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Molecular Mechanism of Beta-Arrestin-dependent ERK Activation Downstream of Protease-Activated Receptor-2

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DEDICATION

To

my late Grandfather,

Byung-So Min

ABSTRACT OF THE DISSERTATION

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by

Jungah Min

Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
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β-arrestins, originally discovered in the context of G protein-coupled receptor (GPCR) desensitization and internalization, also function in signaling of these receptors independently of G protein coupling. These novel functions involve the role for β-arrestins as scaffolds. It has been reported that β-arrestins interact with a number of binding partners including trafficking proteins, cytosolic kinases, cytoskeletal proteins, and non-receptor tyrosine kinase. Downstream of protease-activated receptor-2 (PAR-2), β-arrestin scaffolds the components of the ERK cascade, c-Raf, MEK1, and ERK1/2 with the receptor at the plasma membrane, leading to activation of cytoplasmic/membrane ERK1/2 signaling independently of G-protein coupling. Furthermore, we previously reported that stimulation of PAR-2 induced prolonged activation of ERK1/2 in pseudopodia in a β-arrestin-dependent manner and β-arrestins were required in PAR-2-mediated ERK1/2 activation at the membrane and cell migration in metastatic tumor cell lines, suggesting β-arrestin-dependent ERK1/2 activation might play a role in cell motility. Although a number of recent studies reported that β-arrestins are required for

ERK1/2 activation independently of G protein coupling, molecular mechanism of β-arrestin- mediated ERK1/2 activation via c-Raf has remained unclear. We hypothesized that the ability of β-arrestins to scaffold and prolong MAPK signaling at the membrane, is dependent upon precise molecular interactions that are facilitated by interaction of β-arrestins with PAR-2. To investigate the hypothesis, we determine the sites/domains in β-arrestin-1 that interact with components of the ERK module (c-Raf, MEK1, and ERK1/2) both *in vitro* and in cells using GST pull down assay, sandwich immunoassay, and co-immunoprecipitation and investigate the role of these identified regions of β-arrestin for interaction and activation of ERK1/2 using truncated mutants of β-arrestin or β-arrestin mutants lacking domains. In addition, a mysterious link in functional mechanism of β-arrestin-mediated ERK1/2 activation is how c-Raf relieves autoinhibition engendering a conformation change from a closed, inactive state to an open, active state, without small GTPase Ras, which was known to be critical for c-Raf activation in the classical G protein-dependent ERK activation. We hypothesized that β-arrestins behave similar to Ras for β-arrestin-dependent ERK activation to relieve autoinhibition of c-Raf. We show that β-arrestin-1 binds to the regulatory domain of c-Raf where Ras was indentified to bind. Therefore, binding of β-arrestin-1 to c-Raf might ensure formation of scaffolding complex containing β-arrestin-1 and the ERK cascade and play a critical role to activate c-Raf in β-arrestin-dependent ERK1/2 activation.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

Introduction

1.1. Background

1.1.1. Protease-activated receptor-2 (PAR-2)

Heptahelical G protein-coupled receptors (GPCRs or 7TMRs) are the largest and most diverse superfamily of plasma membrane receptors [1]. Approximately 600 distinct genes combined with alternative splicing account for as many as 1000 to 2000 discrete receptor proteins in human genomes [2, 3]. GPCRs respond to a variety of extracellular stimuli, including light, odor, inflammatory molecules, peptides, neurotransmitters, lipids and hormones [4], directing a diverse array of physiological responses and having relevance to numerous diseases [5]. They all share a common feature in that, upon activation, they can couple to a class of proteins known as G proteins that then transmit downstream signals that govern the ultimate cellular response. The receptors are key controllers of such diverse physiological processes as neurotransmission, cellular metabolism, secretion, cellular differentiation and growth, as well as inflammatory and immune responses. Thus, they have been considered as important targets for the development of new drug candidates with potential application in all clinical fields [6, 7].

Protease-activated receptor-2 (PAR-2) is one of the seven transmembrane G protein-coupled receptors. PAR-2 is the second of four members of protease-activated receptors (PARs) [8-12]. PAR-1, 3, and 4 are activated by thrombin but PAR2 is activated by multiple trypsin-like serine proteases including trypsin, tryptase, coagulation proteases upstream of thrombin (Tissue factors VIIa and Xa) and a membrane-bound serine protease (MBSPII), but not by thrombin [13-18]. PAR-2 is widely expressed in a number

of tissues such as airway, bond epidermis, exocrine glands, gall bladder, immune system, kidney, nervous systems, pancreas, stomach and in various cell types including vascular, immune and epithelial cells, astrocytes and neurons [10, 19-24]. Furthermore, it has been reported that PAR-2 is widely expressed in human tumors suggesting an important role in tumorigenesis and metastasis [25-27]. Numerous studies have shown that the distinct distribution of PAR-2 implicates protective and pathogenic roles in diseases such as asthma, colitis, and cancer.

Activation of PAR-2 occurs through an irreversible proteolytic mechanism. Serine proteases cleave the extracellular N-terminal domain of PAR-2 at specific sites to expose a new N-terminus that acts as a tethered ligand that binds to the second extracellular domain of the receptor and triggers intracellular signaling [20-22]. 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 [9, 28]. 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 [28-30]. More recently, synthetic activating peptide with an N-terminal furoyl group modification (*e.g.*, 2-furoyl-LIGRL-ornithine-NH_2 (2fAP)) has been reported to be equally effective to activate PAR-2 and more potent than SLIGRL-amide [31-33].

Activation of PAR-2 by proteolytic cleavage 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 inner leaflet of the plasma membrane [34, 35]. 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 IP_3 induces intracellular influx of calcium (Ca^{2+}), where DAG and Ca^{2+} activate PKC [36, 37]. Subsequent activation of mitogen-activated protein kinase (MAPK) by PKC leads to activation and nuclear translocalization of ERK1/2. However, unlike other PARs, activated PAR-2 can also elicit signaling independently of G protein coupling through its interaction with β-arrestins for several cellular events including chemotaxis, actin reorganization, prolonged ERK activation and subcellular localization of the ERK1/2 module to the membrane [38-41]. It has been shown that activation of PAR-2 promotes prolonged mitogen-activated protein kinase (MAPK) signaling, regulates activation of PI3K, RhoA, NFκB and cofilin/chronophin via β-arrestin-dependent mechanism in cell type-dependent manner [26, 38-45]. Furthermore, it has been reported that PAR-2-mediated cell motility in breast cancer cell lines requires β-arrestins to elevate ERK1/2 activation, 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 [40].

Despite the irreversible nature of the proteolytic activation, activated PAR-2 is rapidly desensitized and uncoupled from G protein signaling by receptor phosphorylation and β-arrestin binding. G protein-coupled receptor kinases (GRKs) mediate phosphorylation of activated GPCRs which leads to recruitment of β-arrestin to the intracellular c-terminus of the receptor and thereby prevents further the receptor-G protein coupling. Furthermore, the studies indicated that PKC activation can promote

desensitization and internalization of PAR-2. β-arrestin binding involves the receptor endocytosis via clathrin-mediated mechanism. [8, 41, 46-48].

PAR-2 is reported to trigger a wide variety of cellular responses including proliferation, ion transport, exocrine secretion, neurotransmission, electrolyte transport, chemotaxis, tumor cell metastasis in a tissue-specific and cell type-specific manner [10-12]. Ironically and contradictorily, numerous current studies indicate that PAR-2 activation has both protective and pathogenic consequences in cardiovascular, respiratory and gastrointestinal systems. Some of these pro- or anti-inflammatory effects included vasodilation, smooth muscle relaxation, up-regulation of cytokines, or immune cell recruitment [49-53]. There may be a fine balance between PAR-2-mediated responses. Since the activation and signaling of PAR-2 are relatively complex compared to other GPCRs, a greater understanding the effects of PAR-2 triggered signaling events has become more desirable in the study of different diseases. Many therapeutic agents currently in use act by either activating (agonists) or blocking (antagonists) GPCRs; for example, widely used examples are β-adrenergic receptor agonists for asthma and antagonists for hypertension and heart failure, histamine H_1- and H_2-receptor antagonists for allergies and duodenal ulcers, opioid receptor agonists as analgesics, dopamine receptor antagonists as antipsychotics and serotonin receptor agonists for migraine [54]. The current development of such drugs relies heavily on a number of cell-based assays for G protein coupling, as a readout of receptor activation. However, recent evidence has demonstrated that some of these receptors can act independently of G protein coupling [41, 42, 55-58], revealing the need for a greater understanding of these alternate pathways.

The phenomenon that the ability of selective ligands for the same receptor to activate receptor conformations responsible for the activation of particular downstream signaling pathways is referred to as “biased agonism” or “ligand-directed signaling” [59-62]. Such ligands are referred to as biased ligands. Recent studies demonstrated that activation of GPCRs directs two separate pathways: G protein-dependent and β-arrestin-dependent. β-arrestin-biased ligands offer the possibility to design an entirely novel class of therapeutic agents [63]. Bioluminescence resonance energy transfer (BRET) data demonstrated that the existence of G protein-independent and β-arrestin-biased pathways requires that receptors adopt functionally specific conformations: active or ligand-specific conformations [64]. As mentioned earlier, since a number of studies reported the existence of the dual functions of PAR-2 signaling with respect to inflammation [49-53], one can speculate that biased agonism to PAR-2 favor the β-arrestin-dependent pathway over the G protein-coupled pathways, or vice versa to cause the opposite consequences in the cellular context. Therefore, PAR-2 has been considered as a potential target for new drugs that can activate, deactivate or partially activate preferably specific downstream signaling pathways.

1.1.2. β -arrestins

β-arrestins have emerged as multifunctional scaffold proteins that interact with a growing list of molecules, mediating signal transduction and cellular trafficking [65-75]. β-arrestins were initially identified as signal terminators that arrest GPCR signaling and mediate the receptor internalization and degradation [76]. Shortly after the discovery of

β-arrestins as signal terminators, formation of c-src/β-arrestin complex in response to β_2AR activation was observed, leading to the idea that β-arrestins can serve as scaffolds to link receptors to downstream signaling pathways [55]. Subsequently, the function of β-arrestins as scaffold proteins has been demonstrated downstream of PAR-2, type IA angiotensin II receptor (AT1aR), neurokinin-1 receptor (NK1R), vasopressin-2 receptor (V2R), parathyroid hormone receptor (PTH1R), CXCR4 and gonadotropin-releasing hormone receptor (GnRHP) [40-42, 56, 57, 77-79]. Furthermore, β-arrestin-dependent pathways mediate actin cytoskeletal reorganization, cardiovascular regulation, immune responses, metabolic regulation, tumor growth, as well as activation of MAPK cascades [38-42, 55, 74, 79-90]. More recently, numerous putative β-arrestin binding partners have been reported [67, 73, 91, 92]. Among those identified, a well-studied β-arrestin-mediated signaling system leads to activation of MAP kinases: ERK1/2, JNK3, and p38 [41, 42, 55, 79, 85, 90]. In case of the extracellular signal-regulated kinase (ERK) cascade, it has been reported that upon stimulation of β_2-adrenergic receptor (β_2AR), neurokinin1 receptor (NK1R), angiotensin AT1a receptor (AT1aR), and protease-activated receptor-2 (PAR-2), β-arrestin scaffolds the components of the ERK cascade, c-Raf, MEK1, and ERK1/2 with the receptor, leading to activation of cytoplasmic/membrane ERK1/2 signaling. Moreover, for some of these receptors, the β-arrestin-dependent ERK1/2 activation can occur independently of G protein coupling [41, 42, 55-58]. We have showed that β-arrestins were required in PAR-2-mediated ERK1/2 activation at the membrane and cell migration in the metastatic tumor cell lines [26], suggesting β-arrestin-dependent ERK1/2 activation might play a role in cell motility. It has been shown that β-arrestin 2 binds JNK3 and

upstream kinases ASK1 and MKK4 to stimulate JNK3 activation after triggering the angiotensin type 1a receptor (AT1aR) [85, 93] and as well the dual specificity phosphatases (DSPs) MKP7 allowing transient JNK3 activation [94]. In addition, the JNK-interacting protein-1 (JIP-1) scaffold protein targets MAPK phosphotase-7 to dephosphorylate JNK [95]. JNK3 interaction has previously been mapped to the RRS motif of β-arrestin 2 between amino acids 195-202 [85]. More recently, it has been shown that N-terminal extension of JNK3, distinctive from other JNK family, plays a role in interaction with β-arrestin-2 [96]. Furthermore, suppression of β-arrestin-2 expression attenuates CXCR4-mediated cell migration in HEK293 cells mediated by p38 MAPK, but not ERK [79]. Another example has shown that β-arrestin-mediated p38MAPK activation is involved in platelet-activating factor-induced actin bundling for chemotaxis [97]. It appeared that β-arrestin-1 recruits MKK3, forming the scaffolding complex containing ASK1/MKK3/p38 and elicits specific actin bundle formation at the platelet-activating factor receptor (PAFR), suggesting β-arrestin scaffolding complex possibly plays a role in branching out actin filaments in pseudopodia responsible for cell polarity [97]. In addition, kappa opoid receptor-mediated activation of p38 MAPK was required β-arrestin recruitment to the receptor in neurons and astrocytes [90]. Although interactions between β-arrestin-2 and ERK1/2 have previously been reported [41, 57], until recently the site of this interaction on β-arrestin 2 has not been mapped. The results obtained from spot-immobilized peptide array indicated that mutations of positively charged amino acids at the surface limit β-arrestin 2 self-association and interfere with interactions with ERK1/2 MAPKs and with the β_2-adrenoceptor [98]. However, the

domain of β-arrestin-2 which displayed to self-associate and interact with ERK does not have ability to interact with JNK3. Therefore, one can conclude that in response to diverse agonists, β-arrestins can direct to multiple pathways, interacting with one but others or staying self-associated.

Since the evidences that β-arrestins function as adaptors for numerous putative proteins have been emerging, the domains of β-arrestins which interact with molecules such as clathrin, AP2, and c-src have been proposed in agonist-dependent manners [94, 99, 100]. We and other researchers have shown interactions between β-arrestins and each of the ERK module using gel filtration, co-immunoprecipitation and confocal microscopic studies [41, 55-58, 66, 101]. Even though the evidence has been accumulating to indicate that β-arrestins can bind to some MAPKs and facilitate their activation upon stimulation of GPCRs, the role of β-arrestins in regulating MAPK function has not been completely understood. Spot peptide array results showed that MEK-1 binds to 26-50 amino acids in β-arrestin-1 [102] and ERK-2 binds to 270-295 amino acids in β-arrestin-2 [98]. The current data point to a model wherein different receptors, and perhaps the same receptors in different cell types, can elicit specific β-arrestin conformations that expose certain domains to recruit distinct scaffolding complexes. The formation of different scaffolds on β-arrestins can then be determined by the activating receptor and its cellular context, leading to an additional level of signaling specificity.

As introduced above in 1.1.1., since numerous studies have reported that GPCRs directs two separate pathways: G protein-dependent and β-arrestin-dependent pathways.

The recent described concept of “ligand-bias” or “biased-agonism” have been well-accepted. Several recent reviews discussed “liagnd bias” for β-arrestin functions and suggested that β-arrestin-biased ligands might provide opportunities for the development of novel therapies [63, 68, 103].

1.1.3. Mitogen-Activated Protein Kinases (MAPKs)

Intercellular communication in multicellular organisms is achieved by signal transduction pathways. The pathways relay, amplify and integrate signals to elicit appropriate biological outcomes. A number of protein kinases and phosphatases participate in a wide variety of signaling pathways that mediate communication between cells and regulate cellular processes in response to extraordinarily diverse stimuli. One group of protein kinases, the mitogen-activated protein kinases (MAP kinases) are kinases involved in critical signaling pathways such as cell survival, differentiation, proliferation and apoptosis [104, 105]. The unit of mitogen-activated protein kinase (MAPK) module is a three-member protein kinase cascade that establishes a sequential activation pathway comprising a MAPK kinase kinase (MKKK), MAPK kinase (MKK), and MAPK [106-108]. Within the three-kinase module, MAPKs are phosphorylated and activated by MAPK kinases (MKKs). MKKs are characteristically dual specificity kinases which catalyze the phosphorylation of MAPKs on both tyrosine and threonine residues [109-114]. The MKKs are themselves activated by phosphorylation of two residues, either serine or threonine by upstream kinases, MKK kinases (MKKKs) [115-117]. During evolution, many of the components of three-kinase MAPK modules have

been conserved in yeast and in mammalian cells. To date, the combination of twelve member proteins of the MAPKs, seven MKKs, and fourteen MKKKs identified in mammalian cells can be grouped into five subfamilies, on the basis of sequence homology and function. There are examples of families of the multifunctional MAP kinases: five ERKs, three c-Jun NH_2-terminal kinases (JNKs), and four p38s. The ERK pathway was the first signal transduction cascade to be unraveled and delineated from the cell membrane to the nucleus, and its structure is the paradigm for MAP kinase modules in general. Downstream of receptors, the ERK cascade has been known to be activated by small GTP binding proteins (*i.e.*, Ras, Rac, or Cdc42) and specific kinases that could be considered to be MAPK kinase kinase kinases (MKKKKs) regulate the activity of MKKKs, thus controlling the activation of specific three kinase MAPK modules [118-120]. The MKKKs have different regulatory motifs: pleckstrin homology (PH) domains, proline-rich sequences for binding SH3 (src homology) domain, binding sites for GTP-binding proteins, leucine-zipper dimerization sequences, and phosphorylation sites for tyrosine and serine/threonine kinases. Therefore, a number of diverse upstream regulators lead to activation of specific MKKKs for selective signal cascades.

Perhaps the most well-defined MAPK cascade in mammalian cells is the extracellular signal-regulated kinase (ERK) module containing a linear cascade: c-Raf, MEK1/2, and ERK1/2. Depending on the cellular context, activation of this three-tier Raf/MEK/ERK pathway can alter fundamental processes such as proliferation, differentiation, migration and apoptosis [121]. Not surprisingly, abnormalities in the activation of this pathway are associated with several diseases, most notably with cancer

[122, 123]. Since their discovery over two decades ago, regulation of the ERK cascade has been intensively studied. Extracellular signals are transmitted to c-Raf through receptor-induced activation of small G protein, Ras GTPases [124, 125]. Active Ras in the GTP-bound form binds to the NH_2-terminal regulatory domain of c-Raf and recruits it from the cytosol to the plasma membrane, resulting in conformational changes in c-Raf from the autoinhibitory, inactive state to active catalytic state or simply the proper environment for c-Raf signaling [126-129].

One distinctive property of the MAPK cascade is the existence of scaffold proteins to achieve specificity and to coordinate MKKK binding to specific proteins for upstream inputs as well as specific downstream MKK-MAPK complexes in different cell types and in response to diverse stimuli [75, 130-134]. A review by Pawson described three general themes relevant to controlling the cellular outcomes in complex biological systems via the regulation of MAPK cascade: first, large scaffolding complex can bind and organize specific and multiple members of signaling proteins in a network by non-catalytic protein-protein interactions; second, trafficking and sublocalization of signaling complexes within the cell can dictate the biological consequences; and third, the duration of the signal can strongly influence the nature of the biological outcomes [135]. For example, scaffolding proteins in MAPK cascades can give them preferential access to one another, insulate the active kinases from dephosphorylation by phosphatases and localize the grouped components to specific areas in the cell. The orchestration of MAPK cascade components by anchoring or scaffolding proteins can control positive and negative regulation mechanisms. The function of scaffolding proteins in regulation of MAPK

modules has been most clearly investigated in the yeast *Saccharomyces cerevisiae* [89, 136-138]. Ste5 is a scaffold for the mating response MAPK module, functioning to organize a multi-component signaling complex and regulate the mating response. Shortly thereafter, in mammalian cells, several scaffold proteins were identified. Firstly, a protein referred to as MP1 (MEK partner 1) was identified using two yeast hybrid system that appears to be a scaffold protein, selectively promoting signaling from MEK1 to ERK1 [139, 140]. In addition, JIP-1 (JNK interacting protein-1) was initially described as a cytoplasmic protein and subsequently suggested its function as a scaffolding protein for specific component kinases in the JNK pathway [134]. One of these proteins is the kinase suppressor of ras (Ksr) involved in ras-mediated ERK pathway [141-144]. Moreover, a dimeric 14-3-3 protein was identified to support c-Raf activity by binding c-Raf within its amino-terminus [145, 146]. It was speculated that 14-3-3 interaction with c-Raf prevents c-Raf dephosphorylation, thus prolonging its activation [147, 148]. The last example of scaffold protein-dependent MAPK cascade is seen with β-arrestin-1 and 2. As mentioned earlier in section 1.1.2., in addition to their first identified and well-defined role in the desensitization and internalization of G protein-coupled receptors (GPCRs), a growing body of evidence indicates that β-arrestins function as GPCR signal transducers and scaffold proteins that interact with MAPK modules such as ERK, JNK and p38 to regulate both the activity and spatial distribution of ERK signaling, thus eliciting critical biological consequences [41, 55, 75, 79]. β-arrestins interact with each of MAPK cascade components and organize a scaffolded MAPK signaling complex associated with specific receptors. Since a number of scaffolding proteins in regulating MAPK cascades have

been identified so far, the given MAPK cascade scaffolded by specific scaffolding proteins could regulate their signaling in different locations for different durations and functions within specific microdomain of the cell. For example, high affinity β-arrestin binding to selected GPCRs results in retention of the β-arrestin-scaffolded ERK1/2 at the membrane, in contrast KSR-bound ERK1/2 readily dissociates from the scaffolding complex, allowing the ERK to translocate into the nucleus from cytosol. Therefore, function of different scaffolding complexes could elicit diverse cellular consequences.

1.2. Figures and Legends

Fig. 1.1. Schematic drawing of protease-activated receptor-2 (PAR-2) activation. Endogenous proteases such as trypsin, tryptase and coagulation factor VIIa and Xa but not thrombin enzymatically cleave the N-terminus of PAR-2, exposing a tethered ligand domain (NH_2- SLIGRL for mouse and NH_2-SLIGKV for humans) that binds to conserved regions in extracellular loop II of the receptor and leads to receptor activation. Synthetic peptides, such as SLIGRL-amide (mouse), based on the receptor-activating sequence of the tethered ligand, directly binds to the extracellular loop of PAR-2, mimicking the action of endogenous activators and activating the receptor independent of receptor cleavage.

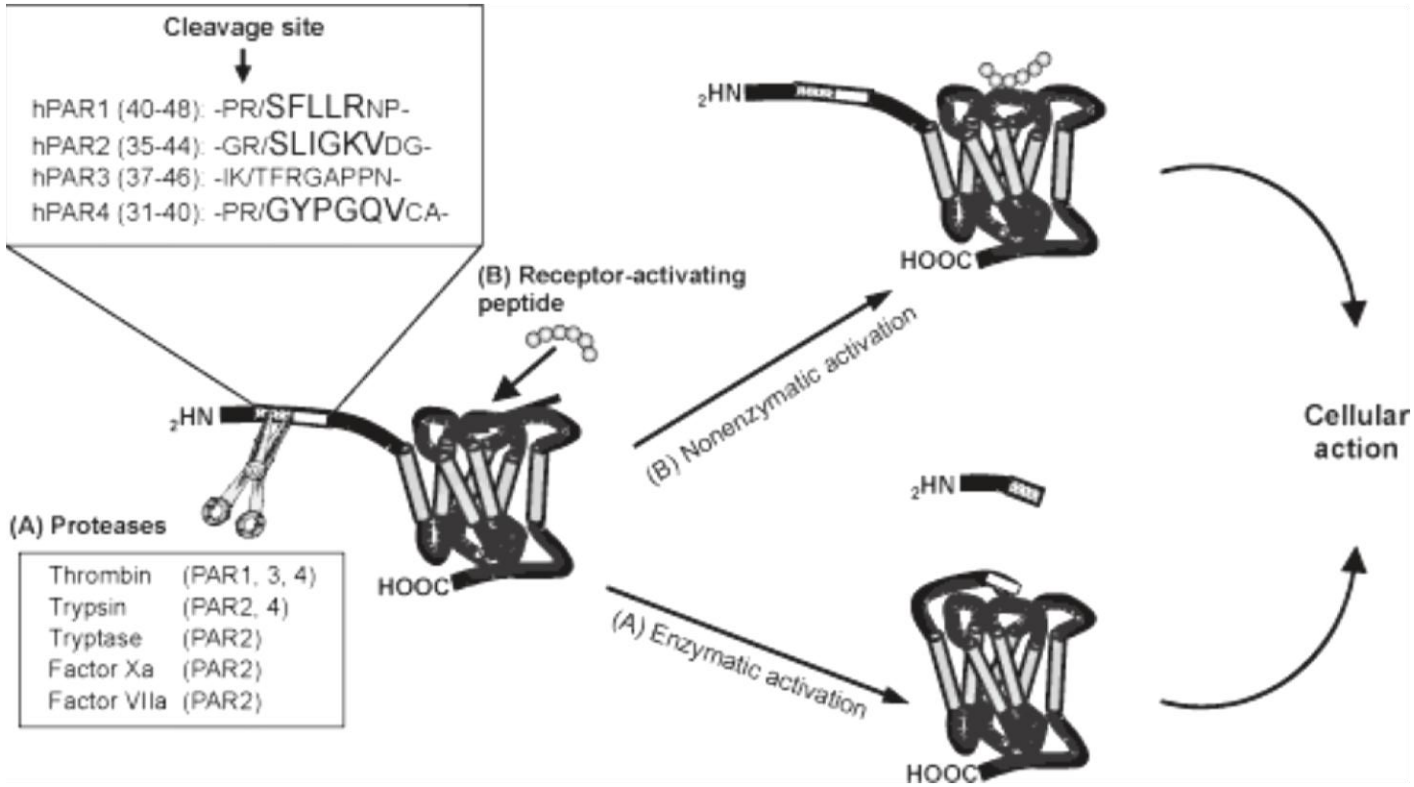

Cleavage site
hPAR1 (40-48): -PR/SFLLRNP-
hPAR2 (35-44): -GR/SLIGKVDG-
hPAR3 (37-46): -IK/TFRGAPPN-
hPAR4 (31-40): -PR/GYPGQVCA-
(B) Receptor-activating peptide
2HN
HOOC
(A) Proteases
Thrombin (PAR1, 3, 4)
Trypsin (PAR2, 4)
Tryptase (PAR2)
Factor Xa (PAR2)
Factor VIIa (PAR2)
(B) Nonenzymatic activation
(A) Enzymatic activation
Cellular action

Fig. 1.2. 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 IP_3 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 receptor-β-arrestin-ERK complexes are localized to endosomal vesicles, and their formation does not result in nuclear translocation of activated ERK1/2 or stimulation of cell proliferation. The function of β-arrestin-bound ERK1/2 is presently unknown, possibly favoring the phosphorylation of plasma membrane, cytosolic or cytoskeletal ERK1/2 substrates.

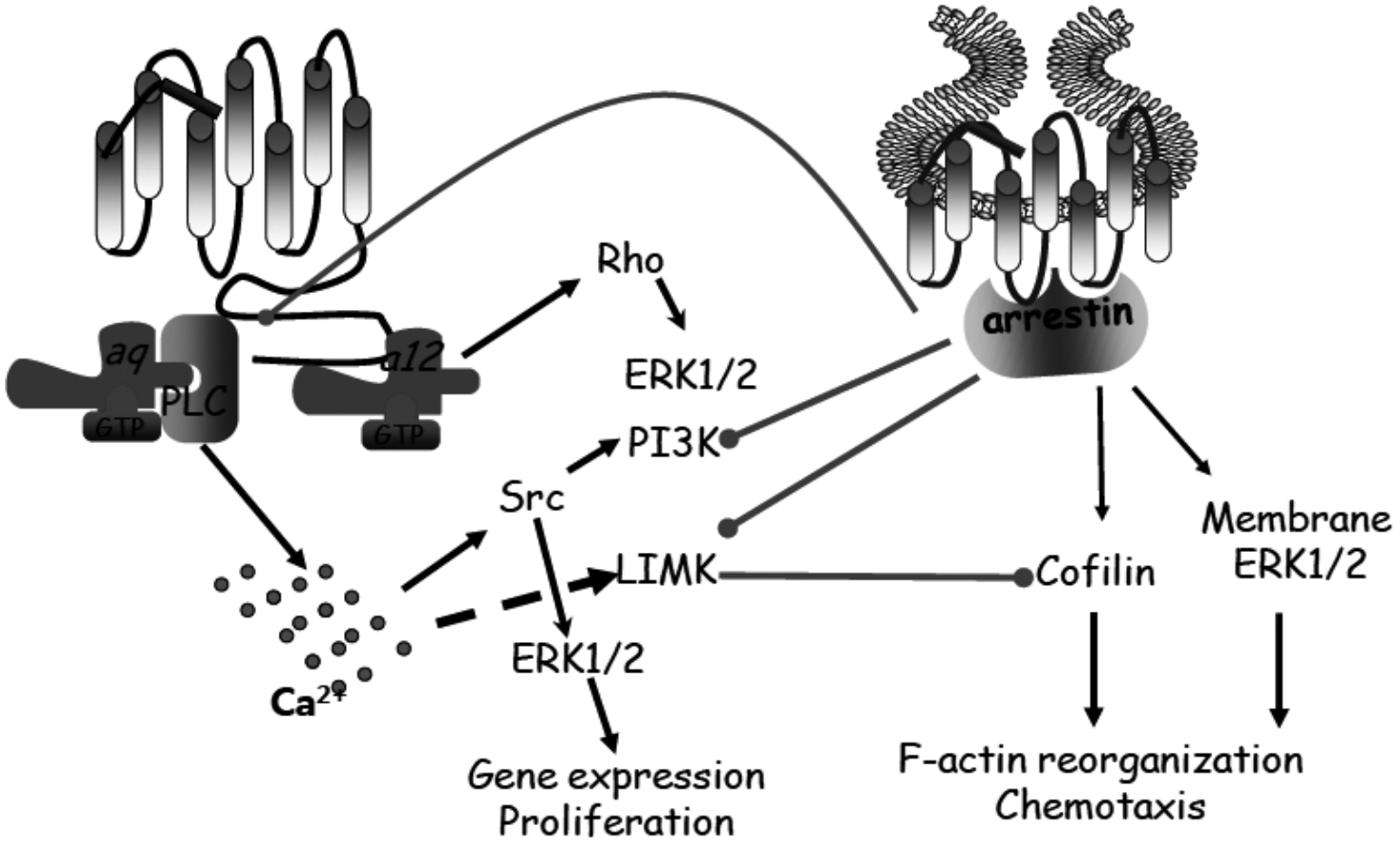

Rho
ERK1/2
aq
PLC
GTP
a12
GTP
arrestin
PI3K
Src
LIMK
Cofilin
Membrane
ERK1/2
Ca2+
ERK1/2
Gene expression
Proliferation
F-actin reorganization
Chemotaxis

Fig. 1.3. 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 poly-proline 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.

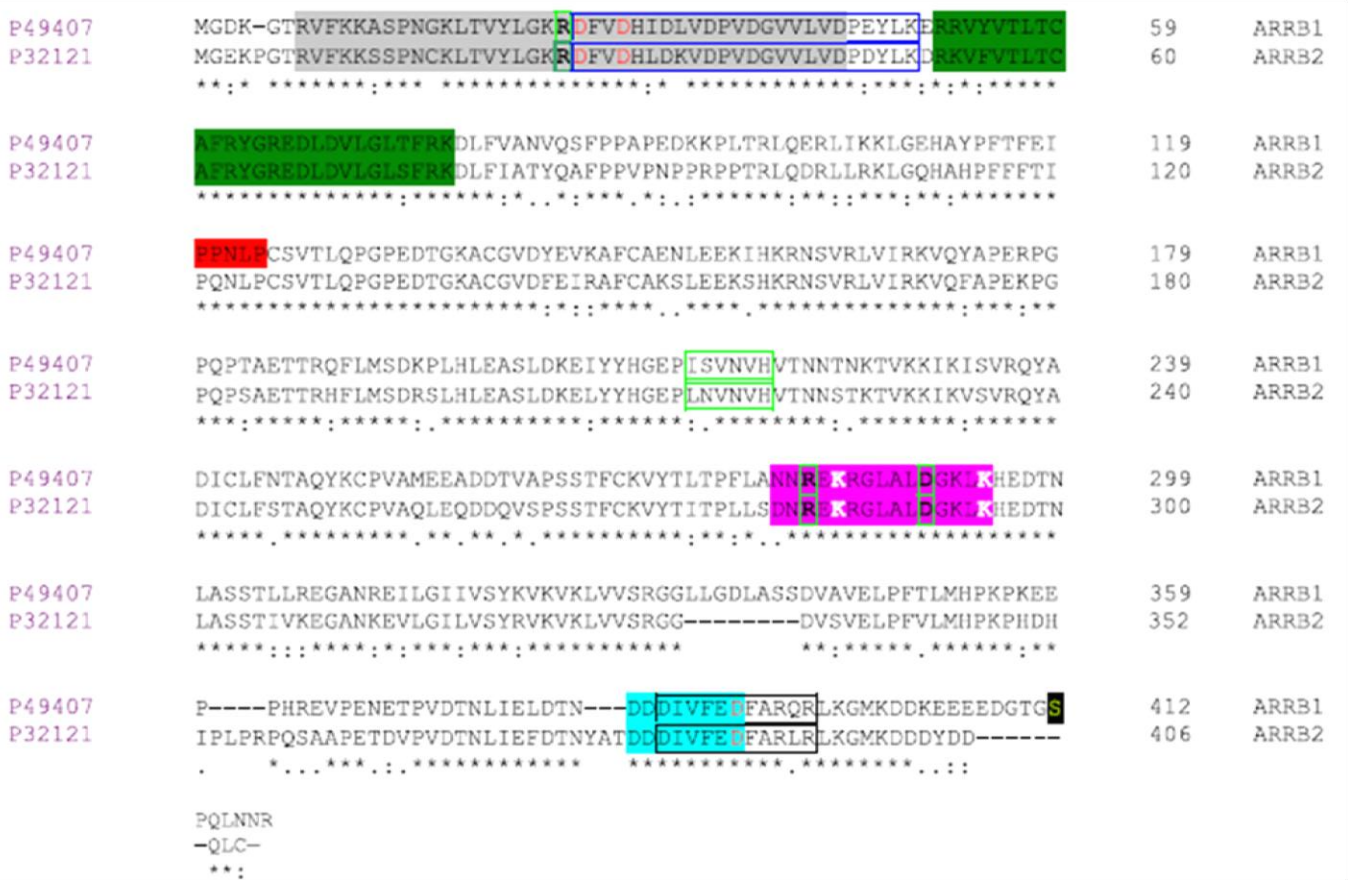

P49407 MGDK-GTRVFKKASPNGKLTVYLGKRDFVDHIDLVDPVDGVVLVDPEYLKERRVYVTLTC 59 ARRB1
P32121 MGEKPGTRVFKKSSPNCKLTVYLGKRDFVDHLDKVDPVDGVVLVDPDYLKDRKVFVTLTC 60 ARRB2
P49407 AFRYGREDLDVLGLTFRKDLFVANVQSFPPAPEDKKPLTRLQERLIKKLGEHAYPFTFEI 119 ARRB1
P32121 AFRYGREDLDVLGLSFRKDLFIATYQAFPPVPNPPRPPTRLQDRLLRKLGQHAHPFFFTI 120 ARRB2
P49407 PPNLPCSVTLQPGPEDTGKACGVDYEVKAFCAENLEEKIHKRNSVRLVIRKVQYAPERPG 179 ARRB1
P32121 PQNLPCSVTLQPGPEDTGKACGVDFEIRAFCAKSLEEKSHKRNSVRLVIRKVQFAPEKPG 180 ARRB2
P49407 PQPTAETTRQFLMSDKPLHLEASLDKEIYYHGEPISVNVHVTNNTNKTVKKIKISVRQYA 239 ARRB1
P32121 PQPSAETTRHFLMSDRSLHLEASLDKELYYHGEPLNVNVHVTNNSTKTVKKIKVSVRQYA 240 ARRB2
P49407 DICLFNTAQYKCPVAMEEADDTVAPSSTFCKVYTLTPFLANNREKKRGLALDGKLKHEDTN 299 ARRB1
P32121 DICLFSTAQYKCPVAQLEQDDQVSPSSTFCKVYTITPLLSDNREKKRGLALDGKLKHEDTN 300 ARRB2
P49407 LASSTLLREGANREILGIIVSYKVKVKLVVSRGGLLGDLASSDVAVELPFTLMHPKPKEE 359 ARRB1
P32121 LASSTIVKEGANKEVLGILVSYRVKVKLVVSRGG--------DVSVELPFVLMHPKPHDH 352 ARRB2
P49407 P----PHREVPENETPVDTNLIELDTN---DDDIVFEDFARQRLKGMKDDKEEEEDGTGS 412 ARRB1
P32121 IPLPRPQSAAPETDVPVDTNLIEFDTNYATDDDIVFEDFARLRLKGMKDDDYDD------ 406 ARRB2
PQLNNR
-QLC-

Fig. 1.4. Structural model and receptor-dependent conformational changes. (A) A ribbon diagram of rat β-arrestin-2 is portrayed using available structures of arrestin and β-arrestin-1. β sheets are colored blue, helix I (indicated) is red, and the connecting loops and C-tail are black. N-domain and C-domain regions are connected by a hinge region. Both the N-terminal β strand I and the C-terminal β strand XX are juxtaposed to helix I. (B) Protein-protein interaction motifs mapped on β-arrestin-2. Prolines at positions 91 and 121 are important for β-arrestin-1-cSrc interaction and are depicted as green space filling spheres. The clathin-binding domain L-I-E-F and the MAPK docking domain R-R-S are shown as cyan and yellow spheres, respectively. AP2 binding requires the arginine residues at positions 394 and 396, shown as magenta spheres. Mdm2 and ASK1 bind to regions delimited by residues, 160 to 300 and 1 to 185 (N-terminal half), respectively. JNK3 binds to the region 185 to 410 (C-terminal half). Both N- and C-domains of β-arrestin interact with 7TMRs. (C) 7TMR-induced conformational changes in β-arrestin. Structural models of 7TMR (rhodopsin) and rat β-arrestin-2 are shown in the basal state on the left side. The C-terminal tail of β-arrestin (blue) is buried and not accessible. Agonist stimulation leads to phosphorylation of serine and threonine residues on the receptor c-tail. The charged domain thus created penetrates and disrupts the polar core of β-arrestin and leads to global conformational rearrangements, which cause the β-arrestin C tail to be released and exposed for protein interactions. The C terminus of β-arrestin contains the clathrin- and AP2-binding regions.

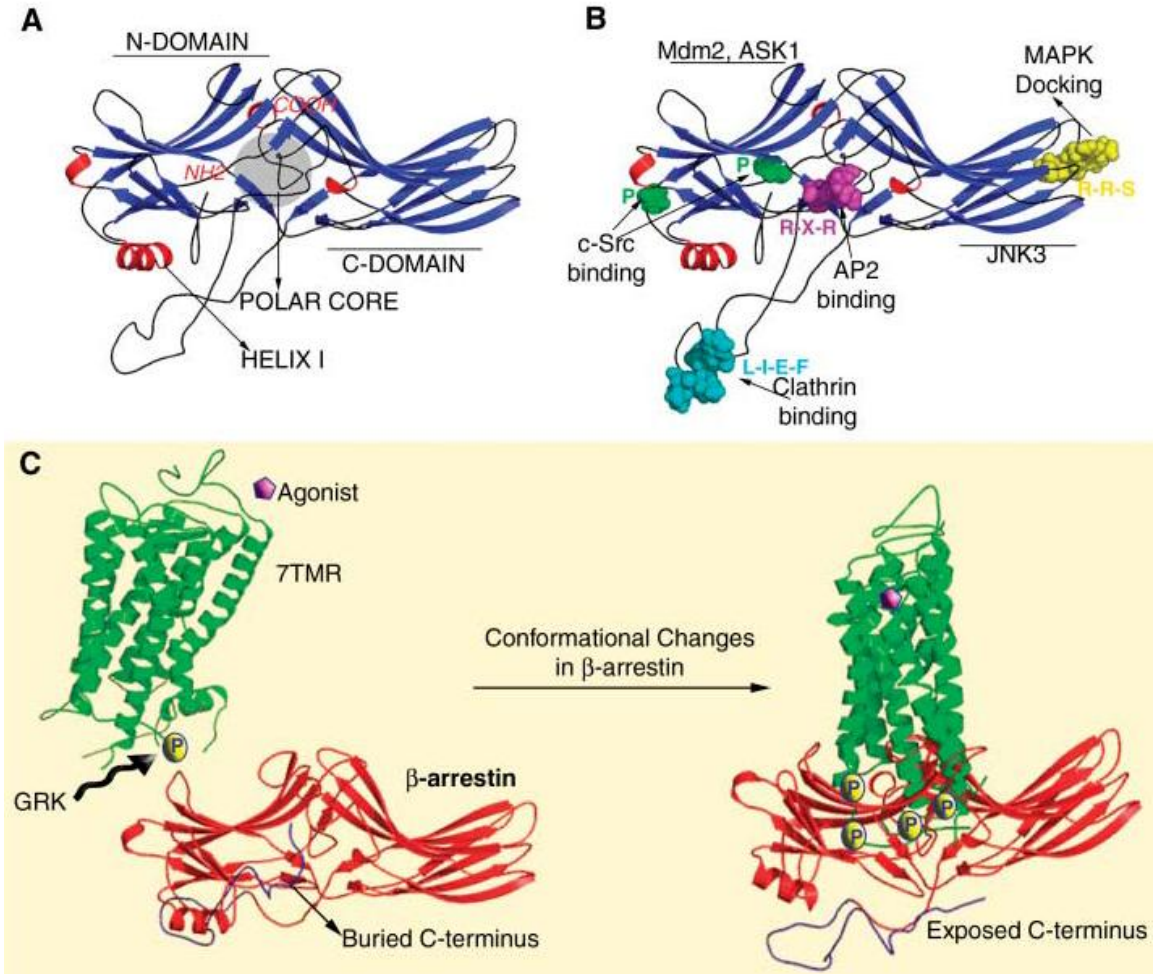

A
N-DOMAIN
COOH
NH2
C-DOMAIN
POLAR CORE
HELIX I
B
Mdm2, ASK1
MAPK
Docking
P
P
R-R-S
R-X-R
c-Src
binding
AP2
binding
JNK3
L-I-E-F
Clathrin
binding
C
Agonist
7TMR
Conformational Changes
in β-arrestin
GRK
β-arrestin
Buried C-terminus
Exposed C-terminus

Fig. 1.5. Differential 7TMR-stimulated G protein- and β-arrestin-mediated signaling. (A) Agonist-stimulated 7TMR signaling via both heterotrimeric G protein β-arrestins. (B) Traditional antagonists bind 7TMRs and block all aspects of 7TMR signaling, prevent agonist-stimulated signaling through both heterotrimeric G proteins and β-arrestins. (C) β-arrestin-biased-antagonists/agonists block agonist-stimulated heterotrimeric G protein signaling while promoting β-arrestin signaling, whereas G protein-biased ligands promote 7TMR G protein signaling in the absence of β-arrestin-mediated desensitization, internalization and signaling.

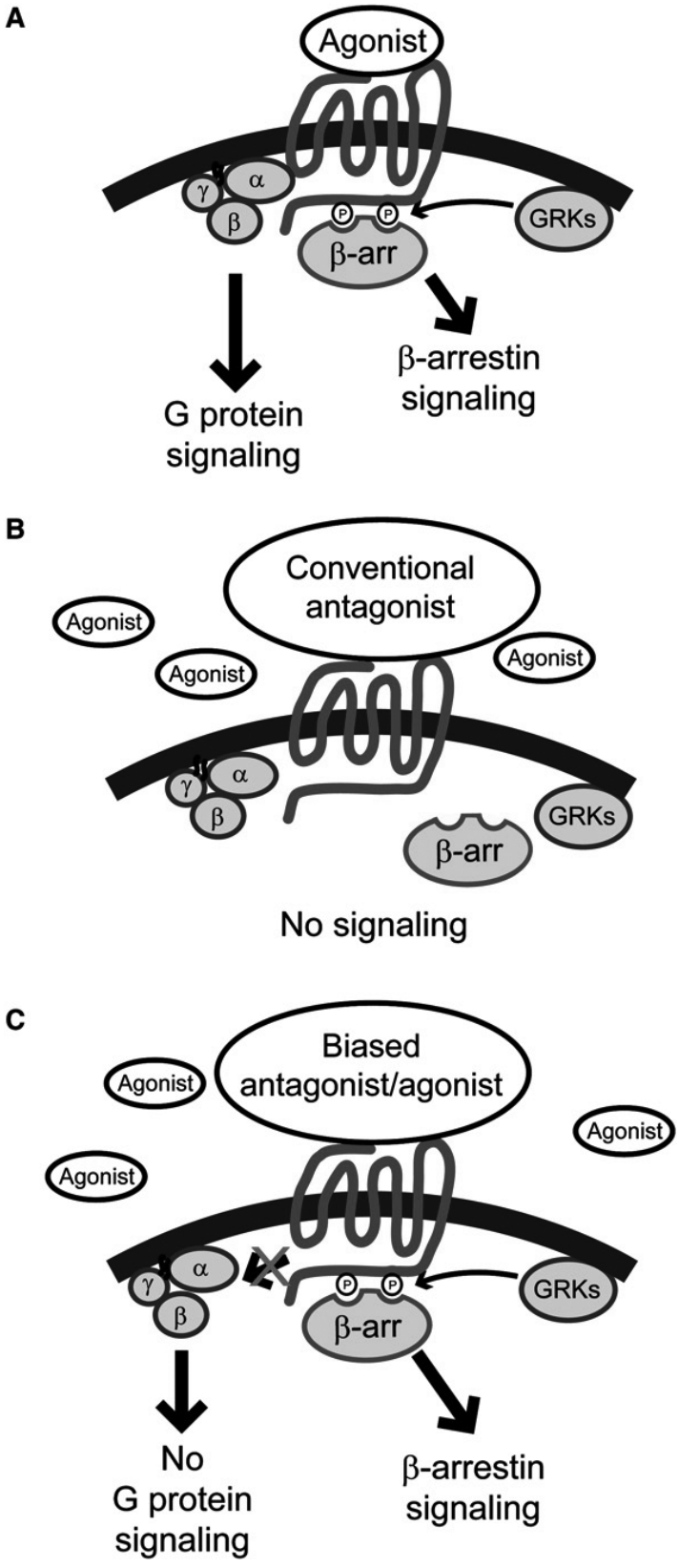

A
Agonist
α
γ
β
GRKs
P
P
β-arr
β-arrestin
signaling
G protein
signaling
B
Conventional
antagonist
Agonist
Agonist
Agonist
α
γ
β
GRKs
β-arr
No signaling
C
Biased
antagonist/agonist
Agonist
Agonist
Agonist
α
γ
β
GRKs
P
P
β-arr
No
G protein
signaling
β-arrestin
signaling

Fig. 1.6. Potential signaling pathways from β-arrestins to MAPKs. Model depicting transient versus stable MAPK complexes. Examples of transient complexes have been reported to scaffold src, leading to cross-activation of EGFR, which would converge on the traditional Ras-dependent activation of Raf and subsequent activation of MEK, ERK2 has been reported in theses complexes along with src, but Raf and MEK have not. This complex is typically associated with nuclear translocation of the MAPKs, resulting gene expression and/or proliferation. Examples of stable ERK1/2 have been reported containing the entire ERK1/2 module (Raf, MEK and ERK1/2) without src (example 2a) or containing the entire module along with src (example 2b). Src-dependent activation of EGFR is important for ERK1/2 activation in example 2a, which may serve to scaffold the ERK1/2 module at the membrane in close proximity to Ras, such that Ras is able to activate Raf. In example 2a, it is unclear how Raf, the upstream kinase in the cascade is activated, leading to ERK1/2 activation.

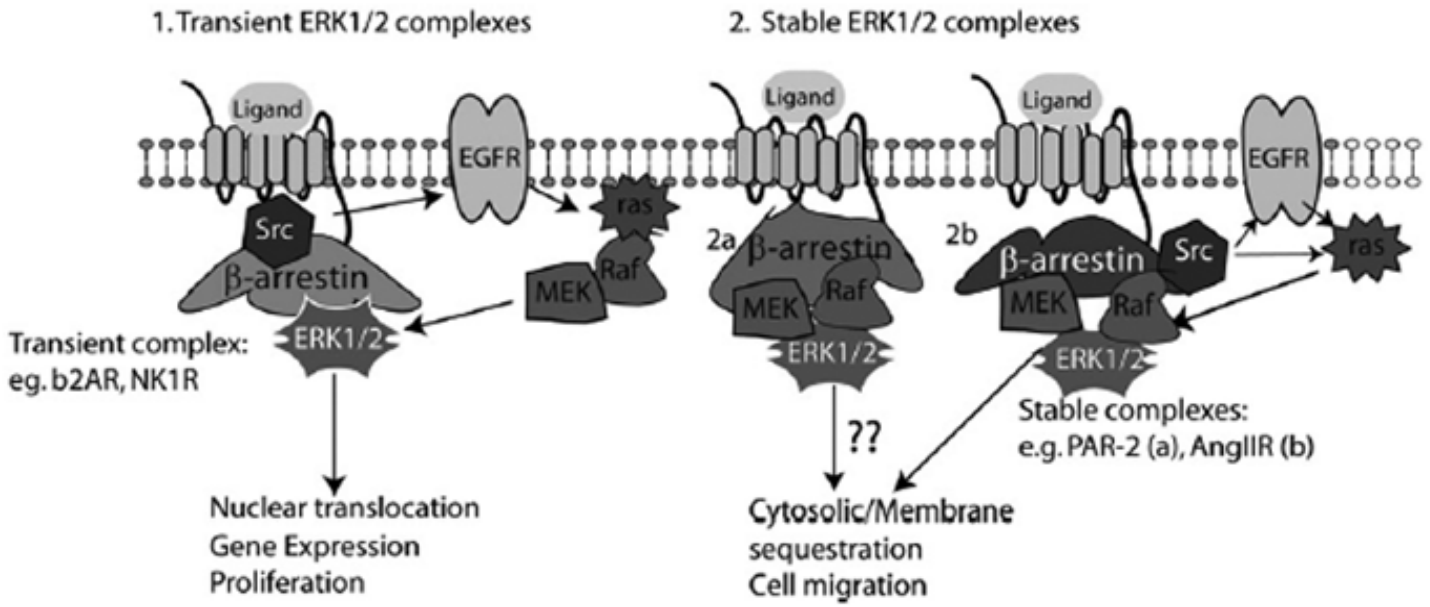

1. Transient ERK1/2 complexes
2. Stable ERK1/2 complexes
Ligand
EGFR
ras
Src
β-arrestin
Raf
MEK
ERK1/2
2a
2b
Transient complex:
eg. b2AR, NK1R
??
Stable complexes:
e.g. PAR-2 (a), AngIIR (b)
Nuclear translocation
Gene Expression
Proliferation
Cytosolic/Membrane
sequestration
Cell migration

Fig. 1.7. Schematic representation of a three-tiered cytosolic kinase (ERK) cascade. Within the three-kinase module, the entry point kinase (MAPKKK) phosphorylates and activates a dual specificity kinase (MAPKK), which in turn passes on the signal to the business end of the pathway (MAPK) on its serine or threonine. MAPK proceeds to phosphorylate a vast range of substrates whose activation engenders both transient and more permanent changes in the behavior of the cell.

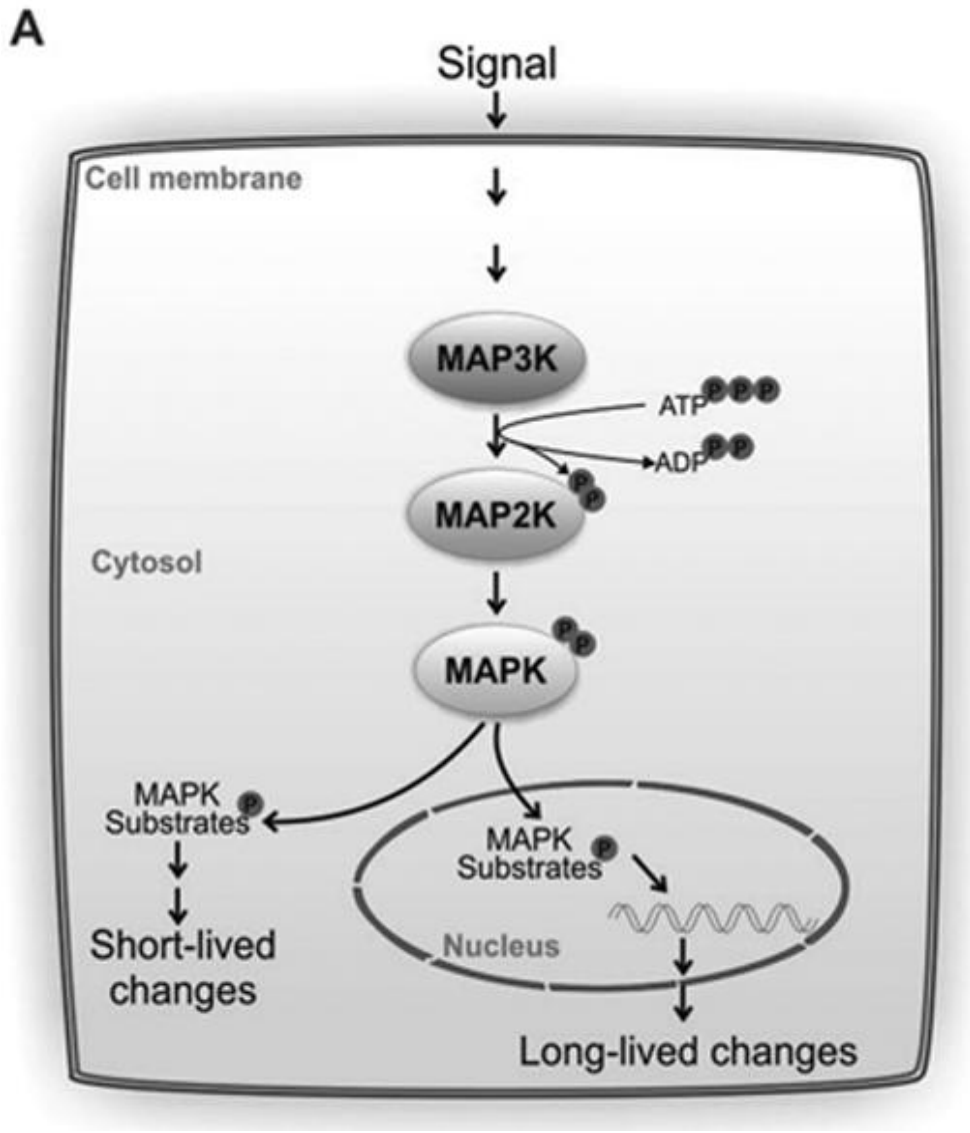
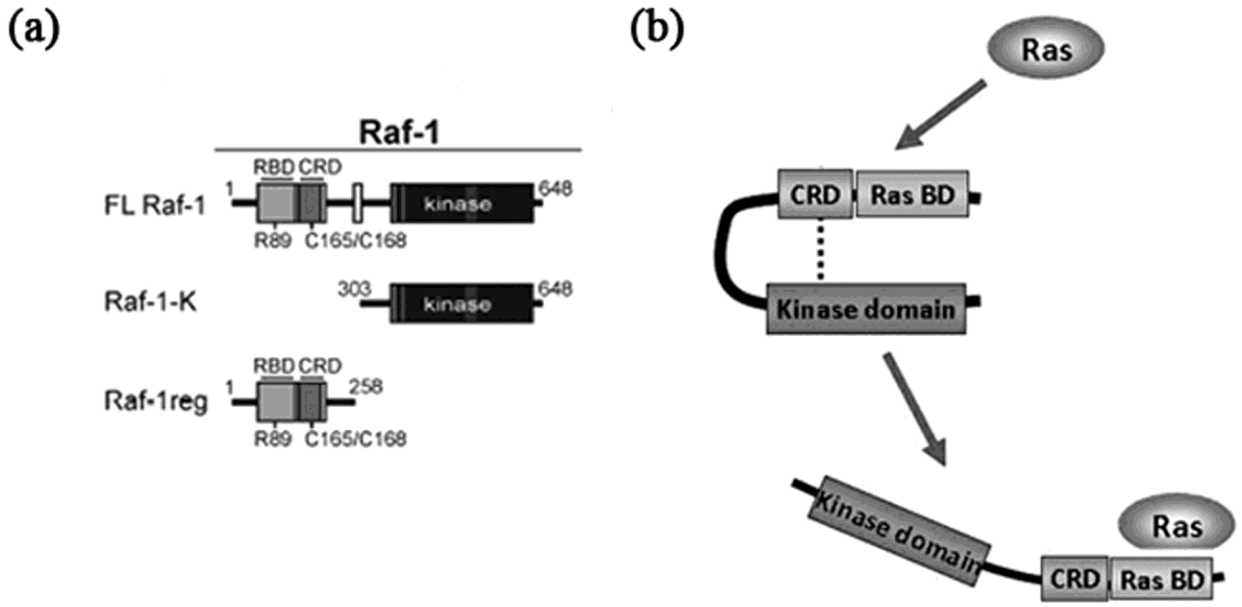

A
Signal
Cell membrane
MAP3K
ATP
ADP
MAP2K
Cytosol
MAPK
MAPK Substrates
Short-lived changes
MAPK Substrates
Nucleus
Long-lived changes

Fig. 1.8. Schematic of c-Raf structure and its activation by Ras binding. (A) Each of the Raf kinases shares a structure featuring three conserved regions (CRs): (1) CR1, with the Ras-binding domain (RBD) and the cysteine-rich domain (CRD) which can bind two zinc ions, (2) CR2, rich in serine/threonine residues which contains a site, when phosphorylated, that can bind to 14-3-3, a regulatory protein, (3) CR3, encompassing the kinase domain. (B) In quiescent cells, the interaction between the CRD and the kinase domain inhibits catalytic activity. Active Ras in the GTP-bound form binds to the NH_2-terminal regulatory domain of c-Raf and recruits it from the cytosol to the plasma membrane, resulting in conformational changes in c-Raf from the autoinhibitory, inactive state to active catalytic state or simply the proper environment for c-Raf signaling.


(a)
Raf-1
RBD CRD
FL Raf-1
1
kinase
648
R89 C165/C168
Raf-1-K
303
kinase
648
RBD CRD
Raf-1reg
1
258
R89 C165/C168
(b)
Ras
CRD
Ras BD
Kinase domain
Kinase domain
Ras
CRD
Ras BD

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CHAPTER TWO

Identification of Interacting Domains in β-arrestins with the ERK1/2 Cascade Module

2.1. Abstract

β-arrestins regulate the signaling by scaffolding molecules and sequestering their activity in microdomain of the cell. Several studies indicated that β-arrestins scaffold mitogen-activated protein kinases (MAPKs) such as ERK1/2, JNK3 and p38 MAPK. We previously observed existence of scaffolding complex containing β-arrestins and c-Raf/MEK1/ERK1/2 upon stimulation of protease-activated receptor-2. However, the molecular mechanism of β-arrestin scaffolding with the ERK cascade downstream of PAR-2 has remained elusive. Here we show multiple lines of evidence that β-arrestin-1 directly binds to each of the ERK1/2 cascade components including c-Raf, MEK1 and ERK2 using GST pull down assay and sandwich immunoassay although it appears that the domains of β-arrestin-1 differentially interact with c-Raf, MEK1 or ERK2. To determine the interacting domains in β-arrestin-1 with the ERK1/2 cascade module, we used truncated mutants of recombinant β-arrestin-1, either N- (1-163 amino acids) or C- (164-418 amino acids) termini of β-arrestin-1. Both N- and C-termini of β-arrestin-1 involves the interaction with the ERK1/2 cascade. Co-immunoprecipitation indicates that multiple domains of β-arrestin-1 exist for interaction with the ERK cascade. Apparently, activation of PAR-2 results in increasing association between each of the ERK components and some mutants of β-arrestin-1, suggesting β-arrestin-1 constitutively interacts with the ERK cascade and can have multiple active conformations depending on the cellular context.

2.2. Introduction

β-arrestins have emerged as pleiotropic scaffold proteins, capable of mediating numerous diverse responses to multiple agonists independently of G protein coupling even though they were originally discovered in the context of G protein-coupled receptor (GPCR) desensitization and internalization [1-4]. Numerous putative β-arrestin binding partners have been reported using spot peptide array and co-immunoprecipitation [5-8]. Among those identified, β-arrestins were reported to mediate assembly of multiprotein mitogen activated-protein kinase (MAPK) cascade such as those for ERK, JNK, and p38 MAPK [9-16]. We and other researchers showed that agonist stimulation of protease-activated receptor-2 (PAR-2) or type IA angiotensin II receptor (AT1aR) resulted in the recruitment of β-arrestins to the activated receptors, scaffolding the components of the ERK cascade, c-Raf, ERK1/2 and possibly MEK1, leading to cytosolic retention of activated ERK1/2 rather than nuclear translocation of phosphorylated ERK and mitogenic signaling [9, 15]. Furthermore, we demonstrated stimulation of PAR-2 led to prolonged activation of ERK1/2 in pseudopodia in a β-arrestin-dependent manner [17] and β-arrestins were required in PAR-2 mediated ERK1/2 activation at the membrane and cell migration in metastatic tumor cell lines [18], suggesting β-arrestin-dependent ERK1/2 activation might play a role in cell motility. Confocal microscopic observation indicated that activation of PAR-2 triggered translocalization of c-raf and β-arrestin from the cytosol to the plasma membrane, suggesting restricting activity of c-Raf at the plasma membrane, subsequently resulting in spatial activation of downstream kinases including

MEK1 and ERK1/2. Recently, the roles of β-arrestins as scaffold proteins in regulation of the MAPK signaling cascade have been mainly investigated by mapping out the interacting sites or domains in β-arrestins with the components of various MAPK cascade modules [5, 19-23]. The results from spot peptide array showed that MEK1 binds to 26-50 amino acids in β-arrestin-1[20] and ERK2 binds to 270-295 amino acids in β-arrestin-2 [22] which overlapped with the domain for β-arrestin-2 self-association. Even though a growing number of studies showed the roles of β-arrestins as scaffold for the ERK1/2 cascade and proposed putative interacting domains in β-arrestins with MEK1 and ERK2 [9, 17, 21], no one has seen if c-Raf directly interacts with β-arrestins. c-Raf is the entry point of the ERK module, and its activation at the membrane starts a cascade in which the signal, in form of phosphorylation, is passed on to the dual specificity kinase MEK1 and from it to the business end of the module, ERK1/2 [24-27]. Therefore, a missing link in this story has to be filled to understand how β-arrestins enhance ERK1/2 activation independently of G-protein coupling. To elucidate the molecular mechanism of β-arrestin-dependent ERK1/2 activation, our aims are as follows: 1) to investigate direct interactions between β-arrestin-1 with each components of the ERK module using GST pull down assay and sandwich immunoassay; 2) to identify specific domains in β-arrestin-1 for its interaction with the ERK1/2 module using truncated/mutated mutants of β-arrestin-1.

2.3. Methods and Materials

Chemicals : All chemicals were from Sigma or Fisher Scientific unless stated otherwise. [^{35}S]-methionine was obtained from Perkin-Elmer. All restriction enzymes were from New England Biolabs. Glutathione sepharose 4B was from GE Healthcare. Glutathione agarose was from BD Biosciences Pharmingen. Activating peptide 2fAP (2-furoyl-LIGRL-Ornithine-NH_2) was synthesized by Tocris Bioscience. TNT® coupled recticulocyte system was from Promega. Protein-G agarose and protein-A agarose were from Upstate. $PVDF_{fl}$ was from Millipore. Recombinant 6xHis tagged MEK1 was obtained from Abcam.

Antibodies: The following antibodies were: rabbit anti-phospho-ERK (Cell Signaling, 1:1000 WB), mouse anti-total ERK (Santa Cruz Biotechnology, 1:1000 WB, 4μg/ml IP), mouse anti-myc 9E10 (Santa Cruz Biotechnology, 1:1000 WB), rabbit anti-myc A14 (Santa Cruz Biotechnology, 1:1000 WB), mouse anti-c-raf (BD Bioscience Laboratories, 1: 300 WB), 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 Transduction Laboratories, 1:1000 WB), and mouse anti-6xHis (BD Biosciences Pharmingen, 1:1000 WB). For sandwich immunoassay, IR-dye-conjugated secondary antibodies (1 μg/ml) were from Rockland Biosciences.

Plasmids: The following plasmids were used in transient expression experiments; Flag-tagged β-arrestin-1 and 2 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. MEK tagged by myc was excised with HindIII and BamHI and subcloned into pcDNA3.1(+). ERK2-GFP was previously described [9]. 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. GST-tagged ERK was excised with BamHIII and subcloned into pGEX4T-1. WT c-Raf was subcloned into pGEX4T-1. For *in vitro* transcription and translation, the following plasmids were used: hRaf-1 cloned into BamHI site of pBluescript, ERK2 subcloned into pBluescript, and His-MEK-1 from Dr. Natalie Ahn (University of Colorado, Denver, CO).

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 RIPA (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitors) for c-Raf and in 1X PBS, 1% NP-40 and protease inhibitors for MEK1 and ERK2. 10μg of protein was analyzed by 10 or 12.5% 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).

***In vitro* pull-down assay:** pBluescript-Raf-1, pRSET-MEK1, and ERK-TNT were *in vitro* translated with [^{35}S]-methionine in rabbit reticulocyte lysate using TnT® Quick coupled Transcription/Translation system (Promega). Recombinant β-arrestin-1 tagged by GST on glutathione-sepharose pulled down with [^{35}S]-Met-labeled c-Raf. Recombinant GST alone was used as a negative control. Beads were washed three times with 20 mM Tris-HCl (pH 7.9), 1 mM β-mercaptoethanol, 3 mM EDTA, 150 mM NaCl, 1% Nonidet P-40 and once with washing buffer without NP-40. The result was obtained by SDS-PAGE followed by autoradiography.

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℃. 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℃ until needed. The protein concentration of eluates 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 both β-arrestin proteins was observed in each preparation, which was quantified by densitometric analysis of 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℃) 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. GST-tagged human WT c-raf and regulatory c-raf

were expressed in *E. coli* Rosetta™ (DE3) BL21 cells (EMD chemicals) which enhance the expression of eukaryotic proteins that contain codons rarely used in *E.Coli*. by induction with 1 mM IPTG and incubation in minimal medium overnight at 22℃.

Sandwich Immunoassay: Recombinant MAPK cascade components either c-Raf, MEK1 or ERK2 were captured by appropriate antibodies against onto the bottom of the 96-well plate. Either 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. Data were analyzed by Li-Cor odyssey.

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 one way ANOVA and Tukey t-tests (to compare between treatment groups).

2.4. Results

2.4.1. c-Raf directly binds to β-arrestin-1 *in vitro*.

As previously stated, a number of studies demonstrated that β-arrestins form scaffolding complex with each of the ERK cascade components using size exclusion chromatography and co-immunoprecipitations [9, 10, 17, 21] and suggested putative interacting domains with MEK1 and ERK2 in β-arrestin-1 or 2 [20-22]. Nonetheless, the mechanism how β-arrestins activate c-Raf in the context of c-Raf/MEK1/ERK1/2 cascade has still remained to be elucidated. Furthermore, it has not yet been reported if c-Raf directly binds to β-arrestins. In order to determine the direct binding of c-Raf with β-arrestin-1, we captured recombinant each of ERK cascade components onto the bottom of 96-well plate using the indicated antibodies and incubated with recombinant GST WT β-arrestin-1. We found that recombinant WT β-arrestin-1 tagged by GST directly bound to c-Raf (Fig. 2.1.A), as did to MEK1 and ERK2 in agreement with previous observations [20, 22] (Fig. 2.1.B and C). In addition, this result was confirmed by cell-free binding assays using GST pull down. Each of the ERK cascade components, c-Raf, MEK1 and ERK2 were expressed in cell-free transcription and translation system in rabbit reticulocyte lysate with [^{35}S]-methionine. c-Raf radiolabeled with [^{35}S]-methionine was only pulled down with β-arrestin-1 tagged by GST, but not MEK1 and ERK2 (Fig. 2.2). However, *in vitro* transcripted and translasted MEK1 or ERK2 with [^{35}S]-methionine appeared to only interact with β-arrestin-1 in the presence of c-Raf (data not shown).

Therefore, these results suggest that c-Raf plays a main role in contact with β-arrestin-1 in forming the scaffolding complex.

2.4.2. Each of MAPK components binds to β-arrestin-1 via its N- and C-terminal domains *in vitro*.

To determine which domains in β-arrestin-1 c-Raf directly binds, recombinant truncated mutants of β-arrestin-1 tagged by GST were generated in bacterial expression system. GST-tagged, truncated mutants of β-arrestin-1 either N- or C-termini were used in sandwich immunoassay as previously described in 2.4.1. We found that both the N- or C-terminal fragments of β-arrestin-1 were involved in interaction with c-Raf, MEK1 and ERK2, consistent with the previous observation by the Gurevich group [21] (Figure. 2.3). c-Raf appeared to interact with full length β-arrestin-1 and both truncations with similar relative affinities. In contrast, the binding curve of MEK1 with N-terminus of β-arrestin-1 was left-shifted than with full length or C-terminus of β-arrestin-1 or the binding curve of ERK2 with C-terminus of β-arrestin-1 was right-shifted than with FL or N-terminus of β-arrestin-1 consistent with the identification of putative binding domains using spot-peptide arrays [20, 22]. Furthermore, the result from cell-free binding assay was consistent that c-Raf directly binds β-arrestin-1. c-Raf was expressed using *in vitro* transcription and translation system with [^{35}S]-methionine in rabbit reticulocyte lysate. We found that c-Raf radiolabed with [^{35}S]-methionine bound with GST tagged wild type and both truncations of β-arrestin-1 (Fig. 2.4). Thus, the data suggest that multiple domains in β-arrestin-1 are involved in binding with the ERK cascade module.

2.4.3. PAR-2 activation triggers interaction of each of c-Raf with β-arrestin-1 via its N-terminus.

We previously showed that β-arrestins were involved in recruitment of c-Raf at the plasma membrane downstream of PAR-2 [9]. Moreover, we found that β-arrestins formed scaffolding complex including c-Raf, ERK1/2 to the activated PAR-2 in pseudopodia [17]. To determine the effect of PAR-2 activation in regulating the interaction of β-arrestin with each of the ERK module, we expressed truncated mutants of β-arrestin-1 tagged by Flag with different ERK module in HEK293 cells and immunoprecipitated Flag-β-arrestin-1 and immunoblotted for individual kinases. Co-immunoprecipitation revealed enhanced interaction of c-Raf with wild type β-arrestin-1 and its N-terminus (1 to 163 amino acids) upon PAR-2 activation whereas constitutive interaction with its C-terminus (164 to 418 amino acids) (Fig. 2.5.A). PAR-2 activation induced increased interaction of MEK1 with wild type β-arrestin-1 and its extreme N-terminal truncation containing 1 to 99 amino acids consistent with the result seen by spot-immobilized peptide array [20] whereas interaction between MEK1 and the truncated mutant of β-arrestin-1 containing 1 to 163 amino acids were constitutive (Fig.2.5.B and C). Furthermore, wild type β-arrestin-1 and truncated mutants of β-arrestin-1 except the mutant 319-418 amino acids were involved in interaction with ERK2. In addition, interaction of the mutant 1-99 amino acids with ERK2 was enhanced by PAR-2 activation. Interestingly, none of the ERK module kinases were shown to interact with C-terminal truncation of β-arrestin-1 which was proved to interact with clathrin and clathrin adaptor AP-2 [28, 29], suggesting that β-arrestin may interact with the ERK module while it is involved in receptor

endocytosis. Since β-arrestins are diverse scaffold molecules dependent upon the activating receptor and spatial availability of binding partners, it is possible that there are multiples domains involved in the interaction with the ERK module. Probably PAR-2 induces the interaction of each of the ERK cascade kinase with specific domains in β-arrestin-1.

2.4.4. *in vitro* translated c-Raf with [^{35}S]-methionine displays enhanced interaction with immunoprecipitated c-terminal domain of Flag β-arrestin-1 upon PAR-2 activation.

To confirm the result of PAR-2 involvement in interaction with c-Raf, we performed binding assays using immunoprecipitated Flag β-arrestin-1 agarose. HEK 293 cells transiently transfected with wild type or the truncated mutants of β-arrestin-1 either containing 1 to 163 amino acids or 164-418 amino acids tagged by Flag were cleared at 90 min after 2fAP treatment to activate PAR-2 and immunoprecipitated with anti-Flag agarose. Immunoprecipitated Flag β-arrestin-1 on agarose pulled down c-Raf expressed in cell-free transcription and translation system in rabbit reticulocyte lysate with [^{35}S]-methionine. *In vitro* translated c-Raf with [^{35}S]-methionine was shown to increasingly interact with C-terminus (164-418 amino acids) of β-arrestin-1 at 90 min after PAR-2 activation (Fig.2.6). Since the co-immunoprecipitation data in 2. 3 showed that PAR-2 induced interaction between c-Raf and N-terminus of β-arrestin-1 and constitutive interaction with C-terminus of β-arrestin-1 at 5min after 2fAP treatment. Possibly, prolonged PAR-2 activation might lead to increased interaction with C-terminus of β-

arrestin-1 rather than N-terminus of β-arrestin-1, suggesting that β-arrestin-1 dynamically adapts favorable conformation to the binding partners depending on receptor activation.

2.5. Discussion

Mitogen-activated protein kinases (MAP kinases) respond to a diverse array of extracellular stimuli and are involved in critical signaling pathways such as cell survival, differentiation, proliferation, apoptosis and cell motility [26, 27]. The unit of mitogen-activated protein kinase (MAPK) module is a three-member protein kinase cascade that establishes a sequential activation pathway comprising a MAPK kinase kinase (MKKK), MAPK kinase (MKK), and MAPK [24, 25, 30]. β-arrestins were shown to scaffold MAPK cascade modules that activate ERK1/2 [9, 15, 17], JNK3 [13], and p38 [16, 31]. Since a number of scaffolding proteins such as MP1 [32, 33], JIP-1 [34], Ksr [35-38], and 14-3-3 [39, 40] as well as β-arrestins [41] in regulating MAPK cascades have been identified so far, the given MAPK cascade scaffolded by specific scaffolding proteins could regulate their signaling in different locations for different durations and functions within microdomain of the cell. Although multiple studies have suggested that β-arrestins form a scaffolding complex containing the ERK cascade components in determining MAPK localization and its activation [9, 10, 15, 17, 21], the mechanism that results in β-arrestin-mediated ERK activation has remained to be uncovered. Therefore, we investigated the molecular details in interaction of β-arrestins with the ERK1/2 cascade.

Previous studies have shown sequestration of β-arrestins with c-Raf at the plasma membrane [9] and with phosphorylated ERK1/2 in a membrane fraction [17], suggesting that β-arrestins recruit the ERK1/2 cascade module to the activated protease-activated receptor-2. In this study, as the first time, we found that β-arrestin-1 directly bound c-Raf

in vitro using sandwich immunoassay and confirmed that β-arrestin-1 directly bound MEK1 and ERK2 which has been shown by spot-immobilized peptide array [20, 30]. Furthermore, we have shown that *in vitro* transcripted and translated c-Raf radiolabeled with [^{35}S]-methionine in rabbit reticulocyte lysate bound GST β-arrestin-1 but not MEK1 and ERK2 in this experiment. It could be explained by the different expression systems where the recombinant ERK cascade components were prepared: bacterial expression for the sandwich immunoassay versus mammalian expression for the GST pull down assay. It is likely responsible for post-translational modification on the ERK1/2 cascade components in rabbit reticulocyte lysates. In addition, we have observed that c-Raf, MEK1 and ERK2 bound via N- and C-terminus of β-arrestin-1 *in vitro*, suggesting that multiple binding domains in β-arrestin-1 could possibly exist or the middle region is involved in interaction with the ERK1/2 cascade module. Considering the β-arrestins are recruited to different receptors, interact with numerous different proteins [3, 5-7], or oligomerize [20, 42-44], β-arrestins could take multiple conformations favorable for interaction with specific binding partners. The current data point to a model wherein different receptors, and perhaps even the same receptors in different cell types, can elicit specific β-arrestin conformations that expose certain domains to recruit distinct scaffolding complexes. Therefore, the formation of different scaffolds on β-arrestins can then be determined by the activating receptor and its cellular context, leading to an additional level of signaling specificity.

Free bovine β-arrestin-1 and 2 were shown to co-immunoprecipitate with c-Raf, MEK1 and ERK2 via their N- and C-domains in COS-7 cells without the receptor

activation [21]. We have also observed that full length and truncated mutants of β-arrestin-1 co-immunoprecipitated with c-Raf, MEK1 and ERK2, consistent with the result observed previously by the Gurevich group. However, we found that PAR-2 activation mainly enhanced interactions of N-terminus of β-arrestin-1 with the upstream kinases, c-Raf and MEK1. Moreover, it appeared that ERK2 interacted with β-arrestin-1 via its N-terminus when immunoprecipitated ERK2 formed complex with β-arrestin-1 whereas ERK2 interacted with β-arrestin-1 via its middle region when immunoprecipitated β-arrestin-1 pulled down with ERK2. It is possible that configuration of the complex might be slightly different from one another when they were immunoprecipitated with antibodies. Nonetheless, interestingly, none of the kinases were shown to interact with the C-terminal truncation of β-arrestin-1 which was proved to interact with clathrin and clathrin adaptor AP-2 [28, 29]. We have shown that β-arrestin-1 mediated early activation of ERK1/2 and rapid internalization of the receptor downstream of PAR-2 [45]. Therefore, it is possible that β-arrestin-1 interacts with the ERK module leading to its trafficking to subcellular locations simultaneously while they are involved in the receptor endocytosis. Taken together, the data show that each of three kinases, c-Raf, MEK1 and ERK2 interact with β-arrestin-1 via multiple domains in a basal level, but PAR-2 activation lead to increased interaction of each of them with N-terminus of β-arrestin-1 which is consistent with the evidence that the conformation of β-arrestins is altered by receptor recruitment.

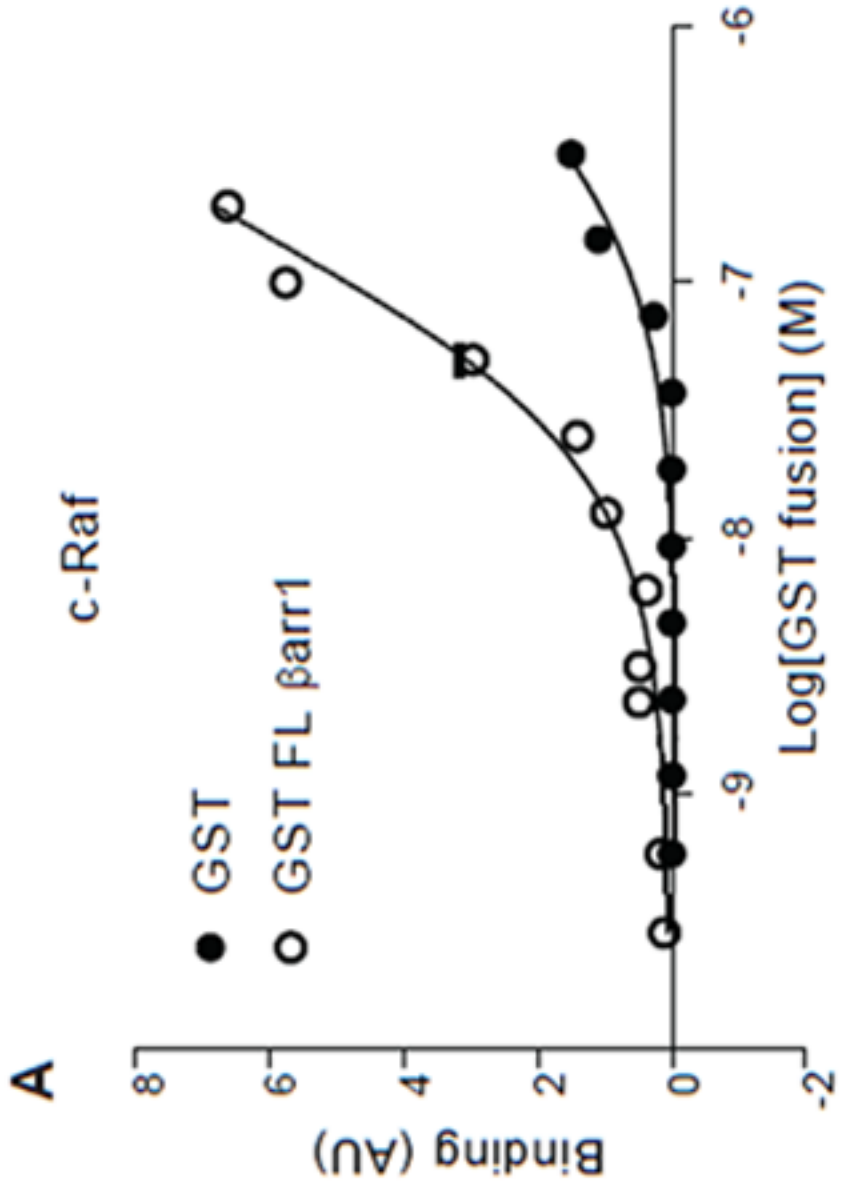
We previously observed the differential effect of β-arrestin-1 and 2 on ERK1/2 activation downstream of PAR-2 that β-arrestin-1 mediated early while β-arrestin-2

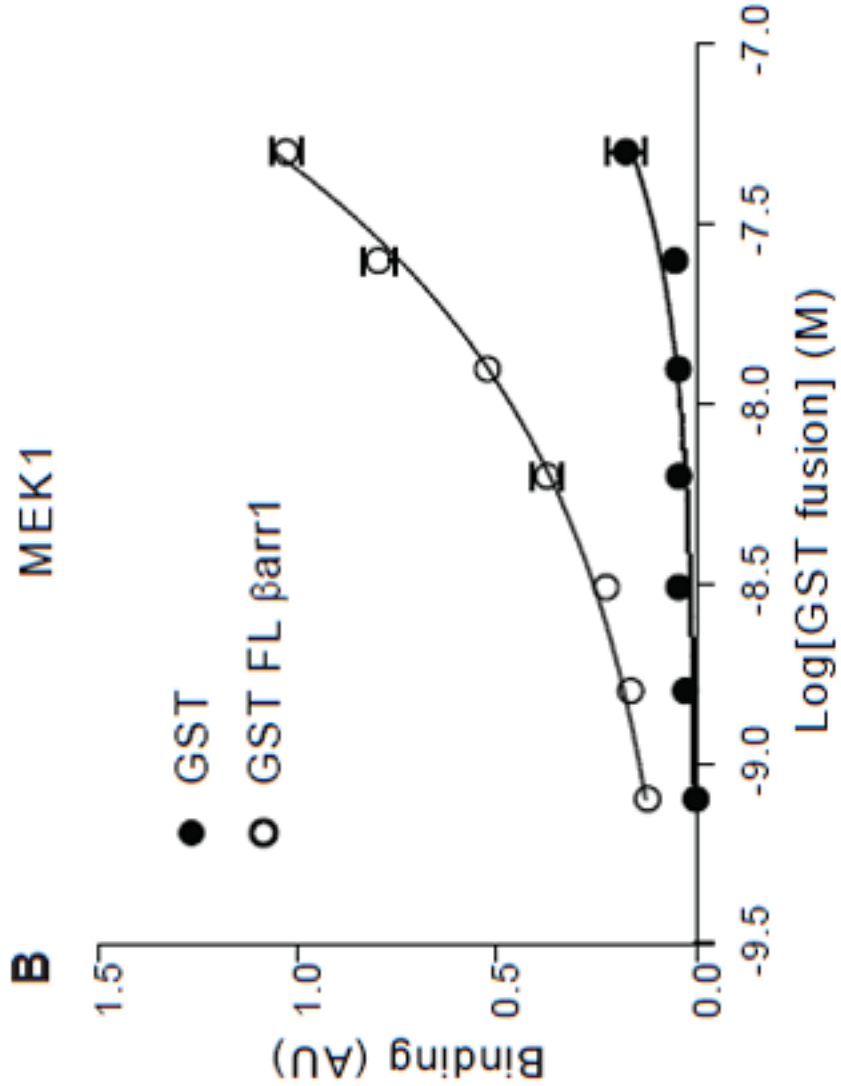
resulted in delayed ERK1/2 activation [45]. Other researchers also reported that function of β-arrestin-1 and 2 are somewhat redundant and simultaneously unique in certain cellular pathways. We found that C-terminal truncation of β-arrestin-1 interacted with *in vitro* translated c-Raf at 90 min after PAR-2 activation. It is possible that β-arrestins undergo a slight change in orientation of c-Raf, MEK1 and ERK1/2 on β-arrestins depending on different cellular environment such as prolonged activation of the receptor or availability of binding partners, suggesting that a single receptor is capable of differentially recruit β-arrestin scaffolding complex in a manner dependent upon agonist activation.

Since β-arrestins are pleiotropic proteins, they are likely to interact with numerous proteins on multiple levels including the ERK cascade modules. Even a slight change in orientation of c-Raf, MEK1 and ERK1/2 on β-arrestins might hinder the conformational changes in one or more kinases making signal propagation impossible, leading to diverse cellular consequences.

2.6. Figures and Legends

Fig. 2.1. Each of the ERK components directly binds to β-arrestin-1 *in vitro*. Recombinant ERK cascade components (c-Raf, MEK-1, or ERK2) were captured by appropriate antibody against each of the ERK cascade or tag proteins onto the bottom of 96-well plates. Subsequently, recombinant WT β-arrestin-1 tagged by GST was incubated. GST alone was used as a negative control. Interaction was detected by anti-GST conjugated with IR-800. (A-C) Binding curves of c-Raf (A), MEK1 (B), and ERK2 (C) with an increasing amount of GST-β-arrestin-1 were shown. Data were analyzed by Li-Cor odyssey. Graphs (A, B, C) were obtained by Graphpad Prism 5.0.


A
c-Raf
GST
GST FL βarr1
Binding (ΔU)
8
6
4
2
0
-2
-9
-8
-7
-6
Log[GST fusion] (M)


B
MEK1
GST
GST FL βarr1
Binding (AU)
1.5
1.0
0.5
0.0
-9.5
-9.0
-8.5
-8.0
-7.5
-7.0
Log[GST fusion] (M)

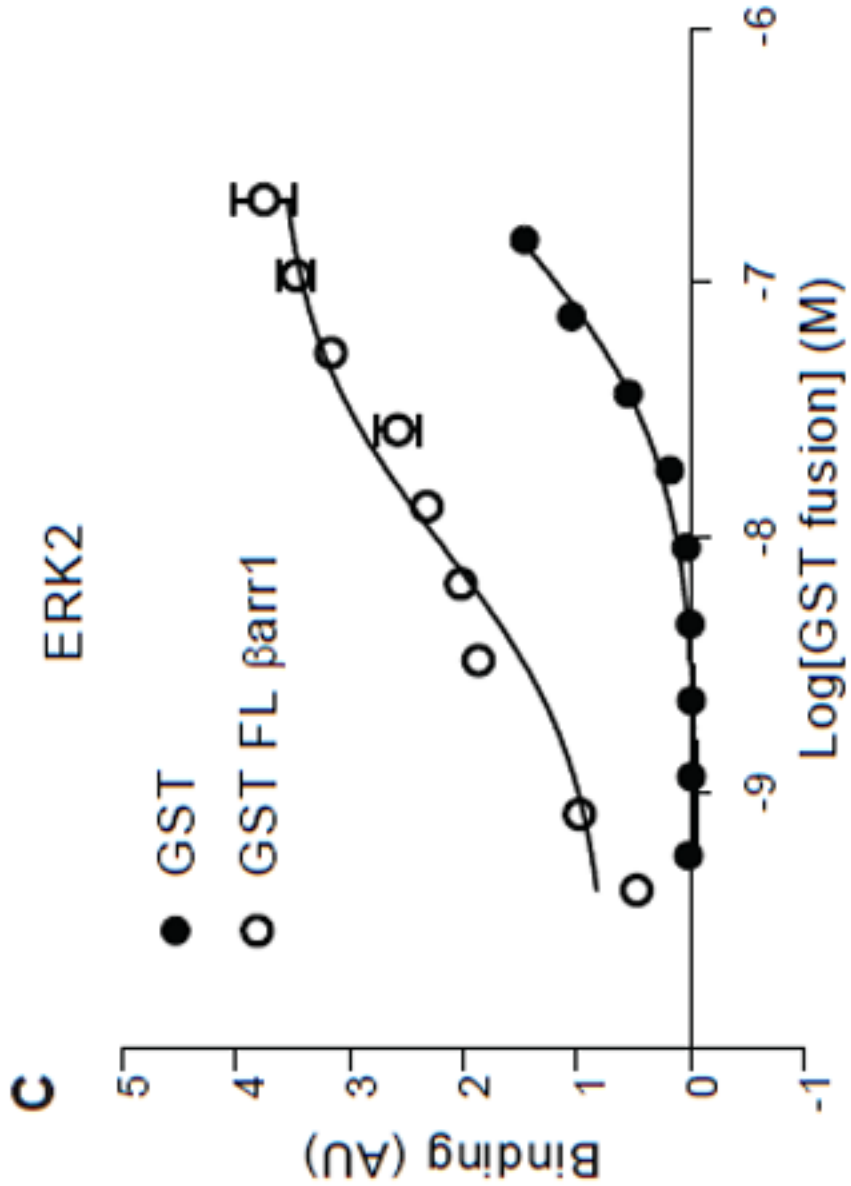

C
ERK2
GST
GST FL βarr1
Binding (ΔU)
5
4
3
2
1
0
-1
-9
-8
-7
-6
Log[GST fusion] (M)

Fig. 2.2. Direct binding of c-Raf to β-arrestin-1 plays a major role in bringing MEK/ERK to the scaffold *in vitro*. c-Raf, MEK1, and ERK2 expressed using *in vitro* transcription and translation with [^{35}S]-Methionine in rabbit reticulocyte lysate. Recombinant WT β-arrestin-1 tagged by GST on glutathione-sepharose pulled down with [^{35}S]-Methionine labeled c-Raf, MEK1 or ERK2. GST alone was used as a negative control. The result was obtained by SDS-PAGE followed by autoradiography.

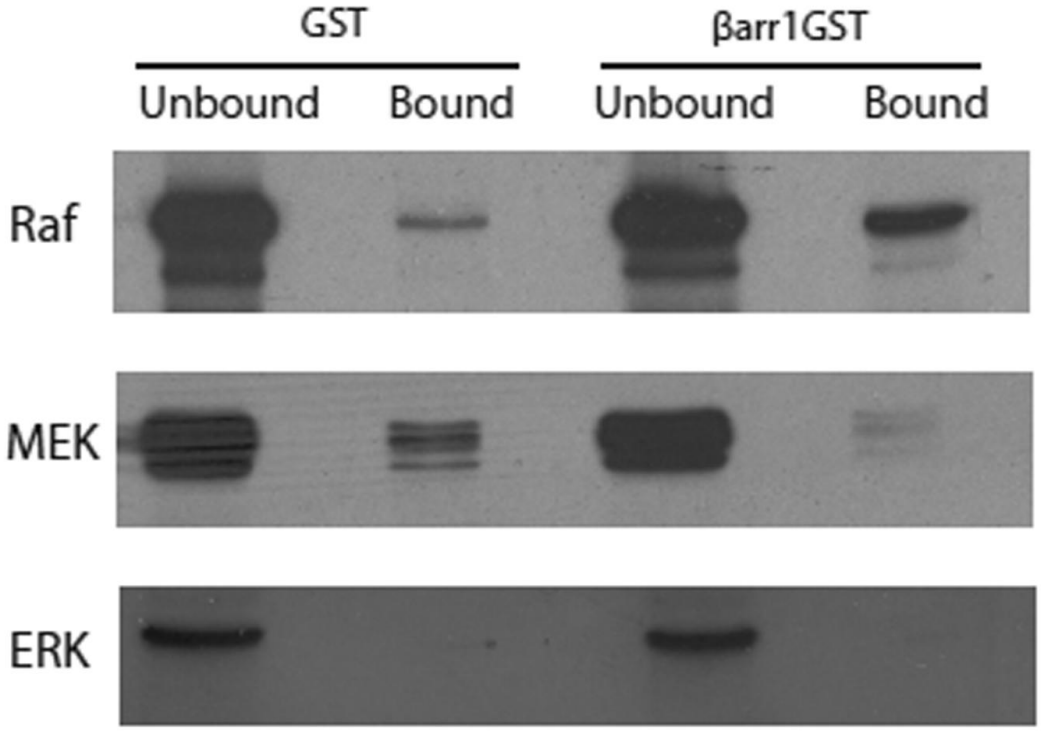
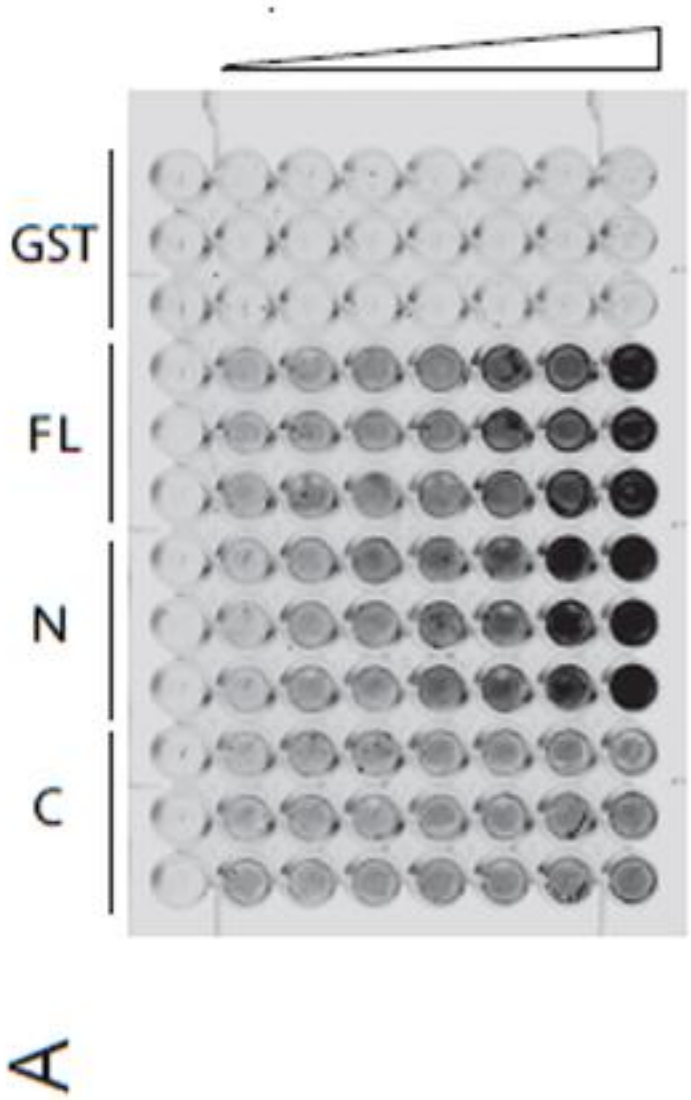
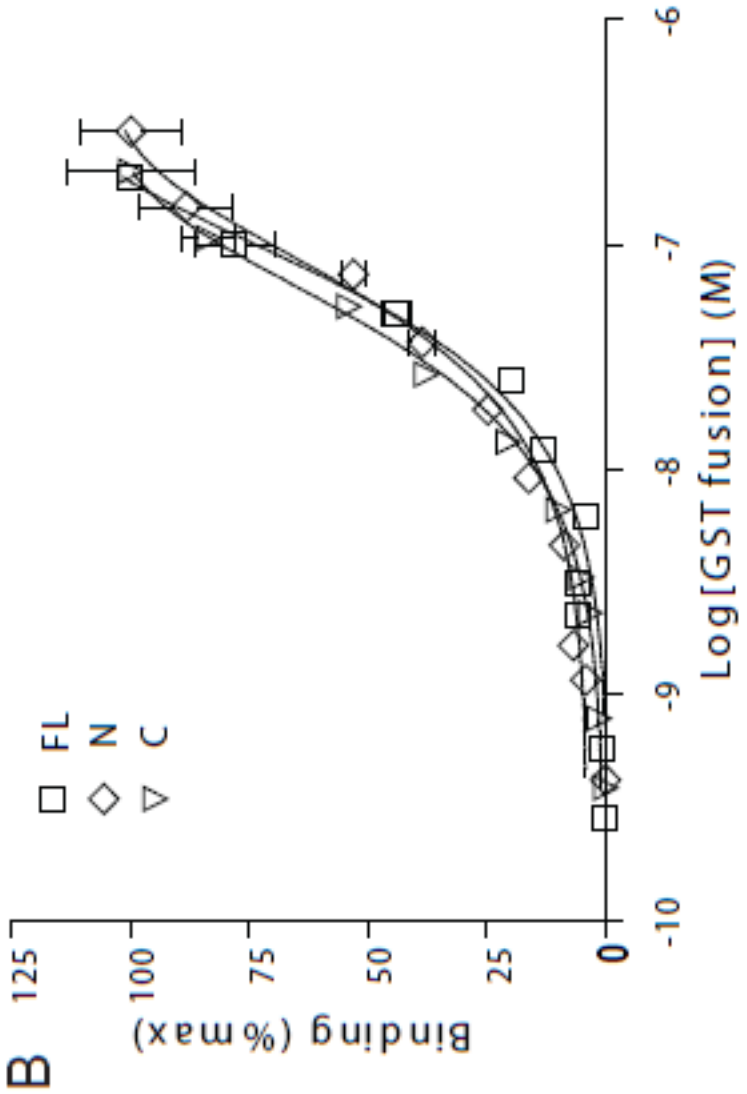
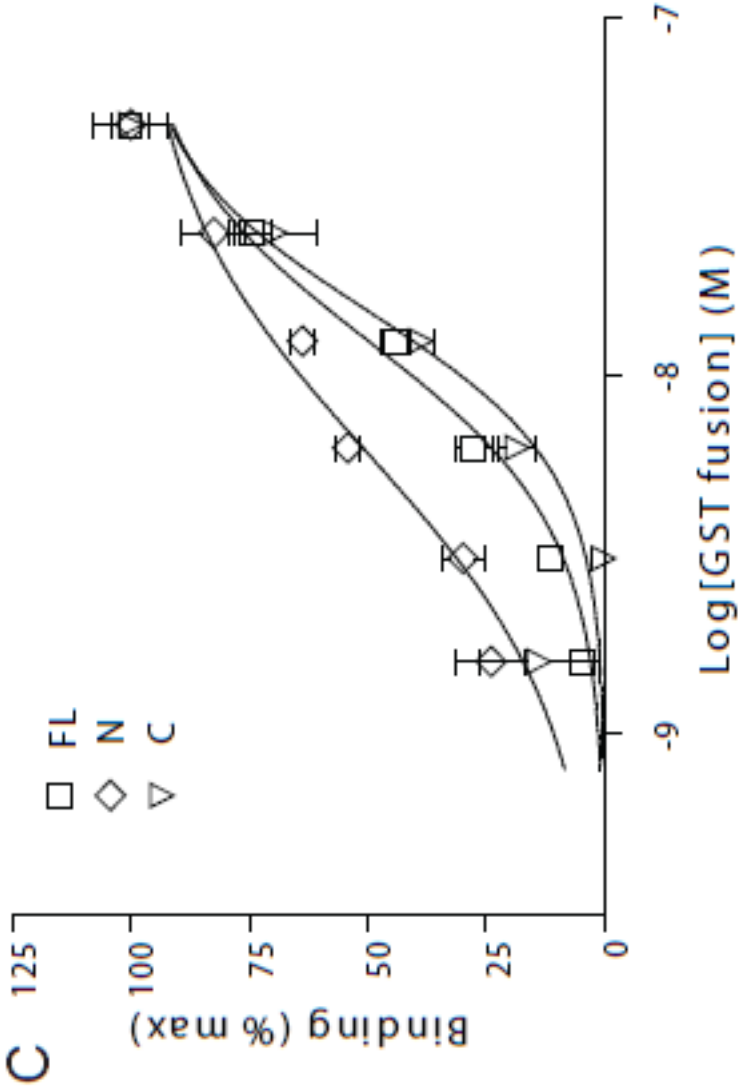

GST
βarr1GST
Unbound
Bound
Unbound
Bound
Raf
MEK
ERK

Fig. 2.3. Recombinant β-arrestin-1 directly binds to each of the ERK cascade module via its N- and C-termini *in vitro*. Recombinant ERK cascade components (c-Raf, MEK-1, or ERK2) were captured by appropriate antibody against each of the ERK cascade or tag proteins onto the bottom of 96-well plates. Subsequently, recombinant WT β-arrestin-1 or truncated mutants of β-arrestin-1 containing 1 to 163 amino acids or 164-418 amino acids tagged by GST were incubated. GST alone was used as a negative control. Interactions were detected by anti-GST conjugated with IR-800. (A) A scanned image of 96-well plate was obtained by Li-Cor odyssey. (B-D) Normalized binding curves of c-Raf (B), MEK1 (C), and ERK2 (D) with an increasing amount of GST-β-arrestin-1 were shown. Data was analyzed by Li-Cor odyssey. The graphs were obtained by Graphpad Prism 5.0.


GST
FL
N
C
A


B
Binding (%max)
125
100
75
50
25
0
-10
-9
-8
-7
-6
Log[GST fusion] (M)
FL
N
C


C
Binding (% max)
125
100
75
50
25
0
-9
-8
-7
Log[GST fusion] (M)
FL
N
C

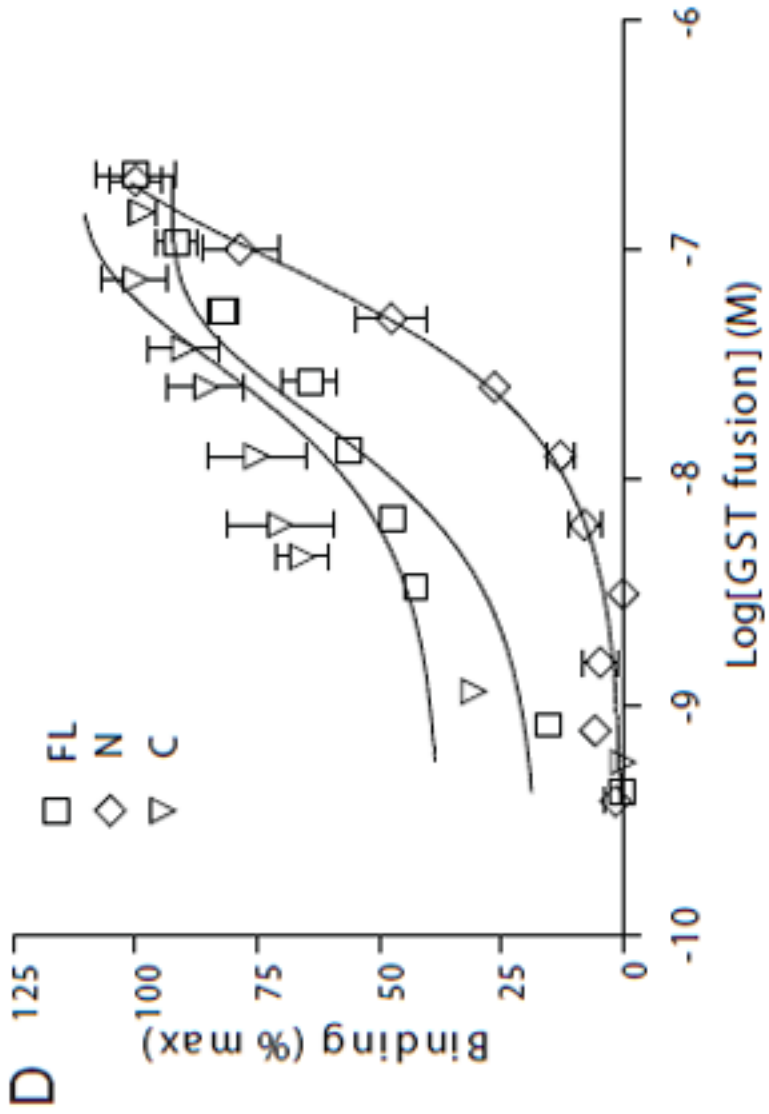

D
Binding (% max)
125
100
75
50
25
0
-10
-9
-8
-7
-6
Log[GST fusion] (M)
FL
N
C

Fig. 2.4. *in vitro* translated c-Raf binds to β-arrestin-1 via both N- and C-termini of β-arrestin-1. c-Raf was expressed in rabbit reticulocyte lysate using *in vitro* transcription and translation system with [^{35}S]-Methionine. Recombinant WT β-arrestin-1 or truncated mutants of β-arrestin-1 containing 1 to 163 amino acids or 164-418 amino acids tagged by GST were captured on glutathione-sepharose and β-arrestin-1-bound glutathione-sepharose pulled down with [^{35}S]-Methionine labeled c-Raf. GST alone was used as a negative control. The result was obtained by SDS-PAGE followed by autoradiography

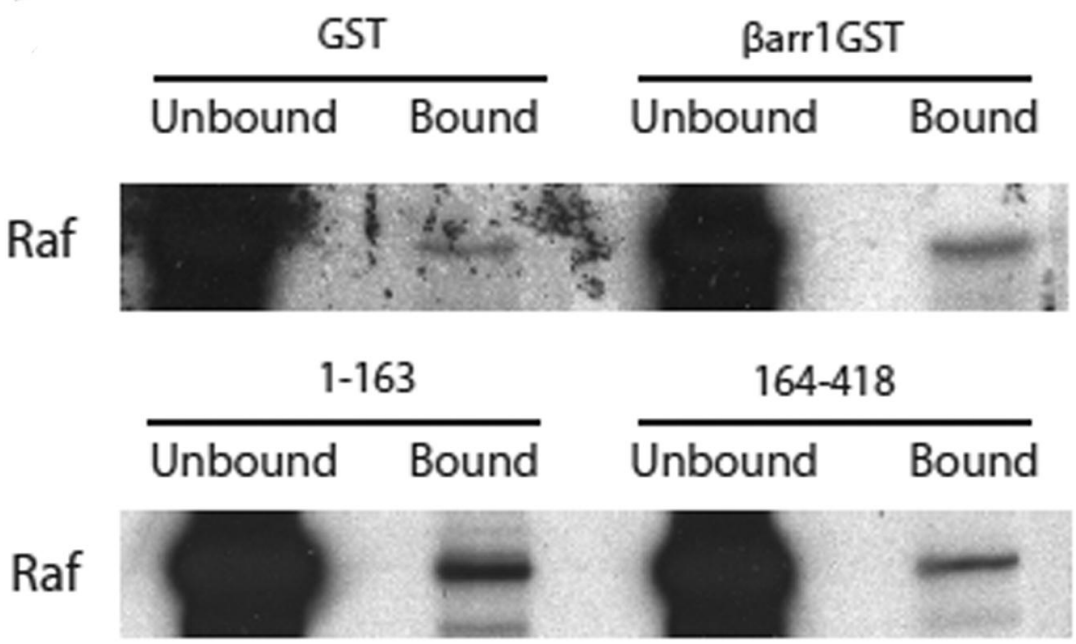
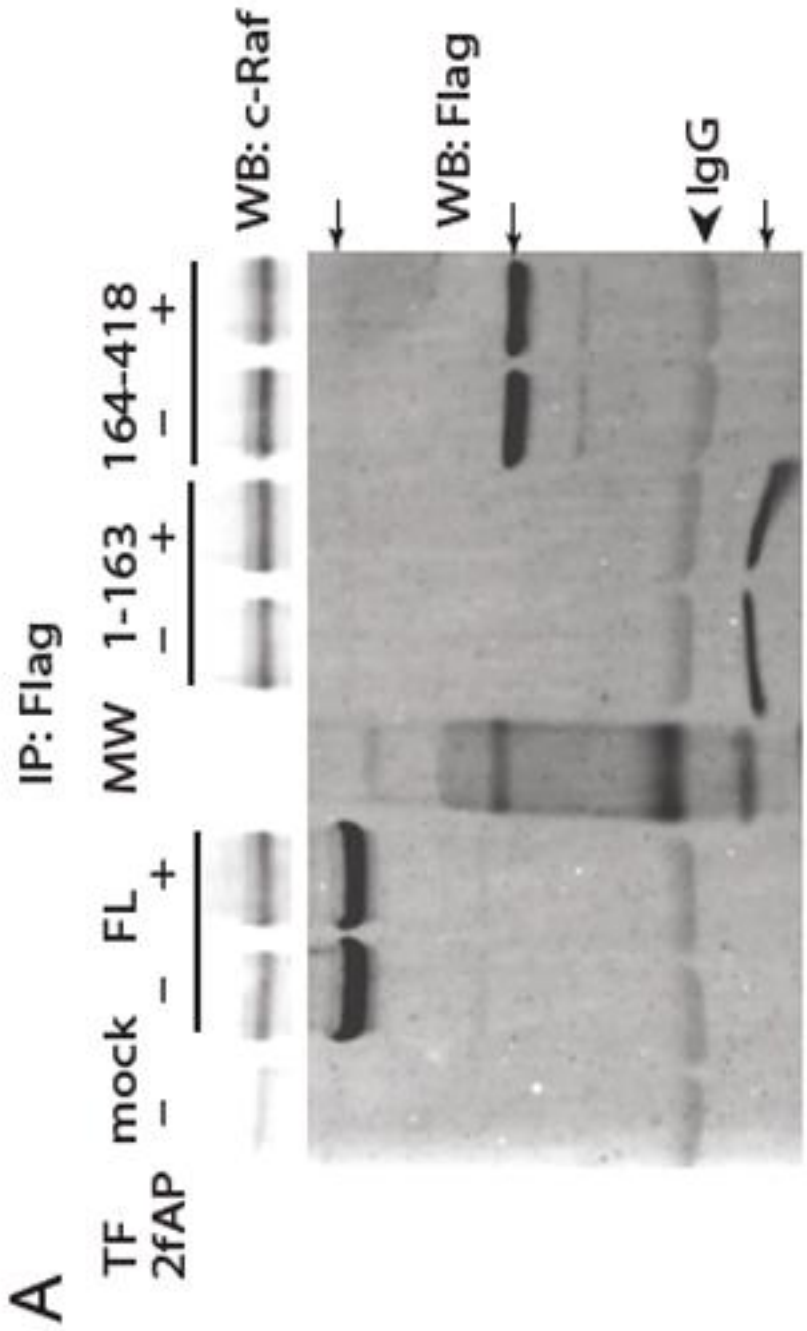
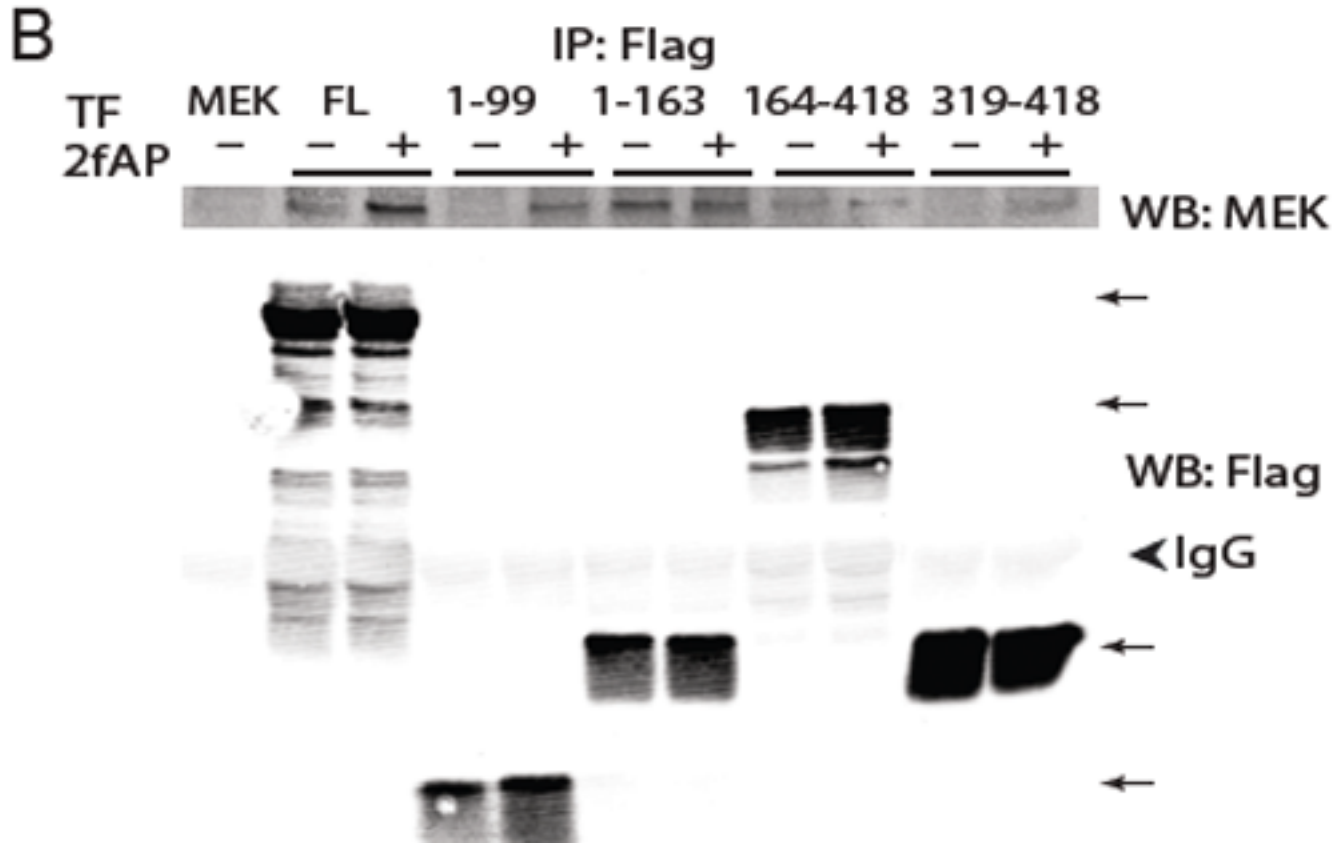
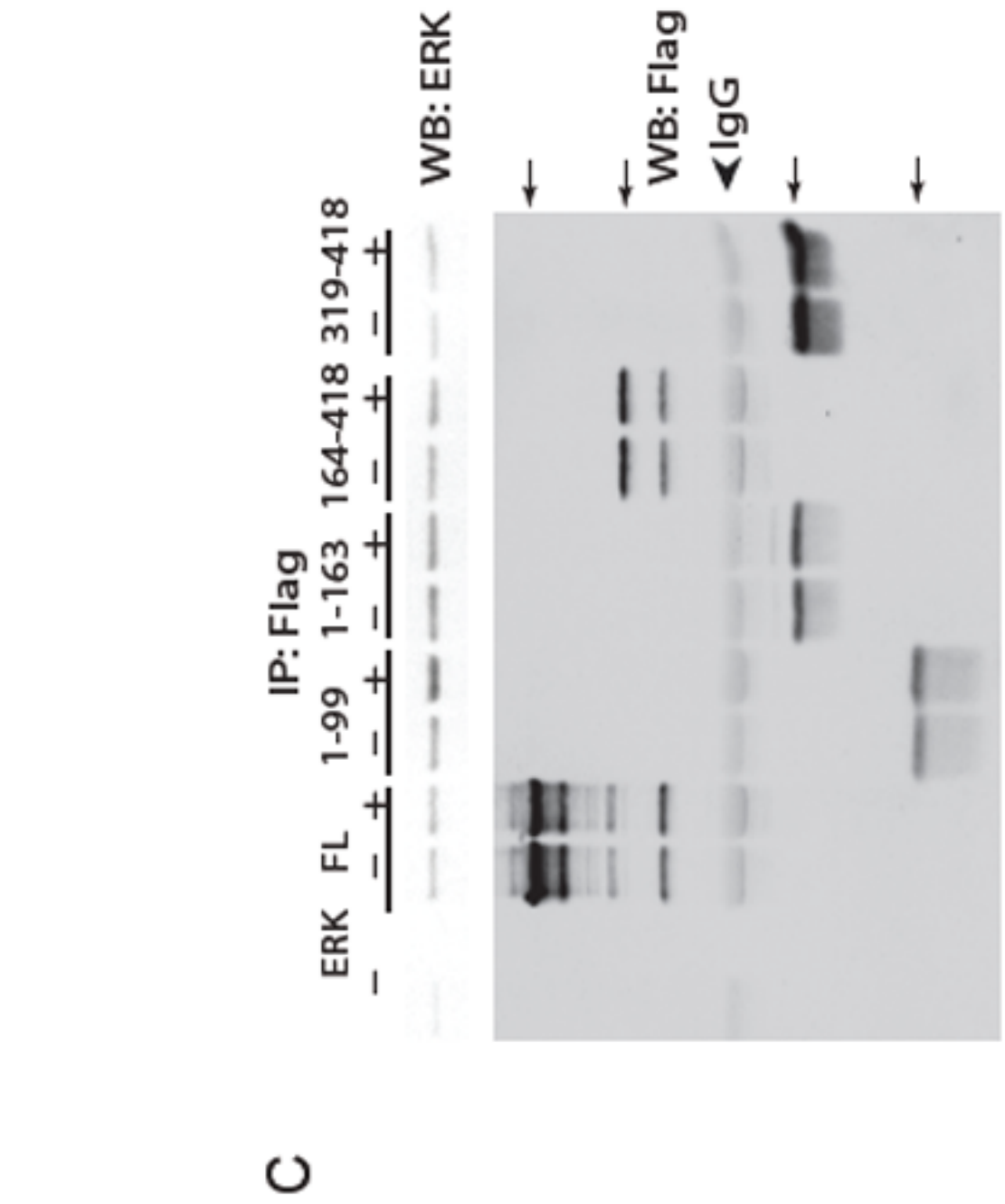

GST
βarr1GST
Unbound
Bound
Unbound
Bound
Raf
1-163
164-418
Unbound
Bound
Unbound
Bound
Raf

Fig. 2.5. PAR-2 activation induces interaction of the ERK cascade module with N-terminus of β-arrestin-1 at 5 min. (A) Both N- and C-domain of β-arrestin-1 bind to c-Raf. HEK 293 cells transiently expressing either N (1-163 amino acids)- or C (164-418 amino acids)- terminal truncations of β-arrestin-1 tagged by Flag were immunoprecipitated with anti-Flag agarose and analyzed by western blot. (B) N-terminus of β-arrestin-1 is involved in interaction with MEK upon PAR-2 activation HEK 293 cells transiently expressing either full length, 1-99, 1-163, 164-418, or 319-418 amino acids terminal truncations of β-arrestin-1 tagged by Flag and MEK tagged by Myc were immunoprecipitated with anti-Flag agarose and analyzed by western blot. (C) N-terminus in β-arrestin-1 is critical for the ERK binding. HEK 293 cells transiently expressing either full length, 1-99, 1-163, 164-418, or 319-418 amino acids terminal truncations of β-arrestin-1 tagged by Flag and ERK tagged by GFP were immunoprecipitated with anti-GFP, captured by protein G-agarose and analyzed by western blot. (D) Supplementary Figure of co-immunoprecipitation of β-arrestin-1 with ERK2. Middle region β-arrestin-1 is critical for the ERK binding. HEK 293 cells transiently expressing either full length, 1-99, 1-163, 164-418, or 319-418 amino acids terminal truncations of β-arrestin-1 tagged by Flag which were immunoprecipitated with anti-Flag immobilized onto agarose, analyzed by Western blot.


A
IP: Flag
TF
2fAP
mock
FL
MW
1-163
164-418
WB: c-Raf
WB: Flag
IgG


B
IP: Flag
TF
2fAP
MEK
FL
1-99
1-163
164-418
319-418
WB: MEK
WB: Flag
IgG


C
IP: Flag
ERK
FL
1-99
1-163
164-418
319-418
WB: ERK
WB: Flag
IgG

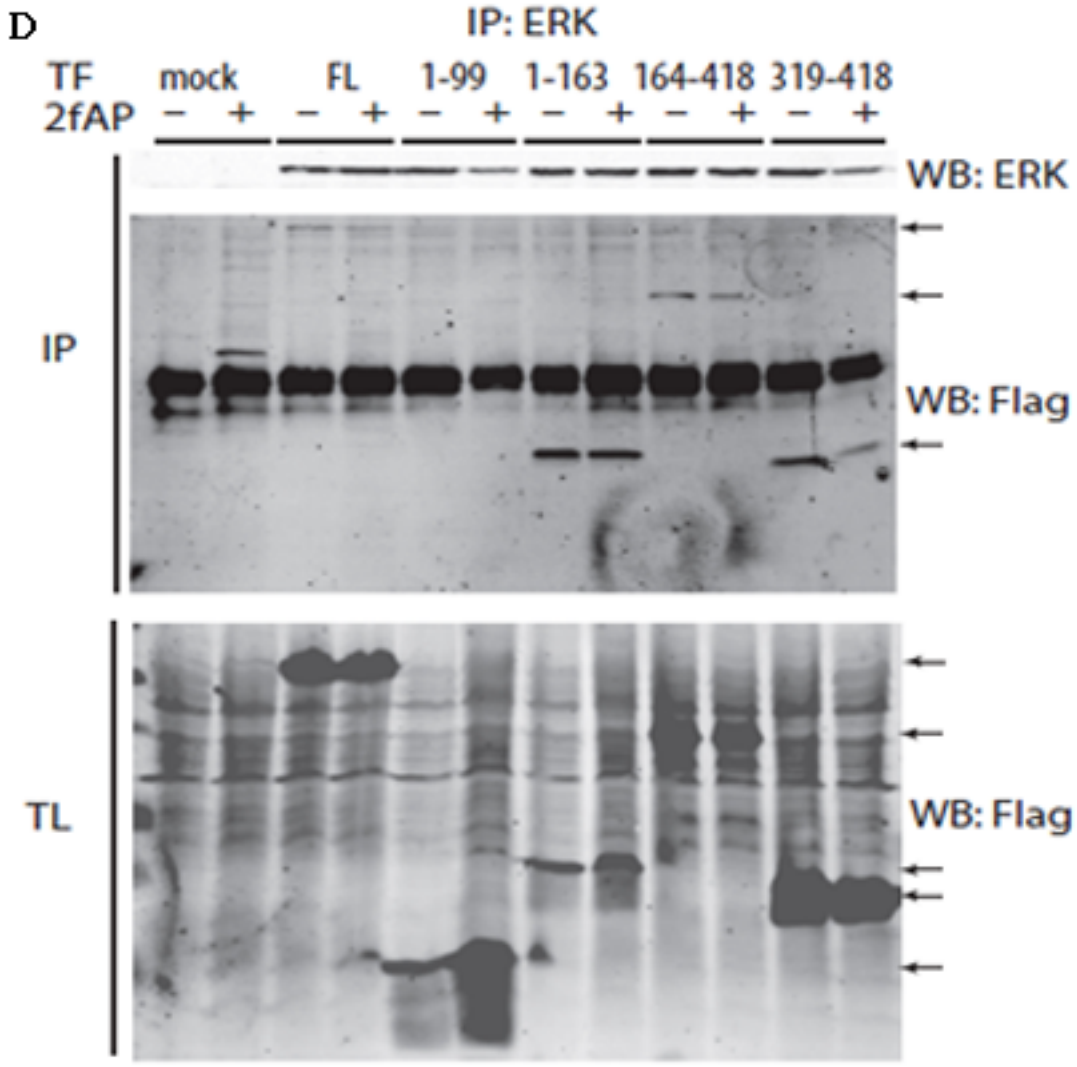
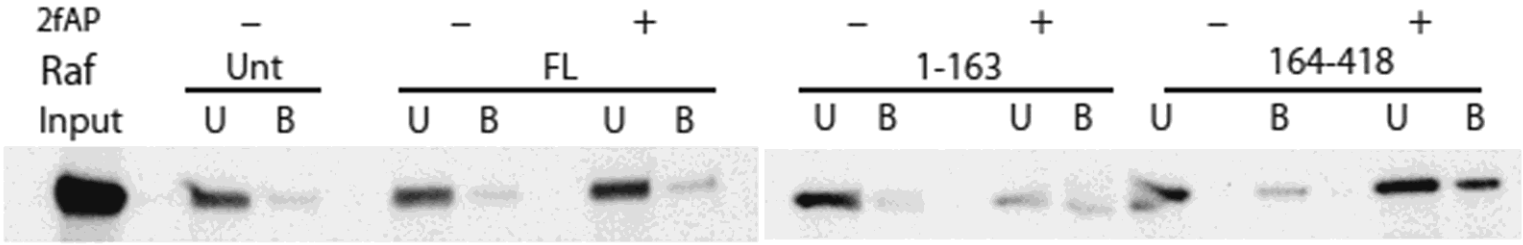

D
IP: ERK
TF
2fAP
mock
FL
1-99
1-163
164-418
319-418
WB: ERK
IP
WB: Flag
TL
WB: Flag

Fig. 2.6. PAR-2 activation enhances interaction of c-Raf with β-arrestin-1 at 90 min. *in vitro* translated c-Raf was pulled down with Flag-tagged β-arrestin-1 upon PAR-2 activation. HEK293 cell were transfected with Flag tagged β-arrestin-1, treated with 2fAP for 0 or 90 min. Clear cell lysates were immunoprecipitated by anti-flag (M2) agarose. Raf-1 was *in vitro* translated with [^{35}S]-Methionine in rabbit reticulocyte lysate. Flag tagged β-arrestin-1captured on anti-Flag agarose pulled down with [^{35}S]-Met labeled c-Raf. GST alone was used as a negative control. The result was obtained by SDS-PAGE followed by autoradiography.


2fAP
–
–
+
–
+
–
+
Raf
Unt
FL
1-163
164-418
Input
U B
U B
U B
U B
U B
U B
U B

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CHAPTER THREE

Elucidation of a Novel Pathway of β-arrestin-mediated c-Raf Activation

3.1. Abstract

The pathway from Ras through Raf and MEK to ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) regulates many fundamental cellular processes. β-arrestins as scaffolding proteins have been identified to sequester the ERK cascade module and prolong their activity within microdomain of the cell. We have previously shown that stimulation of protease-activated receptor-2 (PAR-2) induced sequestration of β-arrestin with the components of the ERK cascade, c-Raf, MEK1, and ERK1/2 with the receptor at the plasma membrane, leading to activation of cytoplasmic/membrane ERK1/2 signaling independently of G-protein coupling. Furthermore, β-arrestin-mediated prolonged ERK activation appeared to be Ras-independent downstream of PAR-2. Since a small GTPase, Ras has been known to be an upstream activator of c-Raf to relieve its autoinhibition, a mysterious link in functional mechanism of β-arrestin-mediated ERK1/2 activation is how c-Raf relieves autoinhibition engendering a conformation change from an inactive to an active state. We hypothesized that β-arrestins behave similar to Ras for β-arrestin-dependent ERK activation to relieve autoinhibition of c-Raf. Here we show that β-arrestin-1 binds to the regulatory domain of c-Raf where Ras was indentified to bind. Therefore, binding of β-arrestin-1 to c-Raf might ensure formation of scaffolding complex containing β-arrestin-1 and the ERK cascade and play a critical role to activate c-Raf in β-arrestin-dependent ERK1/2 activation.

3.2. Introduction

Mitogen-activated protein kinase (MAPK) pathways are signaling platforms through which extracellular signals are routed from the plasma membrane to intracellular targets to trigger different biological outcomes. The basic unit of mitogen-activated protein kinase (MAPK) module is a three-tiered protein kinase cascade that establishes a sequential activation pathway comprising a MAPKKK (MAP kinase kinase kinase), MAPKK (MAP kinase kinase), and MAPK (MAP kinase) [1-3]. The ERK cascade module containing Raf/MEK/ERK determines fundamental cellular processes that include cell proliferation, survival, differentiation, apoptosis, motility and metabolism [4-6]. Obviously, abnormalities in the activation of this pathway are associated with several diseases, most notably with cancer [6].

Activated Raf are the point of entry into a three-tiered kinase cascade and its activation at the plasma membrane starts a cascade in which the signal, in form of phosphorylation, is passed on to the dual specificity kinase, MEK1 and from it to the business end of the module, ERK1/2. Many receptors induce the activation of small GTP binding proteins (*i.e.* Ras, Rac, or Cdc42) and specific kinases that could be considered to be MAPK kinase kinase kinases (MKKKKs) regulate the activity of MKKKs, thus controlling the activation of specific three kinase MAPK modules. [7-9].

Typically, extracellular signals are transmitted to c-Raf through receptor-induced activation of membrane-associated small G protein, Ras GTPases [10, 11]. Active Ras in the GTP-bound form binds to the NH_2-terminal regulatory domain of c-Raf and recruits it

from the cytosol to the plasma membrane, resulting in conformational changes in c-Raf from the autoinhibitory, inactive state to active catalytic state or simply the proper environment for c-Raf signaling [12-15].

Recently a number of scaffolding proteins for MAPK activation have been identified and their important roles in regulating signaling through this pathway are now emerging. β-arrestins have been identified to be versatile adaptor proteins for ERK/MAPK and other signaling pathways although known for their role in desensitizing and internalizing G protein-coupled receptors (GPCRs) [16]. β-arrestins mediate the activation of ERK/MAPK by assembling new signaling complexes comprising c-Raf, MEK and ERK/MAPK [17-21]. In this situation, β-arrestins sequester ERK/MAPK signaling, dispatching ERK/MAPK signaling to different subcellular compartments, mostly excluded from the nucleus. Downstream of protease-activated receptor-2 (PAR-2) β-arrestin scaffolds the components of the ERK cascade, c-Raf, MEK1, and ERK1/2 with the receptor at the plasma membrane, leading to activation of cytoplasmic/membrane ERK1/2 signaling independently of G-protein coupling [17, 18]. Furthermore, we previously demonstrated that stimulation of PAR-2 induced sequestration of c-Raf and activated ERK with β-arrestin at the plasma membrane and β-arrestin-dependent ERK activation at the membrane appeared to be Ras-independent c-Raf activation. Since active GTP-bound Ras, binds and recruits c-Raf from the cytosol to the plasma membrane, setting in motion a multi-step activation process involving dynamic changes in intra- and intermolecular interactions as well as phosphorylation [12-14], we hypothesized that β-arrestins play a significant role in activation of c-Raf, leading to phosphorylation of

ERK1/2. Our aims are: 1) to identify specific domains in c-Raf interacting with β-arrestins; 2) to evaluate the mechanism by which this binding activates c-Raf and the effect of disrupting domains in interactions.

3.3. Methods and Materials

Chemicals : All chemicals were from Sigma or Fisher Scientific unless stated otherwise. [^{35}S]-methionine was obtained from Perkin-Elmer. All restriction enzymes were from New England Biolabs. Glutathione sepharose 4B was from GE Healthcare. Glutathione agarose was from BD Biosciences Pharmingen. Activating peptide 2fAP (2-furoyl-LIGRL-Ornithine-NH_2) was synthesized by Tocris Bioscience. Protein-G agarose and protein-A agarose were from Upstate. $PVDF_{fl}$ was from Millipore.

Antibodies: The following antibodies were: mouse anti-c-Raf (BD Bioscience Laboratories, 1: 300 WB), mouse anti-GFP (Roche, 1:500 WB), rabbit anti-GST (Santa Cruz, 1:1000 WB), rabbit anti-Flag (Sigma, 1:000 WB), mouse anti-β-arrestin-1(BD Transduction Laboratories, 1:1000 WB), and mouse anti-6xHis (BD Biosciences Pharmingen, 1:1000 WB). For sandwich immunoassay, IR-dye-conjugated secondary antibodies (1:45,000 WB) were from Rockland Biosciences.

Plasmids: The following plasmids were used in transient expression experiments; Flag-tagged β-arrestin-1 was provided from Dr. Robert Lefkowitz (Duke University, Durham, NC). WT Raf-GFP. Reg Raf-GFP (1-303) and Kinase Raf-GFP (Δ26-302) were provided from Dr. Manuela Baccarini (University of Vienna, Austria). For expression in bacteria, GST-tagged WT β-arrestin-1 was from Dr. Robert Lefkowitz (DUMC, Durham, NC). GST-tagged ERK was excised with BamhIII and subcloned into pGEX4T-1. WT c-Raf

was subcloned into pGEX4T-1. Reg Raf-GST (1-258 amino acids) was a gift from Dr. Manuela Baccarini (University of Vienna, Austria).

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 RIPA (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitors) for c-Raf. 10µg of protein was analyzed by 10 or 12.5% 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 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℃. 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℃ until needed. The protein concentration of elutes 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 both β-arrestin proteins was observed in each preparation, which was quantified by densitometric analysis of 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℃) 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. GST-tagged human WT c-raf and regulatory c-raf were expressed in *E. coli* Rosetta™ (DE3) BL21 cells (EMD chemicals) which enhance

the expression of eukaryotic proteins that contain codons rarely used in *E.Coli*. by induction with 1 mM IPTG and incubation in minimal medium overnight at 22℃.

Bioluminescence Resonance Energy Transfer (BRET) assay: After 48 hours post-transfection, HEK 293 cells detached with PBS. Approximately 50,000 cells per well were distributed in a 96-well microplate (white bottom costar). Cells were treated with and without 1 μM 2-fAP and the coelenterazine H was added at a final concentration of 5 μM, and readings were collected by the sequential intergration of the signals detected in the 440- to 500-nm and 510- to 590-nm windows. The BRET values were determined by calculating the ratio of the fluorescence signal by emitted by interaction between β-arrestin-1 tagged by Renilla Luciferase (emission at 440-500 and c-Raf tagged by YFP (emission at 510-590). These values were correctd by subtracting the background signal detected when the β-arrestin-1-Rluc or YFP construct were expressed alone.

Sandwich Immunoassay: Recombinant β-arrestin-1 was captured by mouse anti-β-arrestin-1 (BD Transduction Laboratories) onto the bottom of the 96-well plate. Recombinant c-Raf mutant containing the NH_2-terminal regulatory domain (1-258 amino acids) was incubated and followed by probing with anti-GST conjugated with IR-800. Data were analyzed by Li-Cor odyssey.

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 one way ANOVA and Tukey t-tests (to compare between treatment groups).

3.4. Results

3.4.1. PAR-2 activation induces interaction of c-Raf with β-arrestin-1 via its regulatory domain.

Typically, Ras, a small GTPase has been known to activate c-Raf and recruit it from the cytosol to the plasma membrane by its binding to the NH_2-terminal regulatory domain of c-Raf [12-15]. However, we previously observed that stimulation of PAR-2 resulted in sequestration of the ERK cascade module with β-arrestins at the plasma membrane and this appeared to be Ras-independent. We hypothesized that β-arrestin might behave similar to Ras in inducing c-Raf activation, leading to the ERK1/2 activation. To address which domains in c-Raf PAR-2 promotes interaction with β-arrestin-1, wild type or the truncated mutants either the regulatory domain or the kinase domain of c-Raf tagged by GFP were immunoprecipitated from HEK 293 cells transiently expressing c-Raf and β-arrestin-1 after PAR-2 activation with mouse anti-GFP, and immune complexes were analyzed by SDS-PAGE followed by Western blotting with rabbit anti-Flag to detect β-arrestin-1. Both wild type c-Raf and the truncated mutant of c-Raf containing the regulatory domain (Reg-Raf) increasingly associated with β-arrestin-1 upon PAR-2 activation, but not the truncated mutant of c-Raf containing the kinase domain (Kinase-Raf) (Fig. 3.1). Furthermore, association of WT c-Raf, Reg-Raf, or kinase-Raf with β-arrestin-1 was observed even in the absence of receptor activation, suggesting the basal interaction of two molecules. The result thus shows that PAR-2 promotes association of Reg-Raf with β-arrestin-1, suggesting a possible role of β-

arrestin-1 replaced with Ras in inducing c-Raf activation.

3.4.2. The regulatory domain of c-Raf appears to be the key binding element for interaction with β-arrestin-1

To confirm that β-arrestin-1 directly interacts with the regulatory domain of c-Raf (Reg-Raf), we performed sandwich immunoassay using recombinant proteins. Recombinant GST tagged Reg-Raf or GST alone was incubated with wild type β-arrestin-1 and the interaction was detected by anti-GST conjugated with IR-800. Increasing amount of GST Reg-Raf directly bound β-arrestin-1, while GST alone did not (Fig. 3.2). Therefore, it appeared that β-arrestin-1 directly binds Reg-Raf, suggesting that it might directly cause to relieve autoinhibition of c-Raf, leading to its activation.

3.4.3. 27FVD29/27AAA29 β-arrestin-1 displays markedly reduced association with Reg-Raf upon PAR-2 activation.

Based on results obtained from co-immunoprecipition and sandwich immunoassay, interaction of β-arrestin-1 with the regulatory domain of c-Raf might be critical to lead to c-Raf activation. To explore key residues for interaction of β-arrestin-1 with Reg-Raf, we compared sequence alignment between Ras and β-arrestin-1, found the matching amino acids, FVD from 27 to 29 residues, and substituted these with alanines using site-directed mutagenesis (Fig. 3.3.A). HEK 293 cells were transiently transfected with wild type or 27FVD29/27AAA29 mutant β-arrestin-1 tagged by Flag and Reg-Raf tagged by GFP.

Clear cell lysates were immunoprecipitated after PAR-2 activation with mouse anti-GFP, and immune complexes were analyzed by SDS-PAGE followed by Western blotting with rabbit anti-flag to detect β-arrestin-1. PAR-2 activation induced increased interaction of Reg-Raf with wild type β-arrestin-1 whereas abolished enhanced interaction of Reg-Raf with 27FVD29/27AAA29 mutant β-arrestin-1 (Fig. 3.3). It was consistent with the result of immunoprecipitation of β-arrestin-1 using mouse anti-Flag agarose and detection of Reg-Raf with anti-GFP antibody, suggesting that the residues, FVD in β-arrestin-1 might be the key residues for interaction with Reg-Raf.

3.5. Discussion

The pathway from Ras through Raf and MEK to ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) regulates many fundamental cellular processes including cell survival, differentiation, proliferation, apoptosis and cell motility [22, 23]. Downstream of receptors, the ERK cascade has been known to be activated by small GTP binding proteins (*i.e.*, Ras, Rac, or Cdc42) and specific kinases that could be considered to be MAPK kinase kinase kinases (MKKKKs) regulate the activity of MKKKs, thus controlling the activation of specific three kinase MAPK modules [7-9]. We have previously demonstrated that stimulation of PAR-2 resulted in sequestration of c-Raf and activated ERK1/2 with β-arrestin at the plasma membrane and β-arrestin-dependent ERK activation appeared to be mediated by Ras-independent c-Raf activation [17, 18]. Since c-Raf has been known to require Ras in its activation, we hypothesized that β-arrestin plays a key role in activation of c-Raf, leading to ERK1/2 activation.

We found that β-arrestin-1 bound to the regulatory domain of c-Raf similar to Ras and furthermore, this interaction was enhanced by PAR-activation whereas interaction between the kinase domain of c-Raf and β-arrestin-1 was dramatically decreased. In addition, we determined that the residues, FVD from 27 to 29 amino acids in β-arrestin-1 played a critical role in the interaction between Reg-Raf and β-arrestin-1. Possibly, not only these FVD amino acids but also negatively charged amino acids along the matching sequence in Ras and β-arrestin-1might be critically involved in the interaction. MAPK signaling must be activated and inactivated with both temporal and spatial accuracy,

resulting in critical cellular decisions in cell fate. This could possibly be evoked by the non-catalytic docking interactions that involve protein recognition modules such as scaffolding proteins for organization of MAPKs in signaling complexes. β-arrestin scaffolded ERK1/2 cascade leads to prolonged ERK1/2 activity in cytosol and at the plasma membrane, excluded from the nucleus. Obviously, loss of fine tuning of temporal or spatial regulation of MAPK signaling by changes in expression or availability of proteins regulating MAPK singling can make a significant contribution to many different diseases, including chronic inflammation, neurodegenerative diseases and cancer.

We propose a model of β-arrestin-dependent c-Raf activation. In quiescent cells, c-Raf stays in an inactive state with its closed conformation. Once PAR-2 is activated, β-arrestins are recruited and binds to the NH_2-terminal regulatory domain of c-Raf, inducing its conformational change into an active and open state, or simply the proper environment for c-Raf signaling. As a future direction, to monitor the conformational change of c-Raf with binding to β-arrestin, we create c-Raf biosensor which has labeled with *Renilla* Luciferase at the one end of c-Raf and with YFP at the other end. Using a bioluminescence resonance energy transfer (BRET) based assay, we measure directly and in living cells conformational changes of c-Raf and expect to obtain high BRET with basal c-Raf conformation which *Renilla* Luciferase and YFP are close enough for resonance energy transfer, in contrast, when c-Raf binds to β-arrestin, two tag molecules are further apart to generate no specific basal BRET.

Scaffolding proteins organize MKK and MAPK complexes for activation by specific MKKKs and do so in specific locations in the cell. c-Raf associated with the

scaffolding proteins provides selectivity or attenuation for activation by upstream stimuli. The demonstration that β-arrestin induces c-Raf activation is a newly recognized pathway to the control of MAPK networks, different from its classical activation by small GTPases.

3.6. Figures and Legends

Fig. 3.1. The regulatory domain (1-303 amino acids) of c-Raf (Reg-Raf) is the key element to bind β-arrestin-1 in cell. (A) HEK 293 cells transiently expressing either WT, Reg (1-303 amino acids) or kinase domain (Δ26-302) of c-Raf (Kinase-Raf) tagged by GFP and wild type β-arrestin-1 tagged by Flag were immunoprecipitated with mouse anti-GFP, captured by protein G-agarose and analyzed by Western blot with rabbit anti-flag. (B) The graph indicated the quantification of β-arrestin-1/c-Raf association.

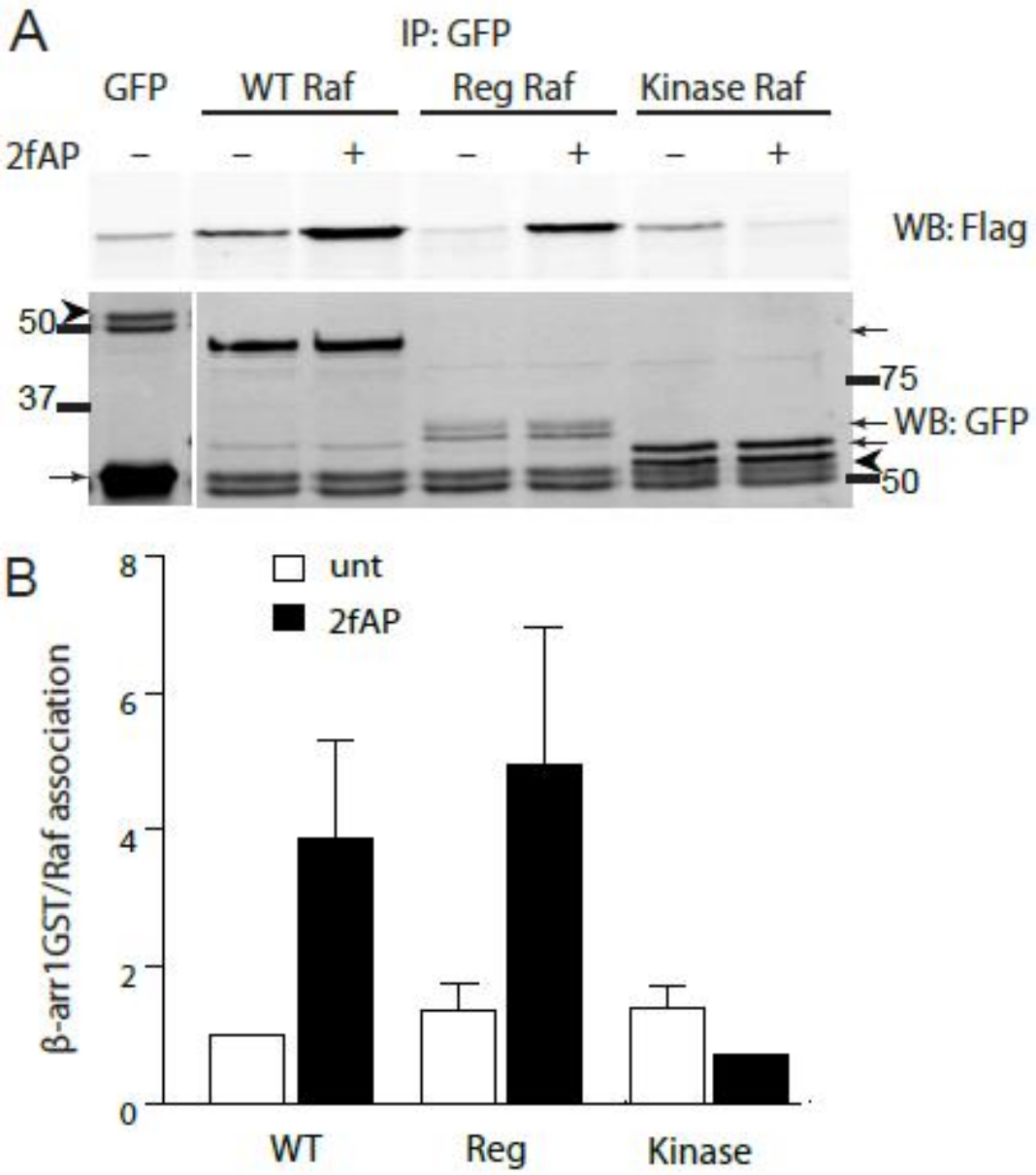
A
IP: GFP
GFP
WT Raf
Reg Raf
Kinase Raf
2fAP
–
–
+
–
+
–
+
WB: Flag
50
37
75
WB: GFP
50
B
unt
2fAP
β-arr1GST/Raf association
8
6
4
2
0
WT
Reg
Kinase

Fig. 3.2. The regulatory domain (1-258 amino acids) of c-Raf (Reg-Raf) directly binds to wild type β-arrestin-1 *in vitro*. Recombinant WT β-arrestin-1 was captured by mouse anti-β-arrestin-1 antibody onto the bottom of the 96-well plate. Either increasing amount of recombinant Reg-Raf tagged by GST or GST alone as a negative control was incubated and followed by probing with anti-GST conjugated with IR-800. Data were analyzed by Li-Cor odyssey.

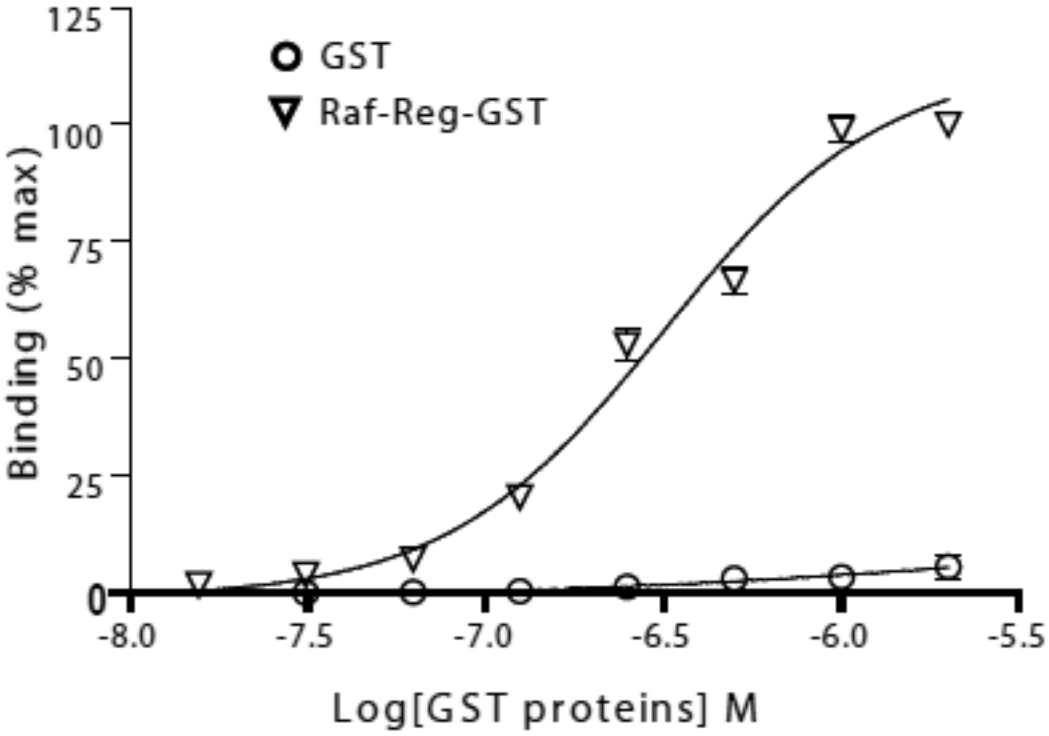
125
100
75
50
25
0
Binding (% max)
GST
Raf-Reg-GST
-8.0
-7.5
-7.0
-6.5
-6.0
-5.5
Log[GST proteins] M

Fig. 3.3. 27FVD29/27AAA29 β-arrestin-1 displays markedly reduced interaction with Reg-Raf. (A) Comparison of N-terminal sequences between human Ras and human β-arrestin-1. Sequence alignment was found that the matching amino acids, FVD from 27 to 29 residues exists between Ras and β-arrestin-1. 27 FVD29 in β-arrestin-1 were substituted with alanines by site-directed mutagenesis. (B) HEK 293 cells were transiently expressed with the regulatory domain (1-303 amino acids) of c-Raf (Reg-Raf) tagged by GFP and wild type β-arrestin-1 or 27FVD29/27AAA29 β-arrestin-1 tagged by Flag. Clear cell lysate were immunoprecipitated with mouse-anti-Flag immobilized on agarose and Western blotted with mouse anti-GFP antibodies (top), or immunoprecipitated with mouse anti-GFP, captured by protein G-agarose and analyzed by Western blot with rabbit anti-Flag antibodies (bottom).

A

```
sp|P01112|RASH_HUMAN    MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGET
sp|P49407|ARRB1_HUMAN   MGDKGTRVFKKASPNGKLTVYLGKRDFVDHID--LVDPVDGVVLVDPEY
```

βarr1AAA (27FVD29_27AAA29)

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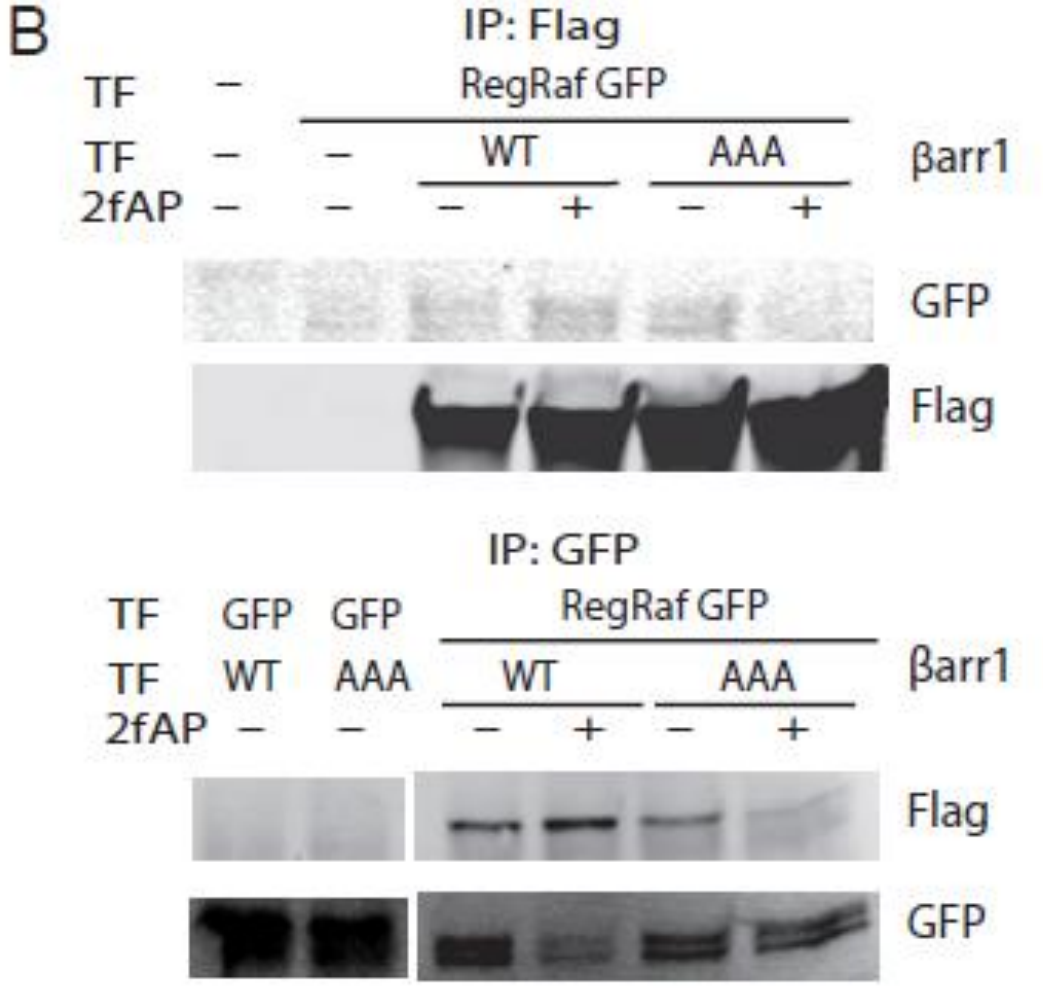
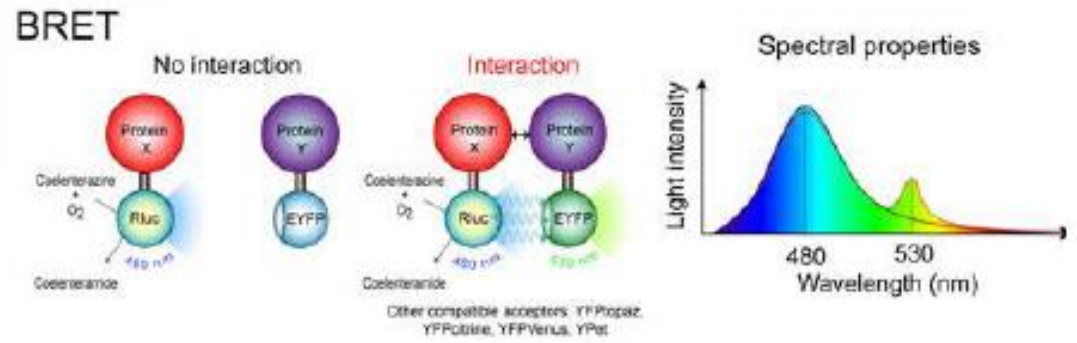

B
IP: Flag
TF
RegRaf GFP
TF
WT
AAA
βarr1
2fAP
GFP
Flag
IP: GFP
TF
GFP
GFP
RegRaf GFP
TF
WT
AAA
WT
AAA
βarr1
2fAP
Flag
GFP

Fig. 3.4. Strategy to monitor conformational change of c-Raf by binding to β-arrestin-1 using Bioluminescence Resonance Energy Transfer (BRET) assay. (A) Scheme of Bioluminescence Resonance Energy Transfer (BRET) assay. To have an energy transfer between a bioluminescent donor enzyme, a variant of *Renilla* Luciferase (Rluc) and a complementary acceptor fluorophore, a variant of green fluorescent protein (GFP), YFP, upon oxidation of a coelenterazine substrate the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor. The energy transfer can occur only when the donor and the acceptor are spatially close. Thus using fusion with protein of interest, BRET signal will be measured when the two proteins studied physically interact. (B) Biosensor c-Raf. Schematic diagram illustrating how c-Raf undergoes conformational rearrangement of c-Raf by binding with β-arrestin-1 measured in BRET. c-Raf biosensor is created by fusing the two tag proteins, Rluc and YFP to c-Raf.


BRET
No interaction
Interaction
Protein X
Protein Y
Coelenterazine
O_2
Rluc
EYFP
Coelenteramide
Other compatible acceptors: YFPtopaz, YFPcitrine, YFPVenus, YPet
Spectral properties
Light intensity
480
530
Wavelength (nm)

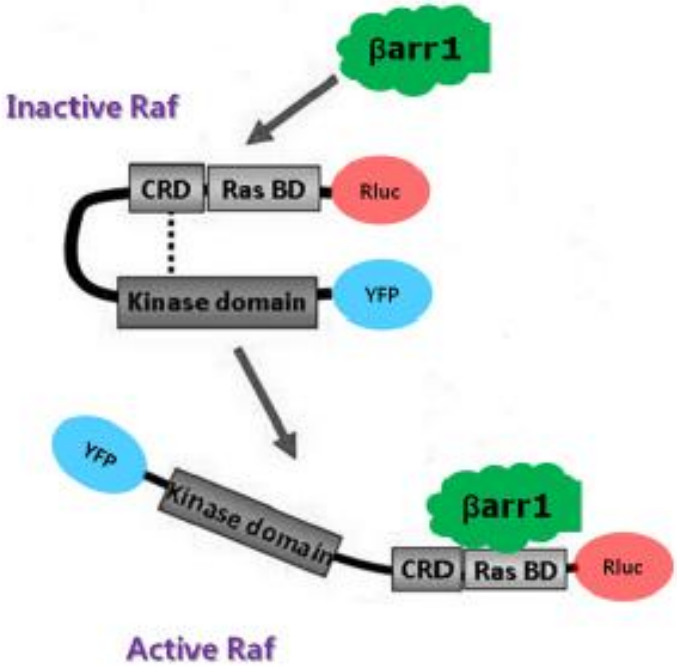
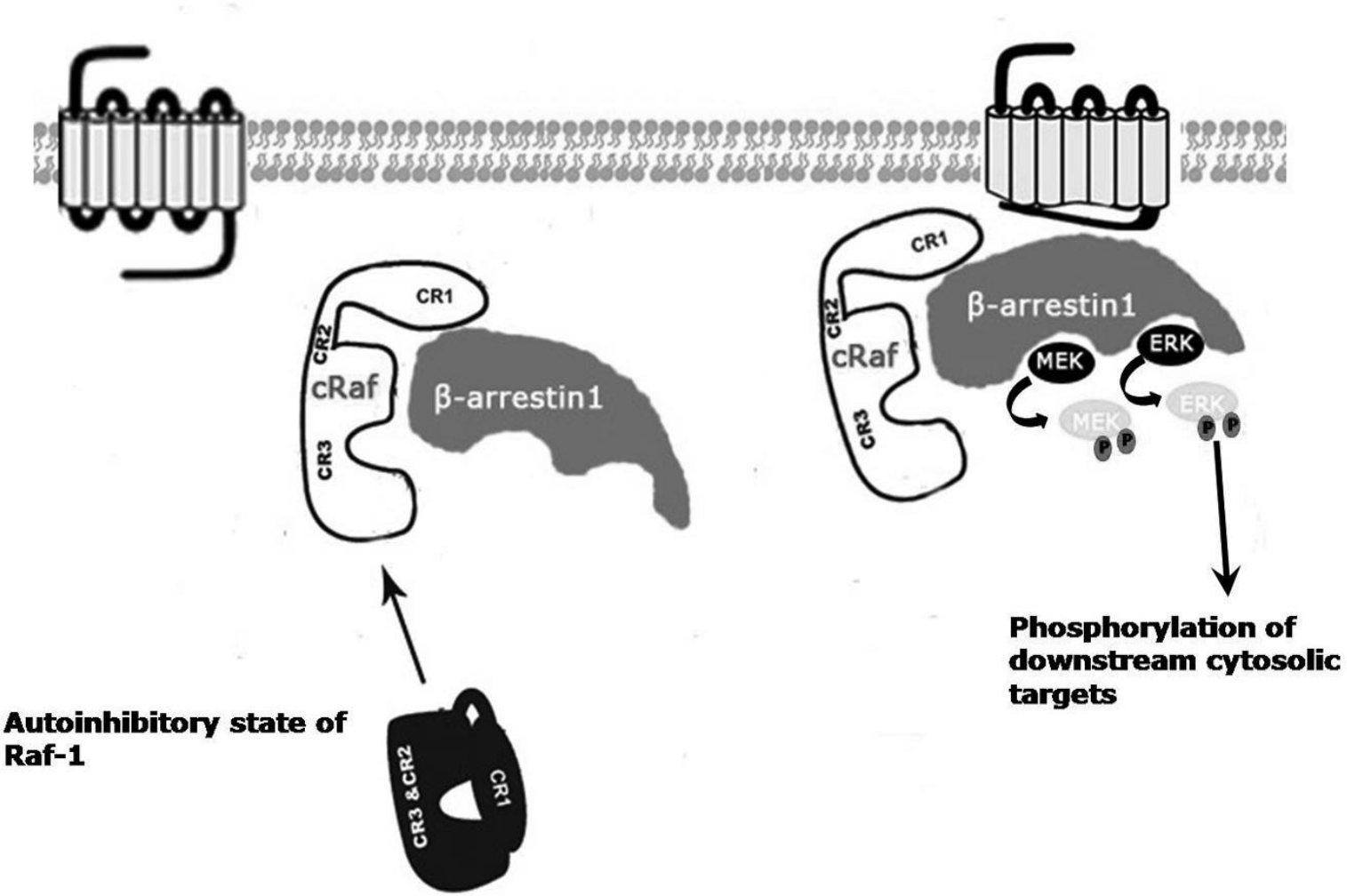

βarr1
Inactive Raf
CRD
Ras BD
Rluc
Kinase domain
YFP
YFP
Kinase domain
βarr1
CRD
Ras BD
Rluc
Active Raf

Fig. 3.5. Proposed model of c-raf activation by binding to β-arrestin-1 leading to prolonged ERK1/2 activation in cytosol. In quiescent cells, c-Raf stays in inactive state by interaction between the CRD and the kinase domain inhibiting catalytic activity. Upon PAR-2 activation β-arrestin-1 binds to the NH_2-terminal regulatory domain of c-Raf and recruits it from the cytosol to the plasma membrane, resulting in conformational changes in c-Raf from the inactive state to active catalytic state or simply the proper environment for c-Raf signaling.


CR1
CR2
cRaf
CR3
β-arrestin1
MEK
ERK
P
Phosphorylation of downstream cytosolic targets
Autoinhibitory state of Raf-1
CR3 &CR2
CR1

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CHAPTER FOUR

Conclusions and Perspectives

G protein coupled receptors (GPCRs) referred to as seven transmembrane receptors (7TMRs) are integral membrane proteins which constitute the largest class of cell surface receptors in the human genome with about 800 different members. These receptors respond to a diverse array of stimuli which include cytokines, hormones, peptides, neurotransmitters, lipids and proteases. Upon activation, they initiate a range of intracellular signaling pathways to elicit appropriate cellular responses. Therefore, they have been considered as major targets for the development of new drugs with potential application in all clinical fields. Many current therapeutic agents in use act by either activating (agonists) or blocking (antagonists) GPCRs. Theses agonists and antagonists have been mostly screened solely on their ability to elicit G protein coupling. However, recent evidence has revealed that some of these receptors including protease-activated receptor-2 (PAR-2) can act via β-arrestin-mediated pathways independently of G-protein coupling. This could be explained by 'Biased agonism', a process by which distinct ligands acting on the same receptor can elicit different signaling responses by stabilizing distinct active receptor conformations. The current data point to a model wherein different receptors, and perhaps the same receptors in different cell types, can elicit specific β-arrestin conformations that expose certain domains to recruit distinct scaffolding complexes and are likely regulated by different mechanisms. The formation of different scaffolds on β-arrestins can then be determined by the activating receptor and its cellular context, leading to an additional level of signaling specificity.

A number of studies have shown that PAR-2 evokes two opposite responses in inflammation: pro-inflammatory or protective responses. Furthermore, PAR-2 is currently

a popular target for new drugs regarding inflammation that can activate, deactivate or partially activate preferably specific downstream signaling pathways. Characterization of molecular mechanisms of two distinct downstream pathways of PAR-2 via G protein-dependent or β-arrestin-dependent pathways will enable the development of new strategies to manipulate PAR-2 signaling and will provide novel targets for the development of drugs for treatment of various inflammatory diseases and prevention of tumor metastasis.