrestin-1 may mediate the cofilin activation directly by disrupting the binding and phosphorylation of LIMK1 to cofilin. β-arrestin-1 may have higher binding affinity to cofilin than the affinity of LIMK1 to cofilin. Thus, when β-arrestin-1 is present, LIMK1 cannot phosphorylate cofilin anymore. On the other hand, the direct binding of β-arrestin-1 to LIMK1 may inhibit its kinase activity by blocking the necessary ATP binding for kinase activity or inducing the bound LIMK1 to an inactive conformation (Figure 5.2).

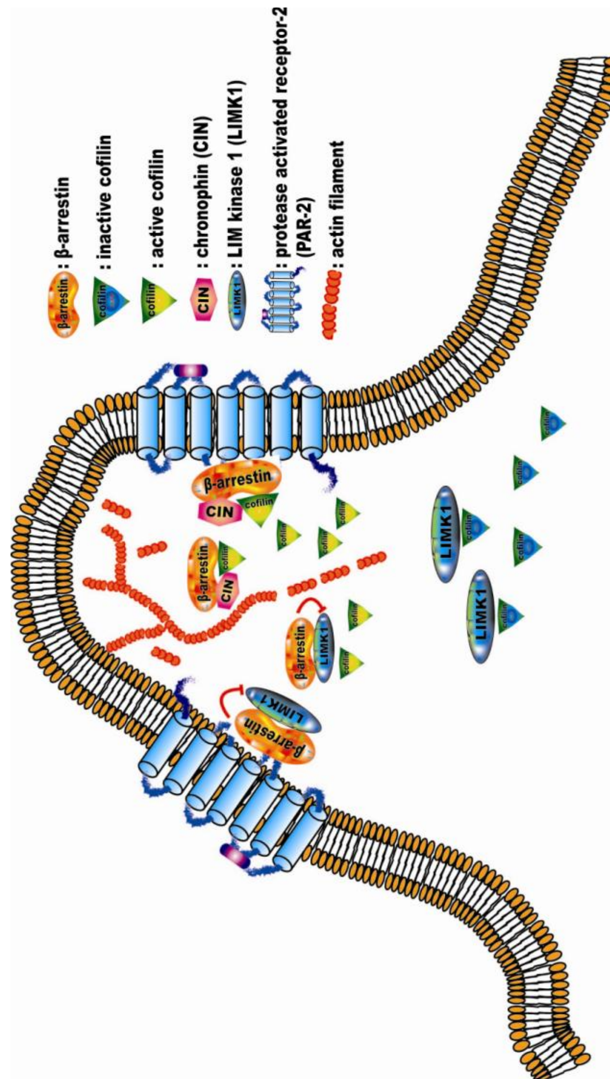


Figure 5.1 the possible mechanism of the regulation of cofilin activation by β-arrestin-1-mediated LIMK1 downstream of PAR-2 activation.

The cartoon figure represents the possible signaling pathways of PAR-2, β-arrestin-1, LIMK1, CIN and cofilin at the cell protrusion. Different pools of β-arrestin-1 molecules localize with different scaffold signal partner in the cell protrusion micro-domains. β-arrestin-1 controls the activation of cofilin spatially at the appropriate area of the actin filaments. Cofilin severs actin filaments and creates lots barbed end for increasing actin dynamics.

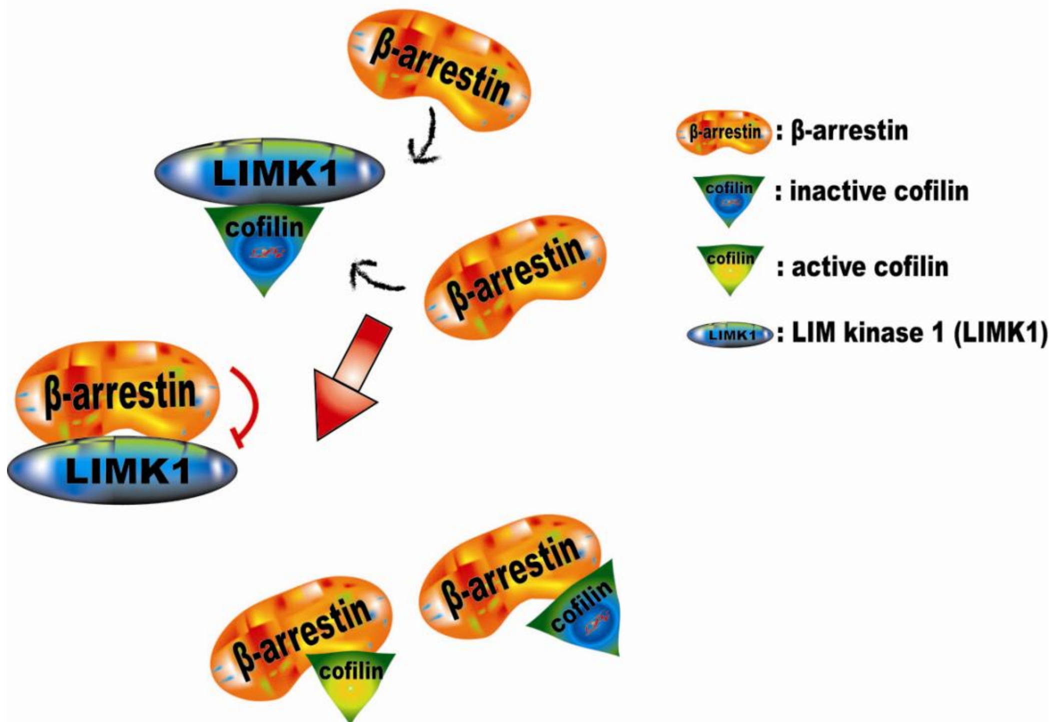


Figure 5.2 the second possible mechanism demonstrates the directly regulation of β-arrestin-1 to the binding of LIMK1 and cofilin.

First, β-arrestin-1 competes with LIMK1 for cofilin. Besides, β-arrestin-1 disrupts the binding of ATP to LIMK1 for its phosphorylation activity.

My research reveals many details about how β-arrestin-1 interacts with LIMK1 or cofilin *in vitro*. Multiple recombinant β-arrestin-1 proteins (the full-length, five truncations, and a deletion mutant) were purified in this study for different experiments including sandwich immunoassay, kinase assay and spot peptide assay. Three-dimensional structures predicted by Jigsaw online server demonstrate the positions of the recombinant β-arrestin-1 truncations in the structure of the wild-type β-arrestin-1(Figure 5.3). In the *in vitro* assays, the conformations of the truncated β-arrestin-1 should not change dramatically. Without the similar conformations of the potential binding pockets, the differences of the *in vitro* protein-protein interactions may be misinterpreted. For example, the highly homologous conformations of the full-length and the deletion mutant of β-arrestin-1 suggest that the deletion including residues 146 to 182 did not significantly change the conformation of β-arrestin-1 (Figure 5.4). Thus, the higher binding affinities of β-arrestin-1-deletion mutant to LIMK or cofilin and the ability of the β-arrestin-1 deletion mutant to inhibit LIMK1 activity are dependent on the differences of the specificities of the binding pockets to LIMK1 or cofilin.

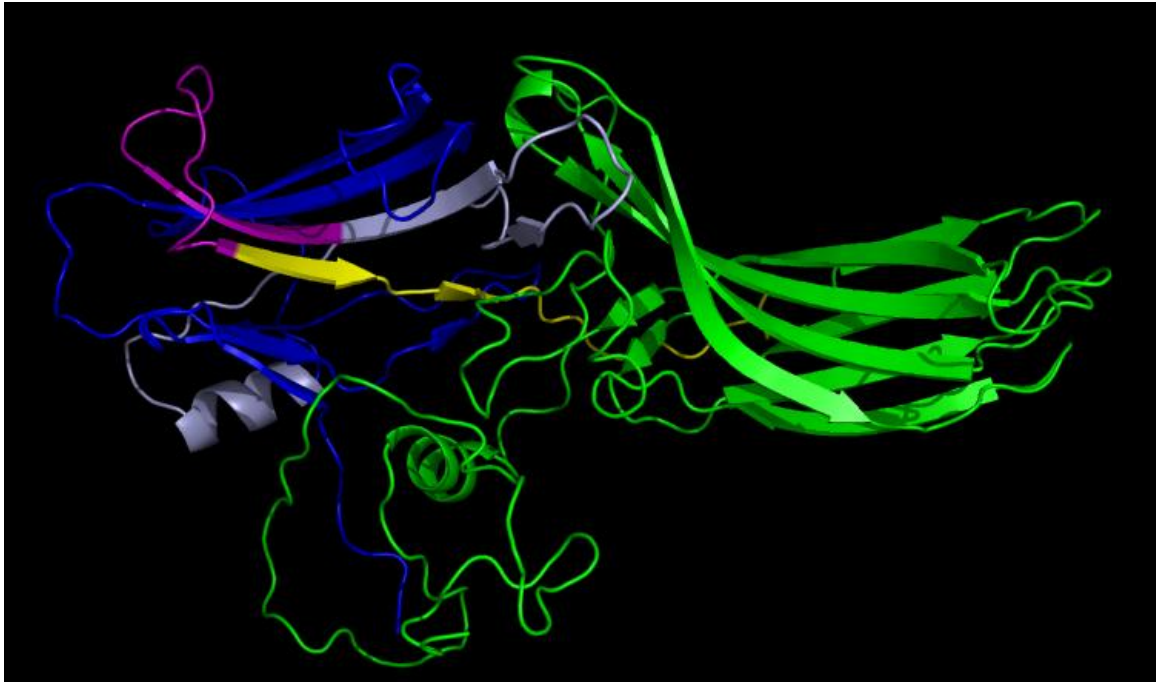


Figure 5.3 multiple recombinant β-arrestin-1 full length, truncations and deletion mutant in the structure of β-arrestin-1.

Truncations labeled with different colors to indicate the positions in the structure of β-arrestin-1. Blue: residues 1 to 99; light blue: residues 100-145; purple: residues 146-163; yellow: residues 164-182; green: residues 183 to 418.Three-dimensional structures were predicted by *3D-Jigsaw comparative modeling*. Protein structures were aligned and graphed by *PyMOL.*

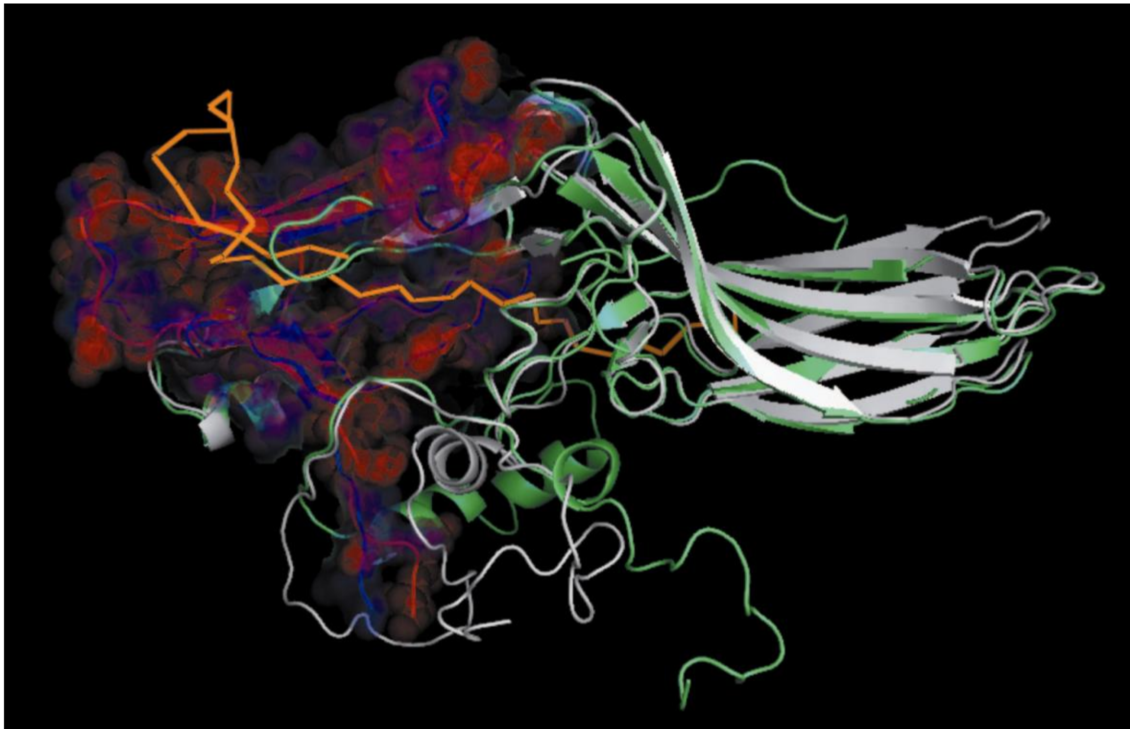


Figure 5.4 β-arrestin-1 full length and β-arrestin-1 deletion mutant (residue 146 to 182 were deleted) have similar conformations.

Cartoon, white: β-arrestin-1 full length; cartoon, light cyan: β-arrestin-1 deletion mutant; surface, red: residues 1 to 99 of β-arrestin-1 full length; sphere, blue: residues 1 to 99 of β-arrestin-1 deletion mutant; ribbon, orange: residues 146 to 182 of β-arrestin-1 full length. Three-dimensional structures were predicted by *3D-Jigsaw comparative modeling*. Two proteins were aligned and graphed by *PyMOL.*

5.2 Prospective

β-arrestins have many specific domains for scaffolding and regulating the activities of downstream signaling molecules. The specific domain of β-arrestin-1, amino acid residues 1 to 99, shows its high affinity and direct inhibitory ability to LIMK1. Some smaller peptide domains have been narrowed down within this region (residues 1 to 99) by results from spot peptide arrays. By revealing the specific binding and kinase regulation domain of β-arrestin-1, we can provide valuable information for the β-arrestin peptide-based therapeutic application. Peptide therapy is a new kind of possible therapies that using specific peptide fragments to interrupt signaling pathways of certain diseases, such as Parkinson's disease and cancer. Peptides are smaller and chemically stable than the monoclonal antibodies, which have shown clinical promise as cancer targeting agents. Some pioneer studies support the potential of peptide therapy in different diseases. The small peptide derived from DJ-1 is capable to arrest the progress of Parkinson's disease by protecting neural cells from dopamine toxicity. Several peptides targeting different tumor cells are currently in clinical trials. For example, [^{90}Y]-DOTA-labeled somatostatin analogues inhibit the growths of tumor cells from pituitary and gastroenteropancreatic tract (Aina, Sroka et al. 2002; Lev, Ickowicz et al. 2009; Lev, Barhum et al. 2012).

Moreover, the emerging knowledge of β-arrestin-dependent signaling pathways downstream of many GPCRs supports the development of a new class of therapeutic targets: the biased agonists. Some ligands can activate one of the two signaling pathways downstream of the same GPCR. Different biased ligands can stimulate the G protein-dependent pathway or the β-arrestin-dependent pathway because the receptor responds to

biased ligands distinctly and changes to many conformations. The β-arrestin-2 biosensor with Luciferase and YFP-tagged at two termini showed different levels of increasing of the BRET ratio when β2AR stimulated by different biased ligands (Shukla, Violin et al. 2008). β-arrestin-biased ligands can act same as the traditional antagonists to block the G protein-dependent signaling, but they can stimulate the β-arrestin-dependent signaling pathway, which may mediate potential benefit effects. For example, we showed that the development of neural growth cones in hippocampus is highly related to β-arrestins. A β-arrestin-biased agonist that can trigger the novel mechanism of cofilin activation downstream of PAR-2 will be potential pharmaceutical targets for abnormal neural development diseases.

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