NON-VISUAL ARRESTINS BIND MITOGEN ACTIVATED PROTEIN KINASES AND REGULATE THEIR SIGNALING

By

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LIST OF ABBREVIATIONS

GPCR	G protein coupled receptor
MAPK	Mitogen activated protein kinase
MEK	MAPK/ERK kinase
ERK	Extracellular signal-regulated kinase
JNK	C-jun N-terminal kinase
β2AR	Beta 2 adrenergic receptor
RGS	Regulators of G protein signaling
PKA	Protein kinase A
PKC	Protein kinase C
cAMP	Cyclic adenosine monophosphate
AP-2	Adaptor protein 2
GRK	G protein coupled receptor kinase
ATP	Adenosine triphosphate
PLCγ	Phospholipase C gamma
DAG	Diacylglycerol

GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
cAMP	Cyclic adenosine monophosphate
MT	Microtubules
$AT_{1A}R$	Angiotensin type 1A receptor
PAR-2	Protease-activated receptor 2
DKO	Double knock out
MEF	Mouse embryonic fibroblast
P-Rh*	Phosphorylated light-activated rhodopsin
WT	Wild type
IGF1	Insulin-like growth factor 1
TGFβRIII	Transforming growth factor beta receptor type 3

ABSTRACT

Arrestins are multifunctional signaling proteins, important for the regulation of signal transduction and the trafficking of G protein-coupled receptors (GPCRs). Recently, GPCR-arrestin interactions have been proposed to be necessary for activation of G-protein-independent signaling pathways, one of which is the activation of mitogen activated protein kinases (MAPKs). To investigate potential arrestin-MAPK interactions, we have used a variety of molecular tools including the co-expression of the individual domains of arrestin with single components of the c-Raf1-MEK1-ERK2 signaling cascade. We found that non-visual arrestins bind all three kinases, assembling c-Raf1, MEK1, and ERK2 along their short axis, with each kinase directly interacting with both domains.

To further investigate the interactions between arrestins and MAPK, we used alanine-scanning mutagenesis of residues on the non-receptor-binding surface of arrestin that are conserved between arrestin-2 and arrestin-3. We found that the substitution of arginine 307 with an alanine significantly reduced arrestin-2 binding to c-Raf1, whereas the interactions of this mutant with active phosphorylated receptors and the downstream kinases MEK1 and ERK2 were not affected. In contrast to wild type arrestin-2, Arg307Ala mutant failed to rescue arrestin-dependent ERK1/2 activation in arrestin-2/3 knockout MEFs. Interestingly, alanine substitution of the homologous arrestin-3 residue (lysine 308) did not significantly affect c-Raf1 binding or its ability to promote ERK1/2 activation. Together, these findings suggest that the two non-visual arrestins

perform the same function via distinct molecular mechanisms. To further elucidate arrestin-MAPK interactions, we performed *in vitro* binding assays using pure proteins, and demonstrated that ERK2 directly binds free arrestin-2 and arrestin-3, as well as receptor-associated arrestin-1, arrestin-2, and arrestin-3. We have also shown that the arrestin-2 and arrestin-3 association with beta2-adrenergic receptors (β2ARs) significantly enhances ERK2 binding, yet has virtually no effect upon arrestins interactions with the upstream kinases c-Raf1 and MEK1.

Arrestins exist in three conformational states: free, receptor-bound, and microtubule (MT)-bound. Using conformationally biased arrestin mutants, we found that ERK2 prefers two conformations: MT-bound, mimicked by "constitutively inactive" arrestin-Δ7, and receptor-bound, mimicked by "pre-activated" arrestin-3A. Both mutants were able to rescue arrestin-mediated ERK1/2 activation in arrestin-2/3 double knockout fibroblasts. Lastly, we found that the arrestin-2 interaction with c-Raf1 is enhanced by receptor binding, whereas the interaction between arrestin-3 and c-Raf1 is not, thus suggesting that the two non-visual arrestins execute similar functions via diverse mechanisms.

CHAPTER I

INTRODUCTION

G protein-coupled receptors

G Protein-Coupled Receptors (GPCRs), a family of seven transmembrane domain proteins, are the most abundant cell surface receptors in the human genome (Lander, Linton et al. 2001; Venter, Adams et al. 2001). They are essential for many cellular functions (Wess 1998) and are involved in numerous physiological and pathological processes (Lombardi, Kavelaars et al. 2002; Rockman, Koch et al. 2002; Premont 2005).

More than 200 of the GPCRs identified have known functions, and when they are defective, or have faulty interactions with G-proteins, diseases such as breast cancer, congestive heart failure and blindness, to name a few, may result (Muller, Homey et al. 2001; Metaye, Gibelin et al. 2005). More than 60% of all prescribed drugs target GPCRs (Papasaikas, Bagos et al. 2003; Overington, Al-Lazikani et al. 2006). Despite the significant level of research focused upon further elucidating biological functions of GPCRs, our understanding is still quite limited, as we have crystal structures of very few GPCRs. These include rhodopsin in the inactive (Palczewski, Kumasaka et al. 2000; Li, Edwards et al. 2004; Standfuss, Xie et al. 2007) and active (Choe, Kim et al. ; Standfuss, Edwards et al. ; Park, Scheerer et al. 2008; Scheerer, Park et al. 2008) state, beta-2 adrenergic receptor (β2AR) (Kobilka and Schertler 2008), beta-1

adrenergic receptor (Moukhametzianov, Warne et al. ; Warne, Moukhametzianov et al. ; Warne, Serrano-Vega et al. 2008), H1 histamine (Shimamura, Shiroishi et al.), A2A adenosine (Xu, Wu et al. ; Jaakola, Griffith et al. 2008), D3 dopamine (Chien, Liu et al.), and CRXR4 chemokine receptor (Wu, Chien et al.). Characterization of the remaining GPCR structures has therefore relied upon sequence homology alignments and mutagenesis studies (Hjorth, Schambye et al. 1994; Ohyama, Yamano et al. 1995).

All GPCRs span the cell membrane seven times with interconnecting intracellular and extracellular loops, and possess an extracellular amino-terminus (N-terminus) and an intracellular carboxyl-terminus (C-terminus) (Baldwin, Schertler et al. 1997). GPCRs range in size from 40-60 kDa and have been classified into five classes (Figure 1). Class A includes the largest group of GPCRs, and is also referred as rhodopsin-like. Examples of this class include beta-adrenergic, rhodopsin, muscarinic and dopamine receptors. Class B, secretin-like, is characterized by a large extracellular N-terminus. Typical examples from this class are glucagon, the corticotropin-releasing factor receptor and the parathyroid hormone receptor (Gurevich and Gurevich 2008). The third class of GPCRs, class C, includes the metabotropic glutamate as well as the calcium-sensing receptors.

GPCRs are stimulated by interactions with extracellular signals such as drugs, hormones, photons, ions, peptides and even proteins. Their interactions induce a conformational change in the protein structure of GPCRs allowing for

transduction of cellular signals to secondary signaling components via G-proteins.

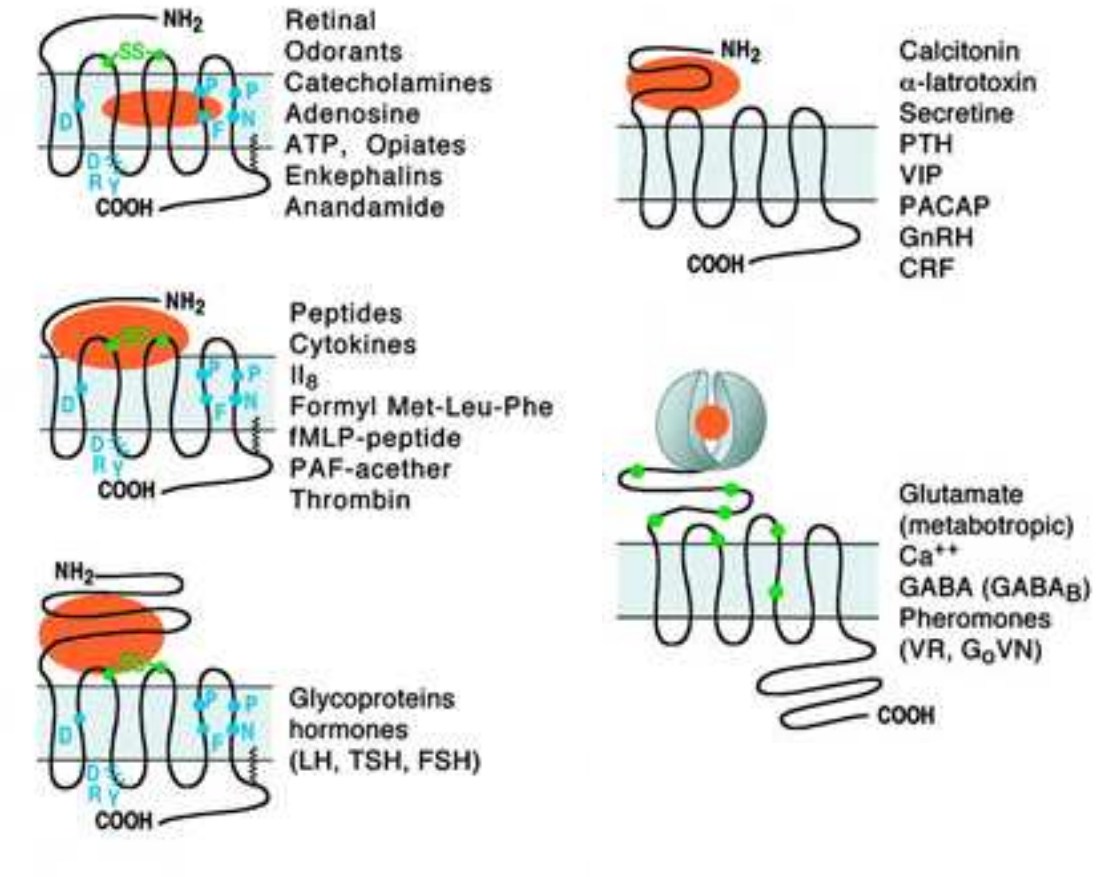

Figure 1. GPCR classification.
GPCRs are classified, based on amino-acids comparison, into five classes:
Class A 'rhodopsin-like"; class B 'secretin-like'; class C 'metabotropic glutamate/pheromone'; class D 'fungal pheromone'; and class E, cAMP receptors. From (Bockaert and Pin 1999)

GPCR/G-protein interactions

Mutagenesis studies have been very useful in the identification of the GPCR amino acids responsible for G-protein selectivity and interactions (Conklin, Farfel et al. 1993; Wess 1998). G-proteins, which are heterotrimeric proteins, consist of alpha (G_α), beta (G_β) and gamma (G_γ) subunits. There are 20 isoforms of G_α, 6 isoforms of G_β and 11 isoforms of G_γ. Based upon sequence homology, $G\alpha$s are divided into four families: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$. Heterotrimeric G proteins are classified based upon G_α subunit characteristics (Lefkowitz 1998). The $G_{\alpha s}$ family members stimulate adenylyl cyclase, which results in increased production of cyclic AMP (cAMP), whereas $G_{\alpha i}$ members inhibit adenylyl cyclase activation. Members of the $G_{\alpha q}$ family stimulate phospholipase C_γ (PLC_γ), which results in the cleavage of phosphoinositol bisphosphate and the resultant production of diacylglycerol (DAG) and inositol triphosphate ultimately leads to an increase in intracellular calcium and PKC activation. The signaling effectors of the $G_{\alpha 12/13}$ family have not been well characterized (Gether and Kobilka 1998).

The interactions between GPCR and G-protein are mediated via the C-terminus of the G_α subunit, assisted by the G_α N-terminus and several other elements, as revealed by the recent crystal structure of β2AR-Gs complex (Rasmussen, Devree et al.). Activation of GPCRs induces exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G_α subunit, resulting in its dissociation from both the receptor and the G-beta-gamma dimer ($G_{\beta\gamma}$). The G_α subunit, as well as the $G_{\beta\gamma}$ dimer, amplify and transduce signals within the cell by

modulating the activity of effector molecules (Lefkowitz 1998; Lefkowitz and Shenoy 2005). Subsequently, signaling initiated by G-proteins is terminated by the intrinsic GTPase activity of G_{α}, which results in re-assembly of the G-protein heterotrimer. This process is accelerated by regulators of G-protein signaling (RGS proteins) (Willars 2006). Following activation of G-protein signaling cascades, a class of serine/threonine kinases leads to “uncoupling” of the G-protein from the receptor. This class includes the second-messenger-regulated kinases, PKA (protein kinase A) and PKC (protein kinase C). These kinases phosphorylate both active and inactive GPCRs resulting in “heterologous” desensitization (Lefkowitz 1998), whereas GPCRs that have been agonist-stimulated are phosphorylated by G protein-coupled receptor kinases (GRKs) and undergo “homologous” desensitization (Figure 2).

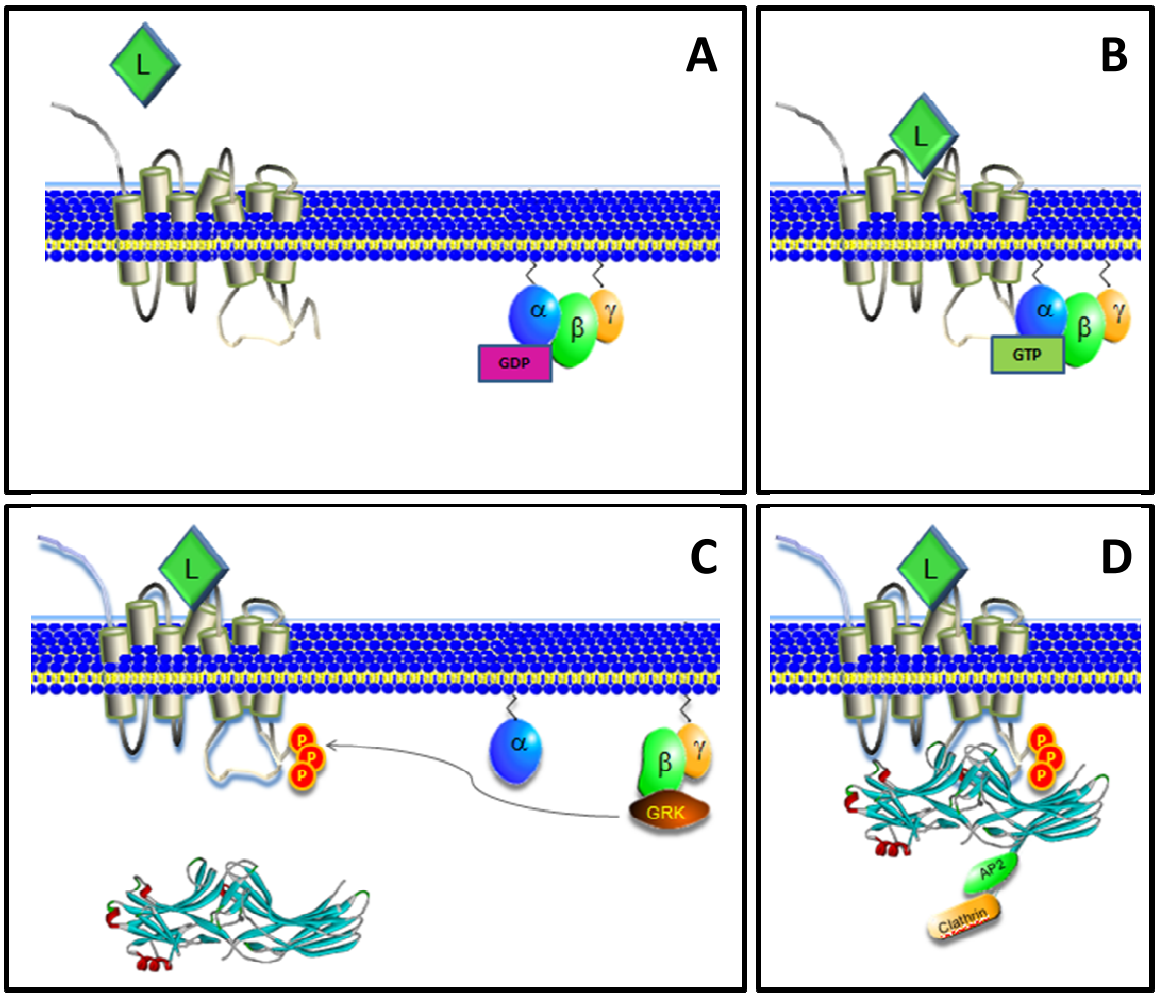

Figure 2. Model of GPCR activation and arrestin recruitment.

(A) Heterotrimeric inactive G-proteins couple to the ligand-activated GPCR. (**B**) The activated receptor causes an exchange of GDP for GTP, **(C)** which results in dissociation of the G-protein trimer. **(D)** Subsequent phosphorylation of the GPCR by GRK induces arrestin recruitment, followed by the release of the C-terminal tail and interaction with adaptor protein-2 and clathrin.

The role of G-protein coupled receptor kinases (GRKs)

Agonist-induced β2AR phosphorylation was found to occur in cells lacking cyclic adenosine monophosphate (cAMP) dependent protein kinases (Strasser, Sibley et al. 1986). The enzyme responsible was purified and named beta-adrenergic receptor kinase (Benovic, Strasser et al. 1986; Benovic, Mayor et al. 1987). It was not until later that the 'beta-adrenergic receptor kinase' was named 'G-protein coupled receptor kinase 2' (GRK2). Identification of the remaining GRK family members followed the cloning of GRK2 cDNA (Benovic, DeBlasi et al. 1989) and rhodopsin kinase, also known as GRK1, (Lorenz, Inglese et al. 1991). Subsequently, all other mammalian GRKs were identified via cloning (Benovic, Onorato et al. 1991; Kunapuli and Benovic 1993; Premont, Koch et al. 1994), and subdivided into three main groups, based on sequence homology: **1)** Rhodopsin kinase, or the visual GRK subfamily (GRK1 and GRK7); **2)** The β-adrenergic receptor kinase subfamily (GRK2 and GRK3); **3)** The GRK4 subfamily (GRK4, GRK5 and GRK6).

The current models of GRK function suggest that following activation by an agonist, GPCRs are phosphorylated exclusively at serine and threonine residues. This results in a decrease in the affinity of GPCRs for G-proteins and an increase in their affinity for arrestins (Fig.2).

Arrestin structure and function

Arrestins are proteins ubiquitously expressed in cells and tissues, and function in the desensitization of most GPCRs. Non-visual arrestins (arrestin-2 and arrestin-

3) were discovered in the late 1980s and early 1990s, from the observation that addition of visual arrestin enhances phosphorylation-dependent reduction of G protein activation by β2AR (Lohse, Benovic et al. 1990). This event suggested that proteins homologous to visual arrestin could exist in non-visual systems. Molecular cloning confirmed the existence of two arrestin isoforms: beta arrestin-1 (arrestin-2) and beta arrestin-2 (arrestin-3)(Attramadal, Arriza et al. 1992; Sterne-Marr, Gurevich et al. 1993). The four mammalian arrestins are highly homologous, with major differences found in the C-termini, where the visual arrestins appear to lack the clathrin binding site. Both arrestin-2 or arrestin-3 knockout mice possess phenotypes comparable to their wild type littermates (Bohn, Lefkowitz et al. 1999), but are deficient in blood pressure regulation (Conner, Mathier et al. 1997). On the other hand, the arrestin-2/3 double-knockout was reported to be embryonic lethal (Kohout, Lin et al. 2001). These data suggest that non-visual arrestins can functionally substitute for one another.

The structures of arrestin-2 and arrestin-3 have been solved by X-ray crystallography (Zhan, Gimenez et al. ; Han, Gurevich et al. 2001). Three-dimensional structures show that arrestins consist of two groups of beta-sheets, referred to as the N-domain and the C-domains, connected by the inter-domain hinge and the C-tail (Figure 3).

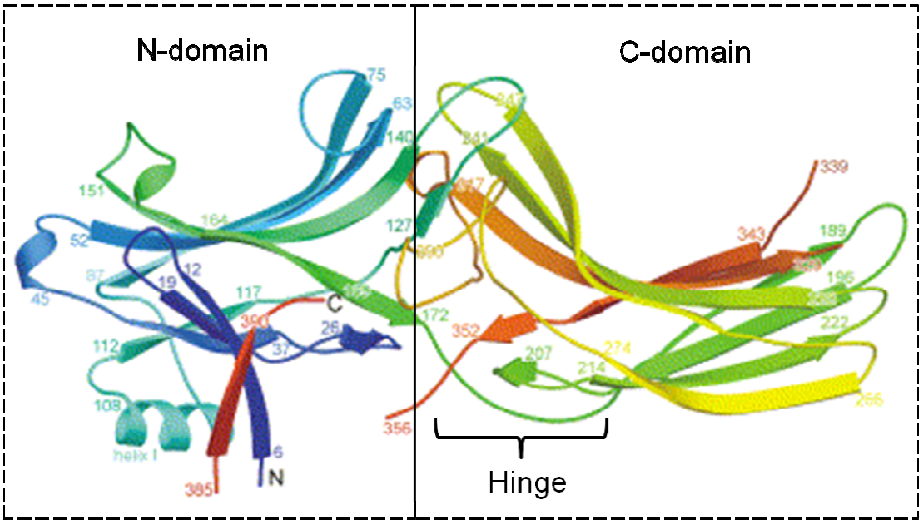

Figure 3. Secondary structure of Arrestin-2

Structural diagram of arrestin-2 basal conformation, showing that arrestins are made up of mainly beta-sheets and one short alpha-helix. N-domain and C-domain are connected by a hinge, as shown. Adapted from (Han, Gurevich et al. 2001)

Localization of receptor binding elements on the concave sides of both domains of arrestin suggests that their movement towards each other, bringing all elements in close proximity with the receptor, is a necessary conformational rearrangement required for arrestin-receptor binding. Movement of arrestin domains toward each other is limited by the length of the inter-domain connector referred to as the "hinge" (Fig.3) (Vishnivetskiy, Hirsch et al. 2002). This hinge is 12 residues long and increasing deletions have been shown to progressively reduce the ability of arrestin binding to the receptor.

Upon binding to the receptor, the C-tail of arrestin is released upon destabilization of the three-element interacting region (Fig. 4A) and the polar core (Fig. 4B). The three-element interaction is a group of three hydrophobic residues (Val11, Ile12, and Phe13) which interact with α-helix I and arrestin C-tail (Hirsch, Schubert et al. 1999). Phosphorylation-independent mutants result from the disruption of this three-element interaction (Gurevich 1998; Vishnivetskiy, Schubert et al. 2000). Extensive mutagenesis studies have been performed on all five residues (Arg175, Asp30, Asp296, Asp303 and Arg382) in the "polar core" and have shown that Asp296 is the most important negatively charged partner of Arg175 (Vishnivetskiy, Paz et al. 1999). Also, the charge reversal of Arg175 (to glutamate) has been shown to confer the ability of arrestin to bind non-phosphorylated GPCRs (Vishnivetskiy, Paz et al. 1999).

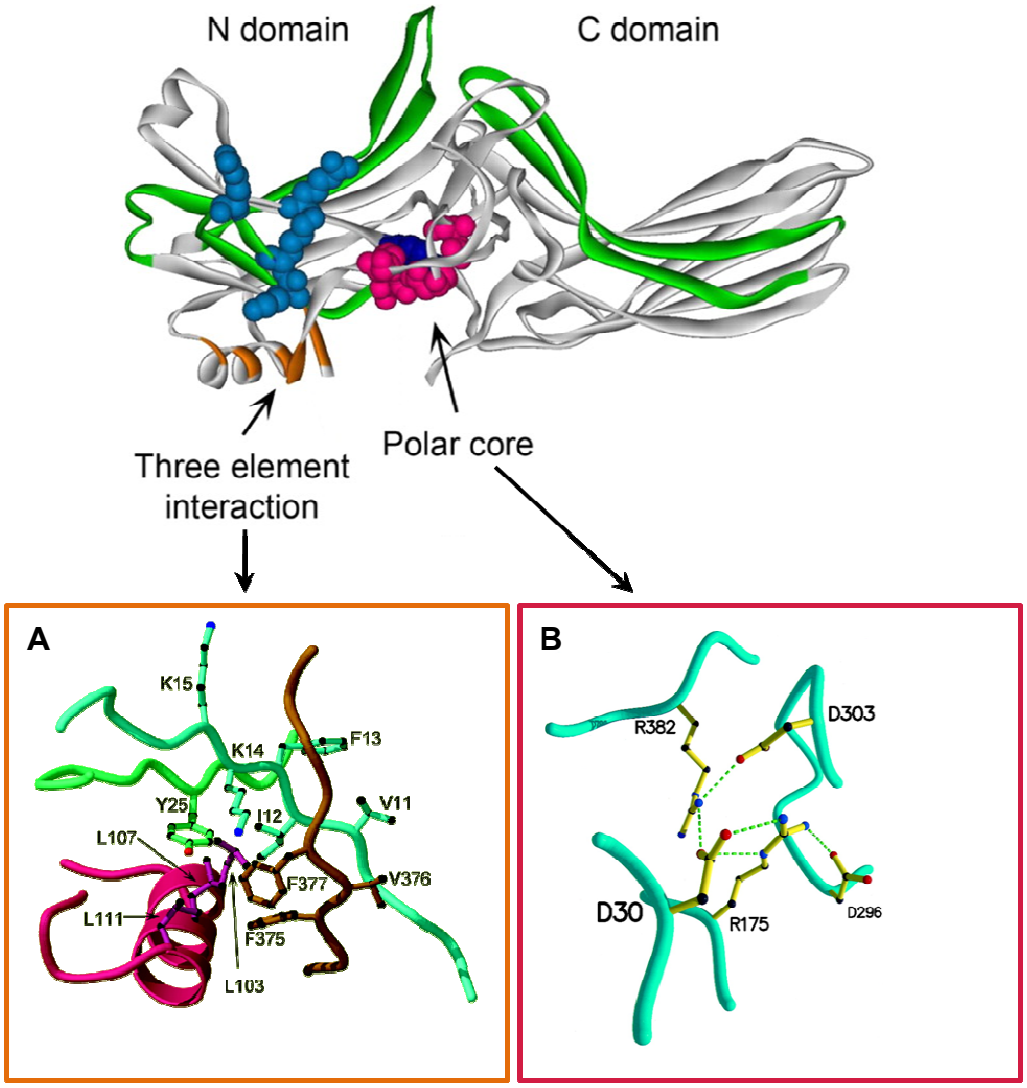

Figure 4. Arrestin polar core and three element interaction

A. Hydrophobic three-element interaction with residues 375–377 (Phe-Val-Phe) of the C-tail and leucines 103, 107, and 111 of the α-helix I. **B.** hydrogen bonds between Arg175, Asp 30, Asp296, Asp303 and Arg382 stabilize the polar core as shown. Adapted from (Vishnivetskiy, Paz et al. 1999; Vishnivetskiy, Schubert et al. 2000; Hanson and Gurevich 2006)

Taken together, these studies suggest that the binding of arrestin to the phosphorylated receptor results in the disruption of the Arg175-Asp296 interaction, destabilization of the three-element interaction and thus results in an increase in the affinity of arrestin binding to the receptor. Subsequently, the release of the arrestin C-terminal tail allows for the interaction of arrestin with the accessory proteins clathrin and adaptor protein 2 (AP-2), ultimately driving internalization of GPCR/arrestin complexes via clathrin coated pits (Goodman, Krupnick et al. 1996; Laporte, Oakley et al. 1999; van Koppen 2001).

Finally, following internalization, the receptor/arrestin complex has one of two fates: 1) degradation or 2) recycling. The GPCR/arrestin complex has been shown to scaffold MAPK signaling cascades including the extracellular signal-regulated kinase 1/2 (ERK1/2) (Luttrell, Roudabush et al. 2001), c-jun N-terminal kinase 3 (JNK3) (McDonald, Chow et al. 2000) and p38 (Bruchas, Macey et al. 2006).

Mitogen Activated Protein Kinases (MAPKs) overview

MAPKs are serine/threonine specific protein kinases which are evolutionary conserved among eukaryotes and play an important role in the transduction of signals from the extracellular environment. Five distinct MAPK families have been identified in mammals (Schaeffer and Weber 1999), and the kinases that have been widely studied and characterized include: a) ERK1/2, b) JNK and c) p38. The c-jun N-terminal kinases, also known as stress-activated protein kinases (SAPKs), and p38 MAPKs are primarily activated by various

stress stimuli, including heat shock, cytokines and ultraviolet (UV) light, and are key to cell differentiation and apoptosis. ERK1/2 proteins have been extensively studied and are the major transducers of growth factor stimulation. ERK1/2 are both known to regulate cell proliferation and cell differentiation. MAPK modules are composed of three kinases: a MAPKKK, responsible for the phosphorylation and activation of a MAPKK, which, in turn, phosphorylates and activates a MAPK. The ERK1/2 cascade is composed of c-Raf1, MEK1 and ERK1/2, where ERK is a MAPK, MEK (MAPK/ERK kinase) is a MAPKK and Raf is a MAPKKK. Once activated c-Raf1 phosphorylates and activates the dual specificity kinase MEK. The interaction between c-Raf1 and MEK is dependent on a proline-rich sequence in MEK (Catling, Schaeffer et al. 1995). While A-Raf has been shown to activate only MEK1 (Wu, Noh et al. 1996), c-Raf1 can activate both isoforms of MEK, MEK1 and MEK2, which, are both capable of phosphorylating tyrosine and threonine residues on ERK1 and ERK2. The serine/threonine kinases ERK1/2, once activated, can either translocate to the nucleus and activate transcription factors via phosphorylation, (Schaeffer and Weber 1999) including Elk-1 and SAP1, or remain in the cytoplasm and phosphorylate other substrates. One common feature of all MAPK modules is that they need to be organized by scaffolding proteins in order to: allow the precise regulation of their signaling; prevent their activation by irrelevant stimuli; and provide spatial and temporal control of signaling.

Arrestin-dependent signaling

The first indication that arrestins could function as signaling adapters arose from studies of receptor internalization defective systems (Luttrell, Ferguson et al. 1999) and arrestin-dependent c-Src recruitment to GPCRs (Luttrell, Ferguson et al. 1999; DeFea, Vaughn et al. 2000; Miller, Maudsley et al. 2000). Mutations of the regions in arrestin that bind SH3 domains (Milano, Pace et al. 2002) were reported to: decrease c-Src binding upon receptor activation; and decrease ERK1/2 phosphorylation; suggesting that arrestin binding to c-Src is critical for the activation of ERK1/2. Recent studies have shown that ERK1/2 activation via stimulation of the β2-AR or the AT_1AR receptor exhibited two individual phases (Shenoy and Lefkowitz 2005; Shenoy, Drake et al. 2006); an early, G-protein dependent phase peaking at around 5 minutes, and a later phase (20min) which was thought to be arrestin dependent. To demonstrate that the second phase was arrestin dependent, the authors inhibited G protein activation using pertussis toxin and they observed a decrease in the early activation phase of ERK1/2. Upon knockdown of arrestins using siRNA, the second phase was found to be decreased.

The activation of the ERK1/2 cascade by G-proteins is thought to induce the translocation of ERK1/2 to the nucleus and initiate transcription of a variety of transcription factors (Tohgo, Pierce et al. 2002). In contrast, when ERK1/2 are activated in an arrestin-dependent manner, ERK1/2 accumulate in the cytosol, where they can activate different effectors and possibly produce different physiological effects than those achieved by G proteins. Finally, the arrestin-2 C-

terminal serine 412 is known to be constitutively phosphorylated by ERK2. Phosphorylation of this residue has been reported to decrease the affinity of arrestin for clathrin and thereby inhibit receptor internalization (Lin, Miller et al. 1999). The biological function of arrestins, in terms of signal transduction, are likely much broader than we currently know. Here, we have explored the mode of interactions of arrestins and MAPK as well as the mechanisms by which these kinases are ultimately activated. Arrestin signaling represents a potentially new therapeutic target for diseases, and should therefore be further elucidated.

CHAPTER II

ARRANGEMENT OF MAPKs ON NON-VISUAL ARRESTINS.

INTRODUCTION

Arrestins are a family of proteins that serve as regulators of G-protein coupled receptors (GPCRs) signaling. Recently it has been proposed that GPCRs coupled to arrestins activate signaling pathways that are G-protein independent. For instance, the activation of the mitogen activated protein kinases (MAPKs) such as extracellular signal-regulated kinase ERK1/2, c-Jun N-terminal kinase 3 (JNK3) and p38 as well as Src has been shown to be arrestin dependent (Luttrell and Lefkowitz 2002; Perry and Lefkowitz 2002; Sun, Cheng et al. 2002). These kinase cascades have been shown to form complexes with both arrestins and GPCRs (DeFea, Zalevsky et al. 2000; Miller and Lefkowitz 2001; Hall and Lefkowitz 2002). The ERK1/2 signaling pathway consists of three kinases: a MAPKKK (RAF), which phosphorylates a MAPKK (MEK) which will sequentially phosphorylate a MAPK (ERK1/2) (Macdonald, Crews et al. 1993). The JNK3 pathway also works on sequential phosphorylation of a MAPKKK (ASK), a MAPKK (MKK4) and a MAPK (JNK3) (Ichijo 1999). ERK1/2 activation occurs on both a tyrosine and a threonine residue (Boulton, Nye et al. 1991), within the activation loop, by upstream kinases (MEK1/2). ERKs activation by GPCR can be a Ras-dependent event or, depending on the receptor type, can be mediated by PKC, some tyrosine kinases (i.e. Src) or by direct interaction with arrestins.

ERK1/2 activation mediated by arrestin will likely lead to different physiological consequences than those achieved by G protein activation. ERK1/2 activation by G proteins leads to the accumulation of these kinases (ERKs) in the nucleus, where they can phosphorylate and activate various transcription factors (Pierce, Luttrell et al. 2001). In contrast, when ERK activation is promoted by arrestins, ERK is found mainly in cytoplasmic compartments (Tohgo, Pierce et al. 2002) of the cell where it may phosphorylate non-nuclear substrates. Several GPCRs have been shown to activate ERKs via their interactions with arrestins. Examples include the angiotensin type 1A receptor ($AT_{1A}R$) (Luttrell, Roudabush et al. 2001) and the protease-activated receptor-2 (PAR-2) (DeFea, Zalevsky et al. 2000). In general, MAPK activation serves to control many cellular functions including cell proliferation, differentiation and apoptosis and can be activated by many extracellular signals such as growth factors and hormones. In order to determine the interactions of arrestin2 and arrestin3 and their individual domains with the components of the c-Raf-1-MEK1-ERK2 cascade we utilized co-immunoprecipitation and found that both arrestin domains interact with c-Raf-1, MEK1 and ERK2.

METHODS

Cell Culture and Transient Transfection

COS-7 African green monkey cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum plus penicillin and streptomycin at 37 °C in a humidified incubator with 5%

CO_2. The cells were plated at 80-90% confluence and transfected with the indicated plasmids using Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA), according to the manufacturers protocol (3 μL of Lipofectamine™ 2000 per 1 μg of DNA). 24 hours post-transfection, cells were serum-starved and lysed with lysis buffer (50mM Tris-HCl pH 7.5, 2mM EDTA, 250mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1mM NaVO3, 10mM N-ethylmaleimide, benzamidine and phenylmethylsulfonylfluoride) on ice for 20 minutes. Cell debris were pelleted by centrifugation for 10 minuntes at 10,000 x g. Arrestins and MAPKs were then immunoprecipitated as described below.

Immunoprecipitation

In experiments involving ERK2, prior to lysis the cells were treated with 1 mM cross-linking reagent dithiobis (succinimidyl propionate) (DSP; Pierce) for 30 min followed by 2 mM Tris-HCl, pH 7.5, for 15 min at room temperature. After centrifugation, supernatants were precleared by 30 μl of protein G-agarose. Then, 600 μl of supernatant was incubated with primary antibodies for 2 hours followed by the addition of 12 μl of protein-G agarose beads for 2 hours or overnight. The beads were washed three times with 1 ml of lysis buffer, and the proteins were eluted with 50 μl of sample buffer, boiled for 5 min, and analyzed by Western blot.

Western Blot

The proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Mouse monoclonal antibodies against FLAG (Sigma), HA (Sigma), GFP (Clontech), and phospho-JNK (Cell Signaling Technology Inc.) were used at 1:1000 or 1:2000 dilution followed by horseradish peroxidase-conjugated anti-mouse secondary antibody. Protein bands were detected by enhanced chemiluminescence (ECL, Pierce) followed by exposure to x-ray film. Immunoblots were quantified using QuantityOne software (Bio-Rad Laboratories).

Statistical Analysis

Quantitative data from at least three experiments were analyzed by one-way analysis of variance with arrestin as a main factor (with Bonferroni-Dunn correction for multiple comparisons).

RESULTS

The Molecular Interactions Mediating Arrestin-dependent Assembly of the ERK Signaling Module

To determine the orientation of c-Raf-1, MEK1, and ERK2 on non-visual arrestins, we co-expressed these kinases with arrestin2, arrestin3, and their separated N- and C- terminal domains in COS-7 cells. We tested the ability of arrestins to co-immunoprecipitate with the kinases, as well as the ability of c-

Raf1, MEK1, and ERK2 to co-immunoprecipitate arrestins and their separately expressed domains (Fig. 5). MEK1 (Fig. 5B) and cRaf-1 (Fig. 5C) were readily detectable in complex with both full-length arrestins. Both kinases demonstrated comparable binding to arrestin2, arrestin3, and individual domains. In contrast, the affinity of the ERK2-arrestin interaction appears to be lower than that of the upstream kinases, therefore cross-linking was required for reliable detection of the arrestin-ERK2 interaction (Fig. 5A). ERK2 also interacts comparably with both nonvisual arrestins and their N- and C- terminal domains (Fig. 5A). Collectively, our data demonstrate that each of the three kinases, cRaf-1, MEK1, and ERK2, binds equally well to both domains of arrestins.

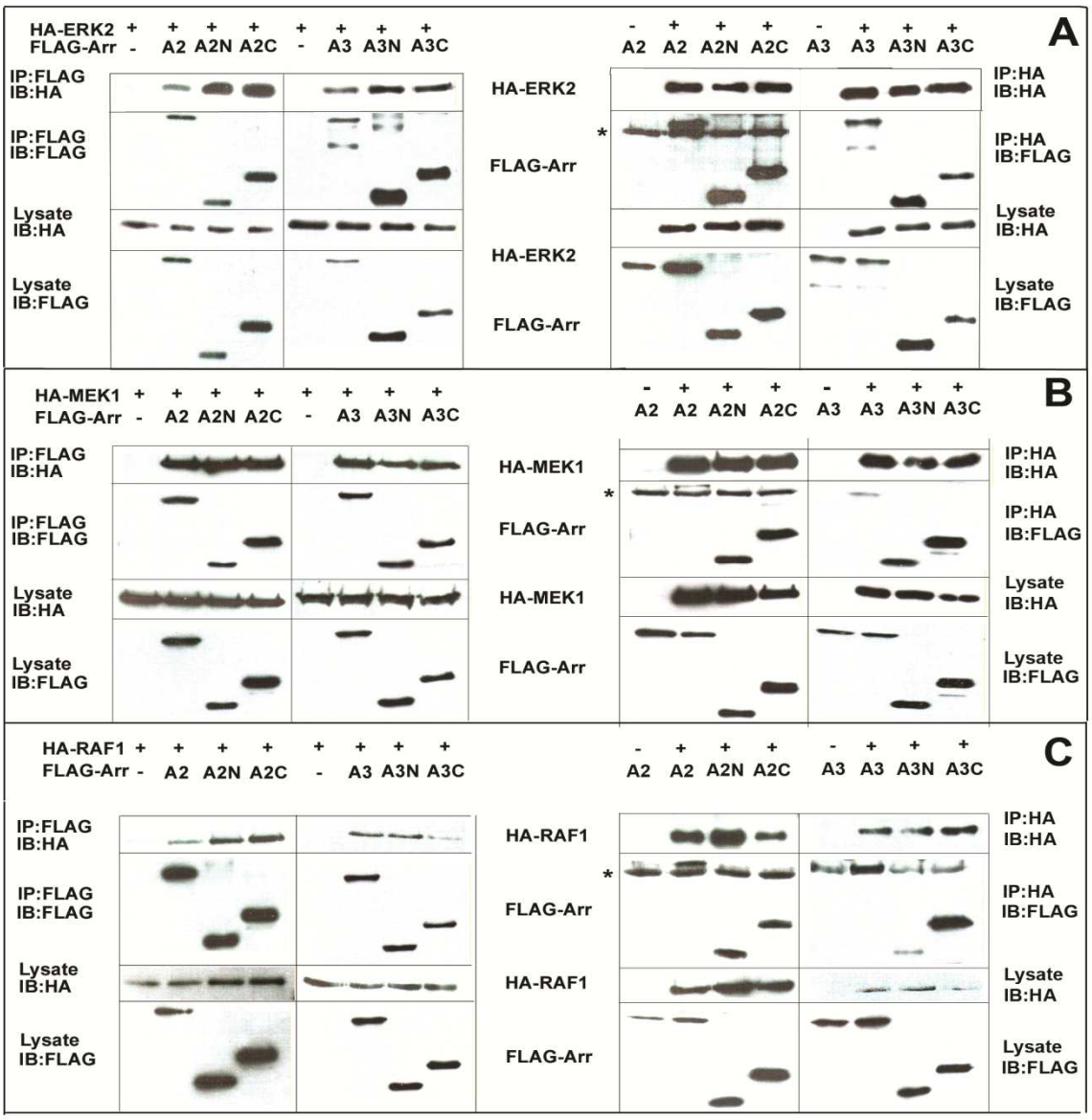

Figure 5. ERK2, MEK1 and cRaf-1 interact with both domains of arrestin2 and arrestin3. COS-7 cells were transfected with plasmids encoding FLAG-tagged arrestin2 (A2), arrestin2 N-domain (A2N), arrestin2 C-domain (A2C), arrestin3 (A3), arrestin3 N-domain (A3N), or arrestin3 C-domain (A3C). HA-tagged ERK2, MEK1, and c-Raf1 were co-expressed with indicated FLAG-tagged arrestins. Arrestins were immunoprecipitated with M2 anti-FLAG antibody, and precipitates were probed for ERK2-HA (*A*), MEK1-HA (*B*) or c-Raf-1-HA (*C*). Alternatively, individually expressed kinases were immunoprecipitated with anti-HA antibody, and the precipitates were probed for arrestins using anti-FLAG antibody (*A*-*C*). The relative expression of each protein was confirmed by immunoblotting cell lysates (shown in the two lower blots in each panel). *Asterisks*, nonspecific band (heavy chain of the antibody).

DISCUSSION

Arrestins have been reported to serve as GPCR activation-dependent scaffolds (Shenoy and Lefkowitz 2003), but the molecular organization of the arrestin-MAPKs complex has not been elucidated. Therefore, we decided to investigate the assembly of the c-Raf-1-MEK1-ERK1/2 signaling cascade on non-visual arrestin proteins. Interestingly, we found that both arrestin domains interact with c-Raf-1, MEK1 and ERK2, but contrary to the ASK1-MKK4-JNK3 cascade (Song, Coffa et al. 2009), where the binding of each kinase is robust, in the c-Raf1-MEK1-ERK2 cascade c-Raf1 and MEK1 demonstrate strong binding, whereas arrestins interaction with ERK2 is very weak (Fig. 5). The subtle differences observed between the two cascades suggest that, JNK3 is more likely to remain in the complex and to phosphorylate cytoplasmic substrates than ERK2 when arrestin is not bound to a receptor. Based on our data, we propose a model of arrestin-dependent assembly of the MAPK signaling module, in which, arrestins bind all three kinases, assembling c-Raf1, MEK1, and ERK2 along its short axis, with each kinase directly interacting with both domains of arrestin (Fig. 6). The members of this MAPK cascade assemble on the arrestin molecule similarly to the components of the ASK1-MKK4-JNK3 signaling module (Song, Coffa et al. 2009). Therefore, this appears to be a universal mode of assembly of the three kinases on arrestin scaffolds.

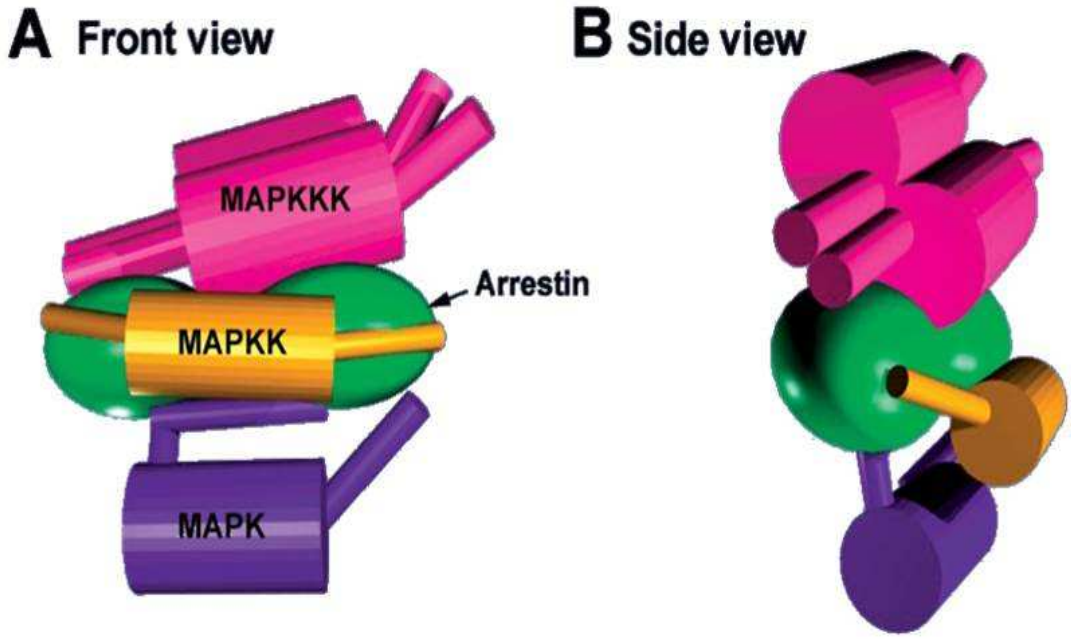

Figure 6. The model of MAPK signaling module organized by arrestin.
The three-dimensional model representing how arrestins assemble MAP kinases is shown as front (Fig.6A) and side views (Fig.6B). Arrestin (purple) is shown as an elongated two-domain molecule. All three kinases are shown to interact directly with both arrestin domains. From (Song, Coffa et al. 2009).

CHAPTER III

A SINGLE MUTATION IN ARRESTIN-2 PREVENTS ERK1/2 ACTIVATION BY REDUCING C-RAF1 BINDING.

INTRODUCTION

Arrestins regulate the signaling and trafficking of G protein-coupled receptors (GPCRs). GPCRs complex both non-visual arrestins, channeling signaling to G protein-independent pathways, one of which is the activation of extracellular signal regulated kinase 1/2 (ERK1/2).

The first indication that arrestins function as signaling adapters came from the studies of arrestin-dependent c-Src recruitment to GPCRs, which resulted in the activation of ERK1/2 (Luttrell, Ferguson et al. 1999; DeFea, Vaughn et al. 2000; Miller, Maudsley et al. 2000). Subsequently, arrestin2 and arrestin3 were shown to scaffold the JNK3 (McDonald, Chow et al. 2000), ERK1/2 (DeFea, Zalevsky et al. 2000; Luttrell, Roudabush et al. 2001), and p38 (Sun, Cheng et al. 2002; Bruchas, Macey et al. 2006) cascades. Despite the fact that arrestins play important roles in regulating MAPK pathways, the mechanism by which arrestins assemble MAP kinases into a signaling complex is not clear. Arrestins facilitate the activation of JNK3 (McDonald, Chow et al. 2000), ERK1/2 (Luttrell, Roudabush et al. 2001), and p38 (Bruchas, Macey et al. 2006). Free and receptor-bound arrestins differentially interact with MAPKs and other signaling proteins (McDonald, Chow et al. 2000; Luttrell, Roudabush et al. 2001; Miller,

McDonald et al. 2001; Bruchas, Macey et al. 2006; Song, Raman et al. 2006). Here we focused on the c-Raf1-MEK1-ERK1/2 cascade, in which all three kinases bind arrestin-2 and arrestin-3 (Song, Coffa et al. 2009). ERK1/2 phosphorylation is facilitated by both non-visual arrestins and is contingent on GPCR activation (Luttrell, Roudabush et al. 2001). These data suggest that: a) the elements conserved between arrestin-2 and -3 play key roles; and b) the well-defined surface occupied by bound receptor (Ohguro, Palczewski et al. 1994; Pulvermuller, Schroder et al. 2000; Vishnivetskiy, Hosey et al. 2004; Hanson, Francis et al. 2006; Hanson and Gurevich 2006; Vishnivetskiy, Gimenez et al. 2011; Zhan, Gimenez et al. 2011) is not involved in the interactions with ERK or upstream kinases. Therefore, we decided to perform alanine-scanning mutagenesis of the conserved residues on the non-receptor-binding surface of arrestin-2 and to compare the ability of wild type (WT) and mutant arrestin-2 to bind receptor, c-Raf1, MEK1, ERK2, and promote ERK1/2 phosphorylation in COS-7 and arrestin double knock out (DKO) mouse embryonic fibroblast (MEF) cells.

We found that the Arg307Ala mutation significantly reduced arrestin-2 binding to c-Raf1, whereas the binding of the mutant arrestin to active phosphorylated receptor and downstream kinases MEK1 and ERK2 was not affected. In contrast to wild type arrestin-2, the Arg307Ala mutant failed to rescue arrestin-dependent ERK1/2 activation via β2-adrenergic receptor in arrestin-2/3 double knockout mouse embryonic fibroblasts. Thus, Arg307 appears to play a specific role in the ability of arrestin-2 to bind c-Raf1, and is indispensable in the

productive scaffolding of c-Raf1-MEK1-ERK1/2 signaling cascade. Arg307Ala mutation specifically eliminates arrestin-2 signaling through ERK, which makes arrestin-2-Arg307Ala the first signaling-biased arrestin mutant constructed. The crystal structure reveals that the Lys308 residue of the side chain of homologous arrestin-3 points in a different direction. Alanine substitution of Lys308 does not significantly affect c-Raf1 binding to arrestin-3 and its ability to promote ERK1/2 activation, suggesting that the two non-visual arrestins perform the same function via distinct molecular mechanisms.

METHODS

Site-directed mutagenesis

Site-directed mutagenesis by PCR (Supplemental Table S1) was performed using pGEM2-based transcription vectors encoding WT bovine arrestin-2 and arrestin-3 with unique restriction sites engineered and previously described (Vishnivetskiy, Hosey et al. 2004; Vishnivetskiy, Gimenez et al. 2011). All constructs were verified by dideoxy sequencing. The coding sequences were excised with EcoR I and Hind III and subcloned into pcDNA3 for expression in cultured mammalian cells and into pFB vector for retrovirus production.

In vitro transcription, translation, and evaluation of protein stability

Plasmids were linearized using a unique Hind III site downstream of the coding sequence. *In vitro* transcription and translation were performed as previously described (Gurevich and Benovic 1992; Gurevich and Benovic 1993).

All arrestin proteins were labeled by incorporation of [^{3}H]leucine and [^{14}C]leucine with a specific activity of the mix of 1.5-3 Ci/mmol, resulting in the specific activity of arrestin proteins within the range of 66-85 Ci/mmol (150-230 dpm/fmol). The translation of every mutant used in this study produced a singly labeled protein band with the expected mobility on SDS-PAGE. Two parameters were used for the assessment of mutant relative stability, as described (Gurevich 1998): its yield multiplied by the percentage of the protein remaining in the supernatant after incubation for 10 minutes at 37^{o}C followed by centrifugation (Supplemental Table S2).

Receptor binding assay

The binding to light-activated phosphorylated rhodopsin (P-Rh*) was performed, as previously described (Gurevich, Dion et al. 1995). Briefly, translated radiolabeled arrestins (50 fmol) were incubated in 50 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 1.5 mM dithiothreitol, 1 mM EGTA, 50 mM potassium acetate with 7.5 pmol (0.3 μg) of P-Rh* in a final volume of 50 μl for 5 min at 37^{o}C in room light, and then cooled on ice. Bound and free arrestins were separated by size-exclusion chromatography on 2-ml columns of Sepharose 2B-CL equilibrated with 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, at 4°C. Rhodopsin-bound arrestins (eluted with receptor-containing membranes in the void volume between 0.5 and 1.1 ml) were quantified by liquid scintillation counting.

Co-immunoprecipitation and Western blotting

Monkey kidney COS-7 cells were transfected with the indicated plasmids using Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA), according to the manufacturers protocol (3 μL of Lipofectamine™ 2000 per 1 μg of DNA). 24 hours post-transfection, cells were serum-starved and lysed with lysis buffer (50mM Tris, 2mM EDTA, 250mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1mM NaVO3, 10mM N-ethylmaleimide, benzamidine and phenylmethylsulfonylfluoride) on ice for 20 minutes. Cell debris were pelleted by centrifugation for 10 minuntes at 10,000 x g. Lysates were precleared with 30 μl of protein G agarose, followed by incubation with a rabbit anti FLAG antibody for 2 hours and by the addition of 30 μl of protein G agarose beads for 2 hours. The beads were then washed 3 times with lysis buffer, and bound proteins were eluted with Laemmli SDS buffer. In experiments involving ERK2, prior to lysis the cells were treated with 1 mM cross-linking reagent dithiobis(succinimidyl propionate) (DSP; Pierce) for 30 minutes followed by 2 mM Tris-HCl, pH 7.5, for 15 minutes at room temperature. The proteins were separated by SDS PAGE (10%) and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Blots were incubated with primary antibodies from Cell Signaling (mouse anti-HA (6E2) mAb #2367, 1:1500; mouse anti-p44/42 ERK1/2 (L34F12) mAb #4696, 1:1000; and mouse anti-p44/42 phospho-ERK1/2 (T202/Y204), (E10) mAb #9106S, 1:1000), or Sigma (mouse anti-FLAG M2, #F3165, 1:1500; rabbit anti-FLAG #F7425), followed by anti-mouse horseradish peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch. Protein bands were visualized by enhanced

chemiluminescence (ECL, Pierce) followed by exposure to X-ray film. The bands were quantified using VersaDoc with QuantityOne software (Bio-Rad Laboratories).

Arrestin-dependent ERK activation

For the production of retroviruses, human embryonic kidney (HEK) 293T cells were transfected using Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA), according to the manufacturer's protocol (3 μL of Lipofectamine™ 2000 per 1 μg of DNA) with the following constructs: pVPack-GP (Stratagene, 217566), pVack-VSV-G (Stratagene, 217567), together with pFB-arrestin-2, pFB-arrestin-2-Arg307Ala, pFB-arrestin-3, pFB arrestin-3-K308A, or pFB-GFP. 24-48 hours post-transfection, media containing the virus produced by HEK293T cells was collected and used to infect arrestin-2/3 double knockout mouse embryonic fibroblasts (MEFs) (a generous gift of Dr. R. J. Lefkowitz, Duke University) (Kohout, Lin et al. 2001). Fresh virus-containing media was used daily for a total of 3 days. Then MEFs were serum starved for 2 hours and subsequently treated with 1 μM ICI118551, a biased ligand of β2-adrenergic receptor (β2AR), which is an inverse agonist of G protein signaling and an agonist of arrestin recruitment (Azzi, Charest et al. 2003), or 10 μM β2AR agonist isoproterenol for 10 minutes at 37^{o}C. MEFs were harvested and lysed in 50mM Tris, 2mM EDTA, 100mM NaCl, 1% Nonidet P-40, supplemented with protease (Roche, 04693124001) and phosphatase (Roche, 04906845001) inhibitors cocktails on ice for 20 minutes.

MATERIALS

[γ-^{32}P]ATP, [^{14}C]leucine, and [^{3}H]leucine were purchased from Perkin-Elmer. All restriction enzymes were purchased from New England Biolabs. Sepharose 2B and all other chemicals were purchased from sources as previously described (Gurevich and Benovic 2000; Vishnivetskiy, Francis et al. 2010). Rabbit reticulocyte lysate was purchased from Ambion, SP6 RNA polymerase was prepared as described (Gurevich 1996). Rhodopsin was phosphorylated and regenerated by 11-*cis*-retinal generously supplied by Dr. R. K. Crouch (Medical University of South Carolina, Charleston, SC), as described (Vishnivetskiy, Raman et al. 2007).

RESULTS

Functional characterization of arrestin-2 mutants by binding to rhodopsin

To identify the arrestin-2 elements involved in the binding of c-Raf1, MEK1, and ERK2, we generated 22 mutants in which conserved residues on the non-receptor-binding surface of both non-visual arrestins were replaced with alanines individually or in groups (Fig. 7, Fig. 8A). Receptor binding is the signature function of arrestin proteins, and can be easily tested using direct binding assays (Gurevich and Benovic 1993; Gurevich, Dion et al. 1995). Both non-visual arrestins demonstrate specific binding to phosphorylated light-activated rhodopsin (P-Rh*) (Gurevich, Dion et al. 1995; Kovoor, Celver et al. 1999; Celver, Vishnivetskiy et al. 2002). Therefore, we used binding to P-Rh* as

the criterion to select proteins that interacted with the receptor normally. To this end, the mutants were expressed in cell-free translation, and their binding to P-Rh* was compared to that of WT arrestin-2 (Fig. 7, Fig. 8B). Twelve mutants showed significantly reduced binding, whereas ten demonstrated normal binding to P-Rh. Proteins showing normal binding were selected for subsequent experiments.

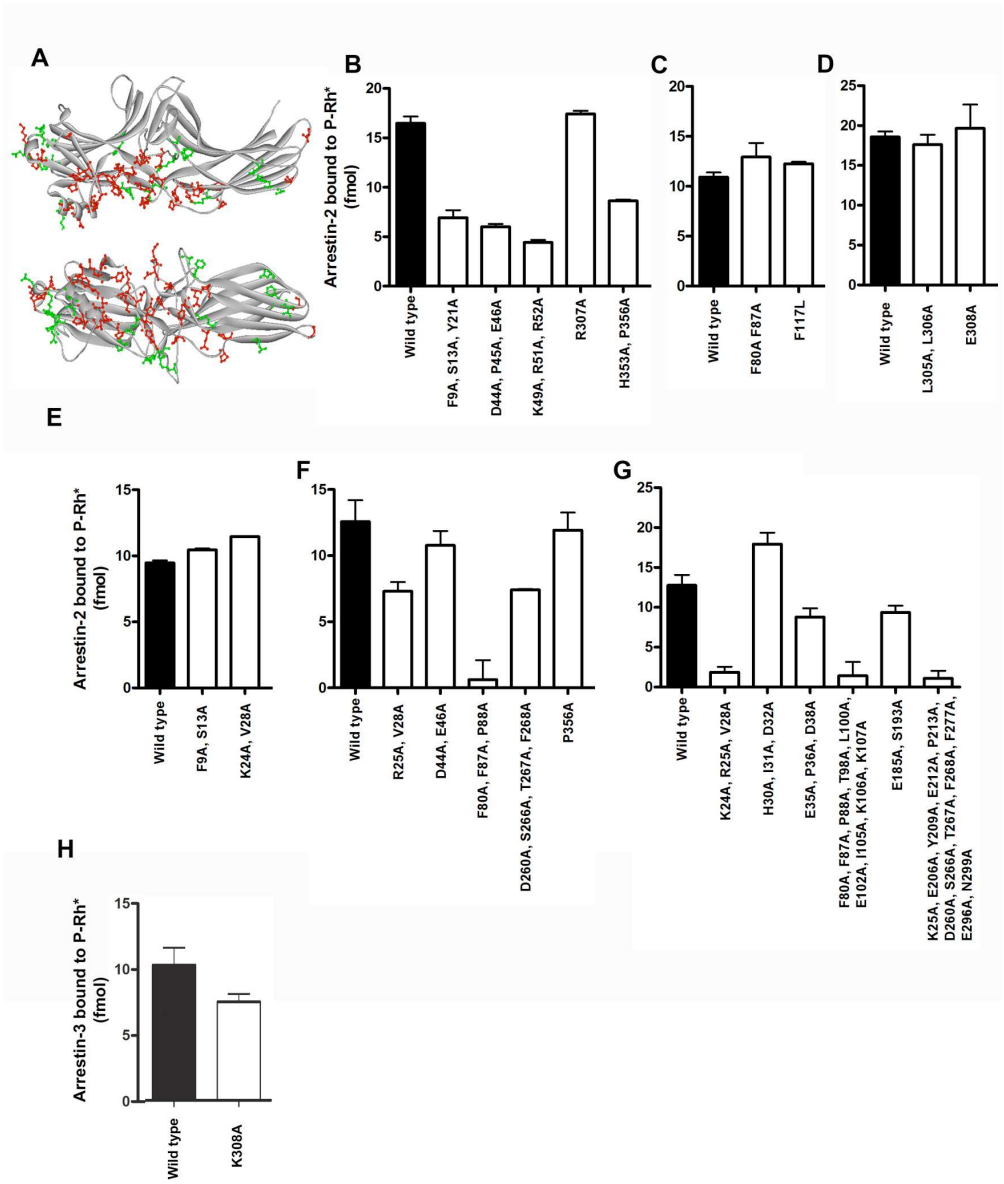

Figure 7. P-Rh* binding of all arrestin-2 mutants.
A. Arrestin-2 structure (upper panel, side view; lower panel, view from concave side of the two domains), with residues mutated in this study highlighted, as follows: identical between arrestin-2 and -3 residues, red; replaced by conservative substitutions, green. **B-H.** All arrestin-2 (**B-G**) and arrestin-3 (**H**) mutants were tested in P-Rh* binding assay. Means + SD of two independent experiments performed in duplicate are shown.

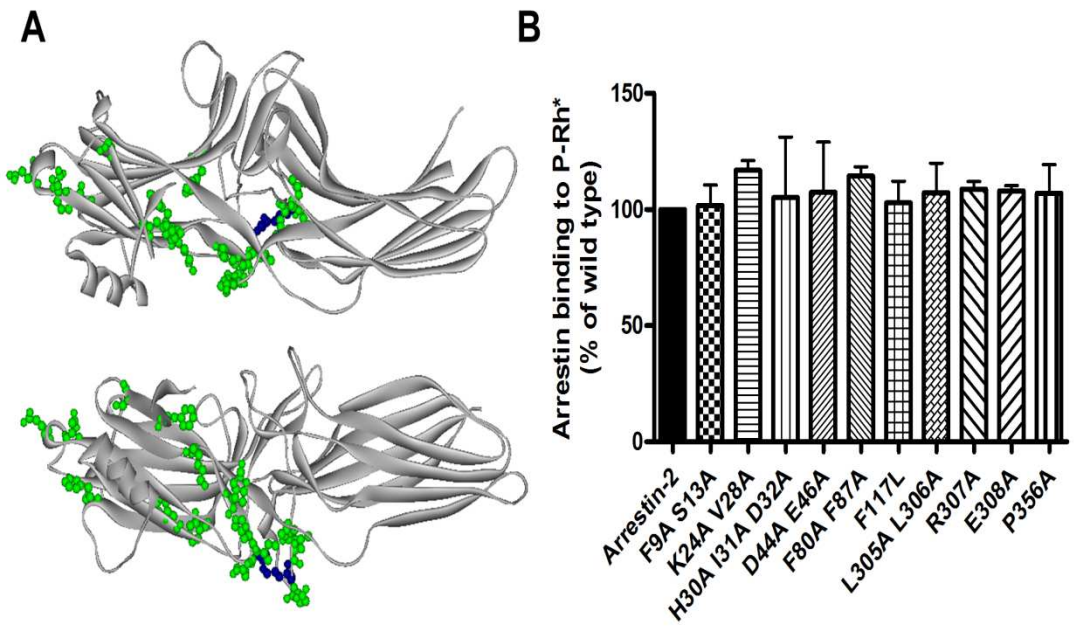

Figure 8. Ten arrestin-2 mutants retain normal receptor binding. A. Arrestin-2 structure (Han, Gurevich et al. 2001) viewed from the side (upper image) or convex surface (lower image). Residues on the non-receptor-binding side, conserved in arrestin-2 and -3 that can be mutated to alanines without affecting receptor binding are shown as green CPK models, Arg307 is shown in blue. **B.** The binding of WT and mutant arrestin-2 to P-Rh*. Means +/- SD of three experiments performed in duplicate are shown. ANOVA with Bonferroni post-hoc test revealed no statistically significant differences between WT arrestin-2 and these mutants.

Identification of a c-Raf1-binding residue on arrestin-2

To compare the binding of WT and mutant arrestin-2 to c-Raf1, MEK1, and ERK2 in the cellular context, we transiently co-expressed Flag-tagged arrestins with HA-tagged kinases in COS-7 cells (chosen because they express very low levels of endogenous arrestins). Arrestins were immunoprecipitated with an anti-Flag antibody, and co-immunoprecipitated kinases were detected by Western blotting using an anti-HA antibody. We found that nine out of the ten mutants bound c-Raf1 essentially as well as WT arrestin-2, whereas the amount of c-Raf1 co-immunoprecipitated with arrestin-2-Arg307Ala was found to be significantly decreased (Figure 9A). Interestingly, none of the mutations tested appeared to affect the binding arrestin-2 to MEK1 (Fig. 9B) or ERK2 (Fig. 9C). Thus, the Arg307Ala mutation selectively reduces the arrestin-2 interaction with c-Raf1, without affecting the binding to the receptor or downstream kinases. Similar to other MAP kinases, c-Raf1 interacts with both domains of arrestin-2 and arrestin-3 (Song, Coffa et al. 2009), which suggests that its binding site includes more than one residue. However, the replacement of multiple residues on the putative kinase-binding surface with alanines reduces receptor binding (Fig. 7). Therefore, their roles in kinase binding and activation could not be assessed by alanine scanning mutagenesis.

Interestingly, an equivalent residue in arrestin-3, which also promotes ERK1/2 activation (Luttrell, Roudabush et al. 2001), is Lys308 (Fig. 10) (Zhan, Gimenez et al. 2011), suggesting that a positive charge in this position could be important.

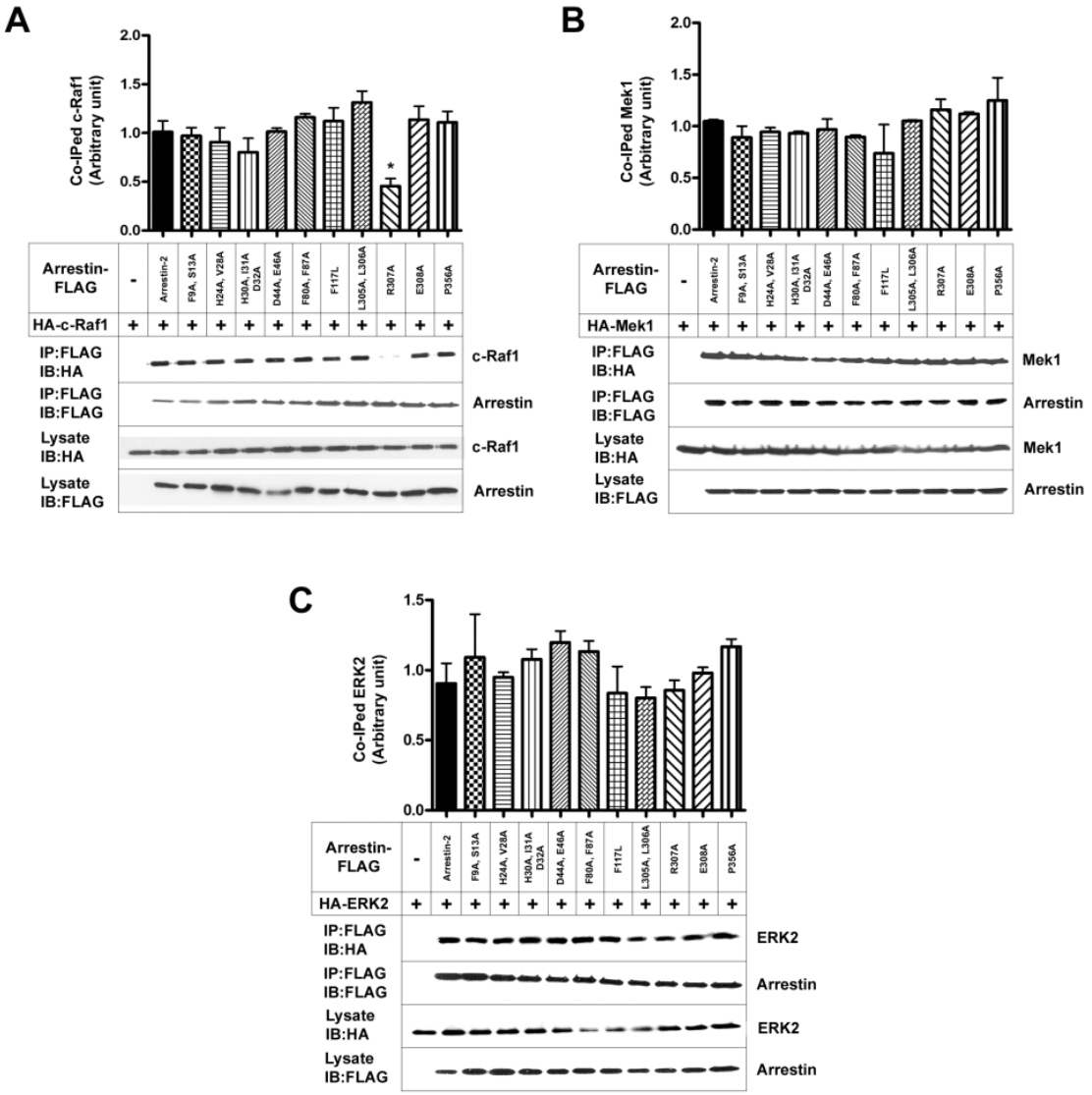

Figure 9. The binding of c-Raf1, MEK1, and ERK2 to WT and mutant arrestin-2. Flag-tagged WT arrestin-2 and indicated mutants were co-expressed with HA-tagged c-Raf1, MEK1, or ERK2 in COS-7 cells. Arrestins were immunoprecipitated with anti-Flag antibody, and co-immounoprecipitated c-Raf1 (**A**), MEK1 (**B**), or ERK2 (**C**) were visualized by Western blot with anti-HA antibody. The binding of all mutants to MEK1 and ERK2 was not different from WT arrestin-2, whereas Arg307Ala mutation significantly decreased the binding to c-Raf1. Means $\pm$ SD of 3-4 independent experiments are shown in bar graphs; representative blots are shown below. * $p<0.05$

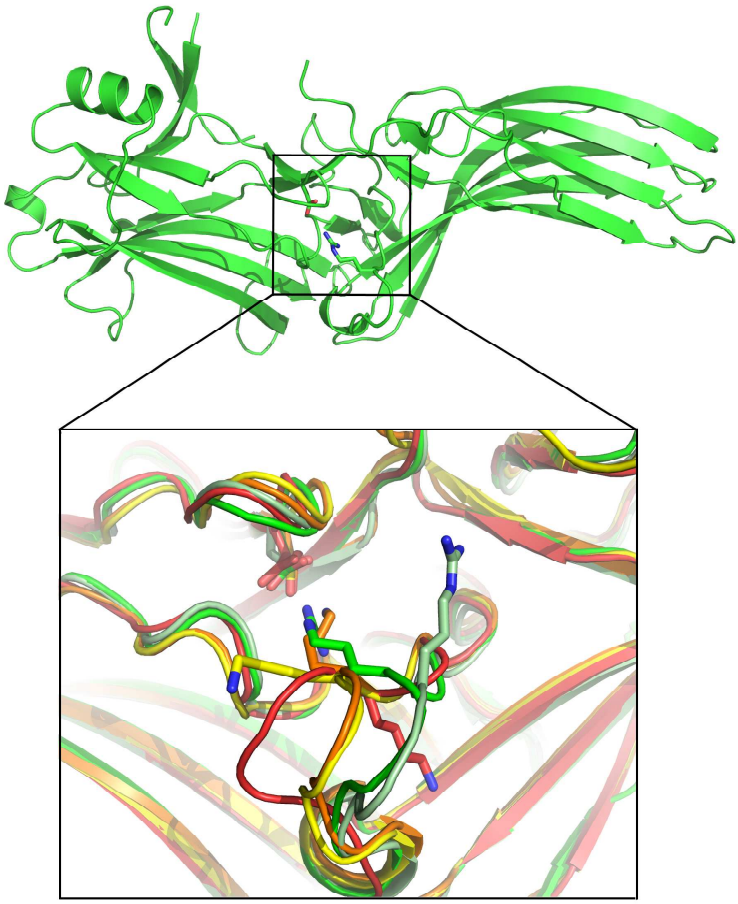

Figure 10. Arginine 307 is a conformationally variable residue. Arg307 can form a long ion pair with Asp29, which bridges the N- and C-domains of arrestin-2. This interaction is likely weak, as evidenced by the lack of its conservation in arrestin-1 (1CF1 shown in yellow), some crystal forms of arrestin-2 (3GDI shown in green and 3JSY shown in light green), arrestin-3 (3P2D shown in red), and arrestin-4 (1AYR shown in orange), and the multiple orientations observed for the loop containing Arg307 in arrestin-2. Note that homologous residue in arrestin-3, Lys308, points in an opposite direction.

Arrestin-dependent ERK1/2 activation induced by ICI118551 via β2AR

Next we assessed whether the reduced c-Raf1 binding of the Arg307Ala mutant affects its ability to promote receptor-dependent ERK1/2 activation. GPCRs activate ERK1/2 via multiple G protein-dependent and independent pathways, one of which involves arrestin scaffolding of the c-Raf1-MEK1-ERK1/2 cascade (Luttrell 2003). Although in some cases the pathways can be distinguished by their time course, with rapid phase of ERK1/2 phosphorylation largely mediated by the G-protein and the slower phase attributable to arrestins (Ahn, Shenoy et al. 2004; Shenoy, Drake et al. 2006). However, in other cases both phases appear to be G protein-dependent (Luo, Busillo et al. 2008). An inverse agonist of β2AR, ICI118551, that blocks G protein activation, was shown to be a biased ligand, acting as an agonist for non-visual arrestins (Azzi, Charest et al. 2003). We confirmed this observation by showing that the robust activation of endogenous ERK1/2 induced by ICI118551 via endogenous β2AR is readily detected in WT MEFs, but completely absent in the arrestin-2/3 double knockout (DKO) MEFs (Fig. 11A). Therefore, we used the ability of arrestin expressed in DKO MEFs to rescue ICI118551-induced ERK1/2 activation as our readout. We found that WT arrestin-2 successfully rescues ERK1/2 activation using ICI118551, whereas the Arg307Ala mutant expressed at the same level failed to do so (Fig. 11B).

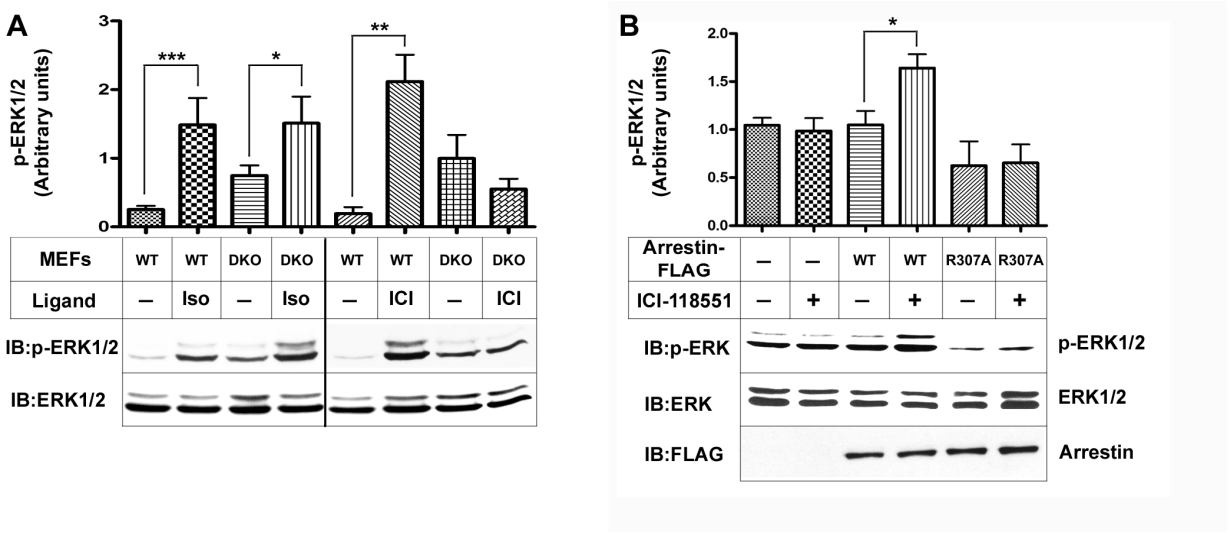

Figure 11. Arg307Ala mutant fails to rescue β2AR-mediated ERK activation in arrestin-2/3 knockout MEFs. A. WT and arrestin-2/3 double knockout (DKO) MEFs were serum-starved and treated with 1 μM ICI118551 (ICI) or 10 μM isoproterenol (ISO) for 10 min at 37°C, then lysed, as described in methods. Cell lysates were analyzed by Western using indicated primary antibodies. **B.** DKO MEFs were infected with retrovirus encoding GFP (control, -), WT arrestin-2 (WT), or arrestin2-Arg307Ala mutant (R307A). 48 hours post-transfection the cells were serum-starved for 2 hours, stimulated with 1 μm ICI118551 for 10 min at 37°C, lysed, and analyzed by Western blot. Means ± SD of 3-4 independent experiments are shown in bar graphs; representative blots are shown below.
* p<0.05, ** p<0.01, *** p<0.001.

Thus, the observed reduction in c-Raf1 binding to the arrestin-2-Arg307Ala mutant (Fig. 8A) translates into a complete loss of the ability to productively scaffold the c-Raf1-MEK1-ERK1/2 signaling cascade, suggesting that Arg307 in arrestin-2 plays a critical role in the binding of c-Raf1 and promoting ERK1/2 activation.

Arrestin-2 and Arrestin-3 scaffold c-Raf1 by different molecular mechanisms

Interestingly, the homologous arrestin-3 residue is a lysine, and Lys308 points in a different direction as suggested by the crystal structure of arrestin-3 (Fig. 10) (Zhan, Gimenez et al. 2011). To test whether this distinct conformation translates into a different role of this residue in the arrestin-3-dependent scaffolding of the c-Raf1-MEK1-ERK1/2 module, we constructed arrestin-3-K308A mutant. We found that this mutation does not significantly affect the ability of arrestin-3 to bind the model receptor, light-activated phosphorhodopsin (Fig. 12A). We then compared the ability of WT arrestin-3 and its K308A mutant to interact with co-expressed c-Raf1 in COS7 cells. We found that both proteins co-immunoprecipitate similar amounts of c-Raf1 (Fig. 12B,C), suggesting that the role of this positively charged residue in arrestin-3 is different. To test whether an equivalent binding of c-Raf1 translates into similar ability of arrestin-3 and K308A mutant to promote the activation of endogenous ERK1/2 in an arrestin-dependent manner, we expressed both proteins in arrestin-2/3 DKO MEFs, and challenged endogenous β2AR with an arrestin-biased agonist ICI118551 (Fig.

13). Our findings confirmed that DKO MEFs do not elicit ERK1/2 activation in response to ICI118551. The expression of WT arrestin-3 and its K308 mutant rescued ERK1/2 response to ICI118551 virtually to the same extent (Fig. 13). Thus, the Lys308 residue in arrestin-3 does not appear to play a critical role in c-Raf1 binding and ERK1/2 phosphorylation (Fig. 12, Fig. 13), in contrast to the homologous Arg307 residue in arrestin-2 (Figs. 9, 11). These data suggest that even though both non-visual arrestins scaffold c-Raf1-MEK1-ERK1/2 cascade, fine molecular mechanisms of their action are distinct.

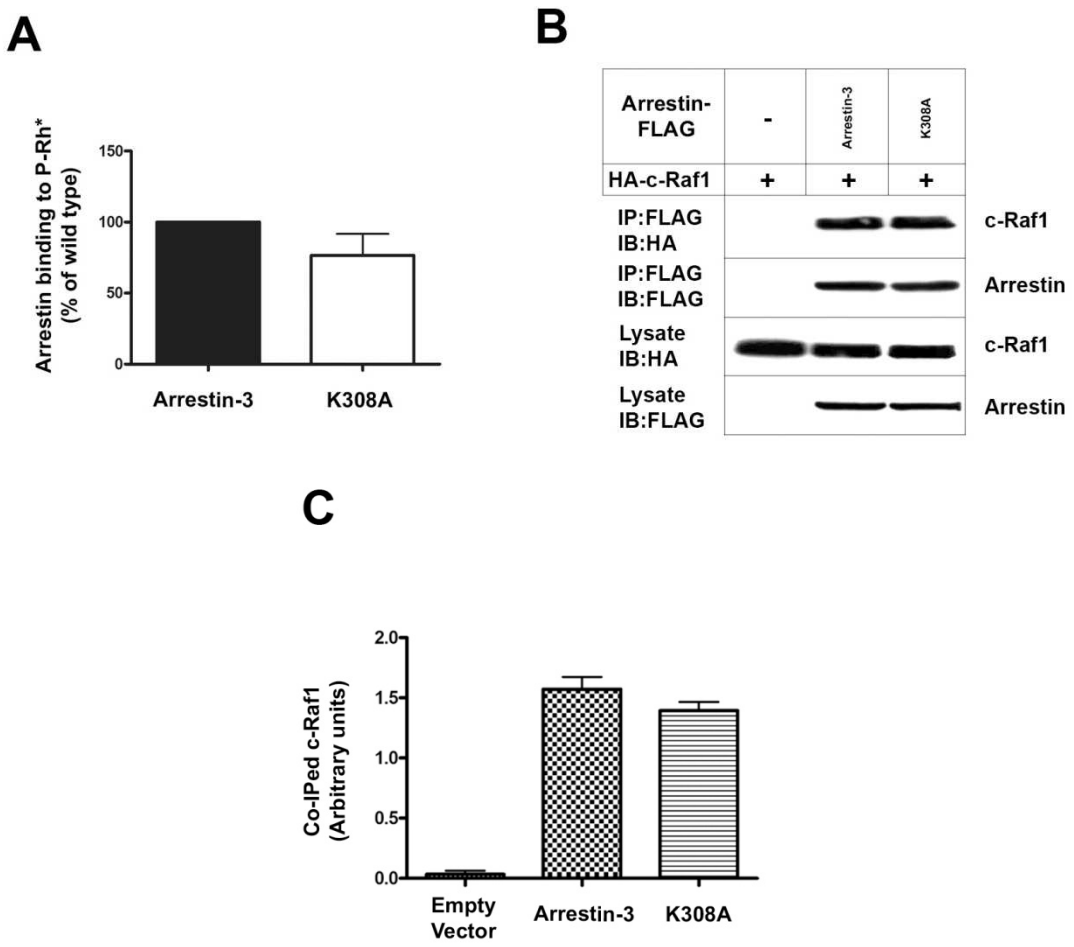

Figure 12. Distinct functional role of homologous positive charge in arrestin-3. A. The binding of WT arrestin-3 and K308A mutant to P-Rh*. Means +/- SD of three experiments performed in duplicate are shown. **B.** Flag-tagged WT arrestin-3 and K308A mutant were co-expressed with HA-tagged c-Raf1 in COS7 cells. Arrestins were immunoprecipitated with anti-Flag antibody, and co-immounoprecipitated c-Raf1 was visualized by Western blot with anti-HA antibody. The results of a representative experiment are shown. **C.** The intensity of c-Raf1 band in the immunoprecipitate was quantified. Means $\pm$ SD of 3 independent experiments are shown.

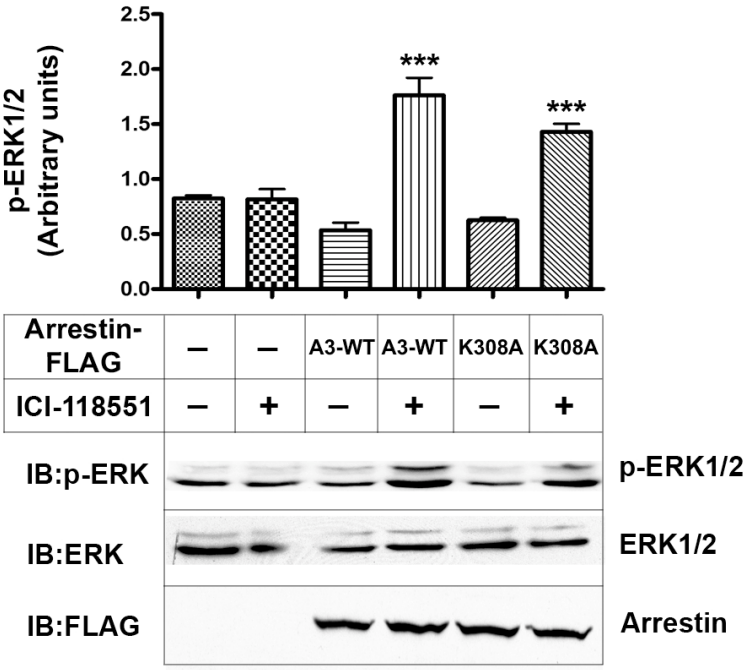

Figure 13. β2AR-mediated ERK activation by arrestin-3 and arrestin-3-Lys308Ala. DKO MEFs were infected with retrovirus encoding GFP (control, -), WT arrestin-3 (A3-WT), or arrestin-3-Lys308Ala mutant (K308A). 48 hours post-transfection the cells were serum-starved for 2 hours, stimulated with 1 μm ICI118551 for 10 min at 37°C, lysed, and analyzed by Western blot. Means ± SD of 3 independent experiments are shown in bar graphs; representative blots are shown below. *** $p<0.001$.

To test whether the mutations in non-visual arrestins change the time course of receptor-dependent ERK1/2 activation, rather than just maximum response, we used DKO MEFS expressing GFP (control), WT arrestin-2, arrestin-2-R307A, WT arrestin-3, or arrestin-3-K308A mutants (Fig. 14). We found that in all cases peak ERK1/2 phosphorylation was observed at 5 min of ICI118551 treatment, and by 20 min ERK1/2 activity returned back to basal (Fig. 14 A,C). In control GFP-expressing DKO MEFs no ERK1/2 activation in response to ICI118551 was detected, once more demonstrating that this effect is strictly arrestin-dependent. Thus, ERK1/2 activation triggered by arrestin-biased β2AR agonist ICI118551 in MEFs is rapid and transient. The time courses confirm that arrestin-2-R307A mutant does not promote ERK1/2 phosphorylation, in contrast to both WT non-visual arrestins and arrestin-3-K308A mutant. Relatively higher ERK1/2 phosphorylation mediated by WT arrestin-2 likely reflects its ~3-fold higher expression level than that of arrestin-3 (Fig. 14B).

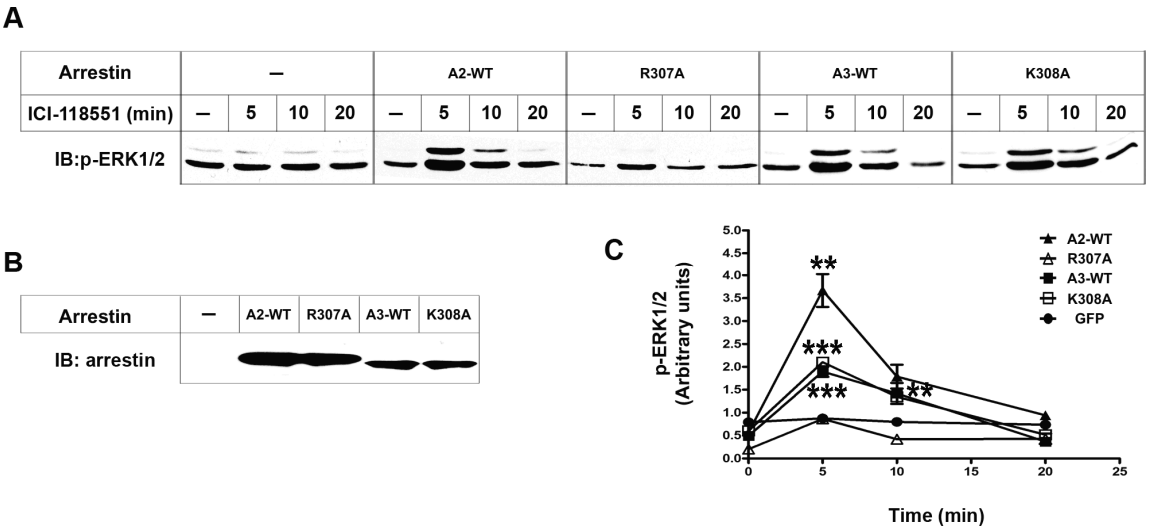

Figure 14. Rapid arrestin-mediated ERK1/2 activation by ICI118551 via β2-adrenergic receptor. A. DKO MEFs were infected with retrovirus encoding GFP (control), WT arrestin-2 (A2-WT), arrestin-2-Arg307Ala (R307A), WT arrestin-3 (A3-WT), or arrestin-3-Lys308Ala (K308A). 48 hours postinfection DKO-MEFs were serum-starved for 2 hours and stimulated with 1 μM ICI118551 for 0, 5, 10 and 20 min at 37oC. Cells were then lysed and analyzed by Western blot. Representative blot for phospho-ERK is shown. **B.** The expression of WT and mutant arrestins was compared by Western blot. Mutants and corresponding parental WT arrestins express at the same level. However, the expression of both forms of arrestin-2 is ~3-fold higher than that of both forms of arrestin-3. **C.** Time course of ERK1/2 activation in DKO MEFs expressing GFP (control) or indicated arrestins. Means + SD of two independent experiments are shown. Statistical significance of the differences (as compared to corresponding zero time point) is shown, as follows: **, $p<0.01$; ***, $p<0.001$.

DISCUSSION

Non-visual arrestins facilitate the activation of several MAP kinases in response to GPCR activation, including JNK3 (McDonald, Chow et al. 2000), ERK1/2 (Luttrell, Roudabush et al. 2001), and p38 (Bruchas, Macey et al. 2006). Multiple GPCRs have been shown to activate ERK1/2 in an arrestin-dependent manner, including β2AR (Shenoy, Drake et al. 2006), angiotensin type 1A receptor (Ahn, Shenoy et al. 2004), μ-opioid receptor (Macey, Lowe et al. 2006), and the protease-activated receptor-2 (DeFea, Zalevsky et al. 2000).

Based upon our identification of kinase-binding elements in arrestins, we have proposed a novel model of arrestin-dependent assembly of the MAPK signaling module. Arrestin binds all three kinases, assembling MAPKKK, MAPKK, and MAPK along its short axis, with each kinase directly interacting with both domains of arrestin. The relative size of arrestin and the kinases suggests that an individual arrestin molecule can assemble only one MAPK signaling module.

Our studies, as well as others, have shown that free arrestins also bind ASK1, JNK3, MKK4, c-Raf1, MEK1, and ERK2 (Miller, McDonald et al. 2001; Scott, Le Rouzic et al. 2002; Wang, Wu et al. 2003; Song, Raman et al. 2006; Song, Gurevich et al. 2007; Li, Macleod et al. 2009; Meng, Lynch et al. 2009; Song, Coffa et al. 2009). Interestingly, while the interaction of ASK1, MKK4, and JNK3α2 with free arrestin-3 translates into JNK3 phosphorylation (Miller, McDonald et al. 2001; Song, Coffa et al. 2009), receptor-independent ERK activation by free arrestins has not been observed. This may be due to a very low

affinity of ERK for free arrestin2 and -3 (Song, Coffa et al. 2009) (Fig. 5). Structurally, arrestins are elongated molecules with two cup-like domains (Hirsch, Schubert et al. 1999; Han, Gurevich et al. 2001; Milano, Pace et al. 2002; Sutton, Vishnivetskiy et al. 2005; Zhan, Gimenez et al. 2011). All identified receptor-binding elements are localized on the concave side of both domains (Ohguro, Palczewski et al. 1994; Gurevich and Benovic 1995; Gurevich, Dion et al. 1995; Pulvermuller, Schroder et al. 2000; Vishnivetskiy, Hosey et al. 2004; Hanson, Francis et al. 2006; Hanson and Gurevich 2006; Vishnivetskiy, Francis et al. 2010; Vishnivetskiy, Gimenez et al. 2011), suggesting that binding partners recruited to the arrestin-receptor complex engage the opposite convex surface of the molecule (Fig. 8A, Fig. 10).

Although the structure of arrestin suggests that the functions mediated by different surfaces can be modulated independently of each other by specific mutations (Pan, Gurevich et al. 2003; Gurevich and Gurevich 2010), alanine-scanning mutagenesis of the conserved residues on the non-receptor-binding side of arrestin-2 revealed numerous mutations that significantly affected receptor binding (Fig. 7). Thus, targeted design of arrestins with desired functional characteristics may be a more complex endeavor than previously thought: it appears that the basal conformation of arrestin-2 is stabilized by an intricate network of interactions that spans both the receptor- and kinase-binding surfaces. The functional coupling of these two interfaces needs to be further explored experimentally. Therefore, for the analysis of arrestin-2 interactions with c-Raf1, MEK1, and ERK1/2 we only used ten mutants that demonstrated normal

receptor binding (Fig. 8B), indicative of proper folding. Nine of these showed essentially WT interactions with all three kinases in the c-Raf1-MEK1-ERK1/2 pathway, whereas c-Raf1 binding was selectively impaired by the Arg307Ala mutation (Fig. 9). Although these data do not suggest that Arg307 is the only residue involved in c-Raf1 interaction, it appears to be an important docking point for this kinase.

Arg307 is located at the inter-domain interface, and in most structures of arrestin-2, this residue interacts with Asp29 forming an inter-domain bridge (Fig. 10). This interaction is quite long, ranging from 3.5 to 5.8 Å, and likely contributes little energy to the crystallographically observed conformations. In arrestin-2 structures G4M (Han, Gurevich et al. 2001), G4R (Han, Gurevich et al. 2001), 1ZSH (Milano, Kim et al. 2006), and 3GDI (Kang, Kern et al. 2009) the interaction is present, but it is absent in 3GC3 (Kang, Kern et al. 2009) and 1JSY (Milano, Pace et al. 2002). The interaction is observed in 1AYR, the only arrestin-4 structure (Sutton, Vishnivetskiy et al. 2005). Arrestin-1 and -3 do not form this interaction in the crystals, although Arg307 is substituted by lysine in arrestin-3 and arrestin-1, preserving positive charge (Wu, Hanson et al. 2006). Our observation that Arg307Ala is impaired in c-Raf1 binding and unable to promote detectable ERK1/2 activation indicates that in the c-Raf1 bound state, Arg307 interacts with c-Raf1, rather than with Asp29. Arg307 is localized on the periphery of the interface between the N- and C- domains (Fig. 10). Its interaction with Asp29 can bridge the two domains, but is likely one of many weak

interactions that stabilize the basal arrestin conformation and are broken upon receptor binding (Gurevich and Gurevich 2004).

We have shown that ERK1/2 activation via endogenous β2AR stimulated by ICI118551 in MEFs is strictly arrestin-dependent (Fig. 11A). Using this model, we demonstrated that impaired c-Raf1 binding results in the inability of the Arg307Ala mutant to scaffold productively the c-Raf1-MEK1-ERK1/2 cascade (Fig. 11B), despite its normal ability to bind receptor, MEK1, and ERK2 (Figs. 8, Fig. 9). Interestingly, Arg307Ala shows a tendency to act as a dominant-negative mutant, reducing overall ERK1/2 activity in MEFs (Fig. 10B), likely via sequestering MEK1 and/or ERK1/2, both of which bind normally to this mutant (Fig. 9B,C). Thus, as far as ERK1/2 activation is concerned, arrestin-2-Arg307Ala is the first signaling-biased arrestin constructed. Importantly, this function of arrestin-2 was selectively suppressed by a point mutation, which did not appreciably affect arrestin-2 binding to MEK1, ERK2, or receptor. However, we did not test many other reported arrestin-2 functions, so it is conceivable that its interactions with some other partners were also affected. The analysis of the time course of arrestin-mediated ERK1/2 activation via endogenous β2AR stimulated by ICI118551 in MEFs shows that WT arrestin-2 and arrestin-3, as well as arrestin-3-Lys308Ala mutant, comparably rescue ERK1/2 activation at all time points tested, whereas arrestin-2-Arg307Ala is consistently inactive (Fig. 14). Thus, the differences between arrestin-2-Arg307Ala and arrestin-3-Lys308Ala mutants to facilitate ERK1/2 phosphorylation at 10 min (Figs. 11, 12) reflect their inherent activity, rather than different kinetics of the response.

Interestingly, we found that ERK1/2 activation by ICI118551 in MEFs, which we showed to be strictly arrestin-mediated (Fig. 11A), is transient: the response reaches the peak at 5 min and rapidly declines, returning to the basal level by 20 min (Fig. 14). ERK can be activated by GPCRs via distinct G protein- and arrestin-mediated mechanisms (Luttrell 2003). Several previous studies using over-expressed angiotensin II (Ahn, Shenoy et al. 2004), β2AR (Shenoy, Drake et al. 2006), and parathyroid hormone receptors (Gesty-Palmer, Chen et al. 2006) suggested that ERK activation via G proteins is rapid and transient, whereas arrestin-mediated ERK1/2 activation is slow, but prolonged. However, ERK1/2 activation by endogenous M3 muscarinic receptor via Gq in HEK293 cells was recently shown to be as long-lasting as previously reported arrestin-mediated activation (Luo, Busillo et al. 2008). We found that arrestin-mediated ERK1/2 phosphorylation in MEFs is rapid and fades away in less than 20 min (Fig. 14), i.e., essentially as quickly as previously reported G protein-mediated ERK1/2 activation in other cell types. Importantly, in our experiments ERK1/2 was activated in response to the stimulation of β2AR, the same receptor that was previously reported to induce prolonged arrestin-mediated ERK1/2 activation in HEK293 cells (Shenoy, Drake et al. 2006). Previous studies used four different GPCRs (Ahn, Shenoy et al. 2004; Gesty-Palmer, Chen et al. 2006; Shenoy, Drake et al. 2006; Luo, Busillo et al. 2008), so that distinct kinetics of G protein-mediated ERK1/2 activation could be explained by the use of different receptors (Gurevich and Gurevich 2008). However, here we used the same β2AR as Shenoy et al (Shenoy, Drake et al. 2006), yet found very different timing of

arrestin-mediated ERK1/2 phosphorylation (Fig. 14). The fact that we activated ERK1/2 via endogenous β2AR expressed at fairly low level, whereas Shenoy et al (Shenoy, Drake et al. 2006) over-expressed WT and mutant β2AR could have contributed to this difference. Conceivably, cellular context also affects the time course of ERK1/2 activation via a particular pathway at least as much as the subtype of activated GPCR. Our finding that alanine substitution of the homologous positively charged residue in arrestin-3, Lys308, does not affect the ability of this subtype to bind c-Raf1 and promote ERK1/2 activation (Figs. 12, 13) is the first demonstration that when both non-visual arrestins perform the same function, the two subtypes employ distinct molecular mechanisms. Structurally, several elements in arrestins appear to be fairly flexible, assuming distinct conformations not only in different arrestin subtypes, but even in different monomers within crystal oligomer (Zhan, Gimenez et al. ; Hirsch, Schubert et al. 1999; Han, Gurevich et al. 2001). Therefore, these differences are often dismissed as mere indication of the plasticity of certain elements in the protein. However, our data suggest that subtle structural differences between arrestin-2 and -3 (Fig. 10) (Zhan, Gimenez et al.) revealed by the crystal structures can have significant functional consequences.

CHAPTER IV

ARRESTIN CONFORMATION AND RECEPTOR BINDING DETERMINE THE RECRUITMENT OF c-Raf1, MEK1, AND ERK2 ACTIVATION.

INTRODUCTION

Arrestins were first discovered as proteins that bind active phosphorylated G-protein coupled receptors (GPCRs) and stop ("arrest") G protein-mediated signaling (Wilden, Hall et al. 1986) due to direct competition with G proteins for the cytoplasmic tip of the receptor (Wilden 1995; Krupnick, Gurevich et al. 1997). In the last 15 years arrestin interactions with many non-receptor partners have been described, suggesting that arrestins serve a versatile signaling regulators in the cell (reviewed in (Gurevich and Gurevich 2006; DeWire, Ahn et al. 2007)). Crystal structures of all four vertebrate arrestins (Hirsch, Schubert et al. 1999; Han, Gurevich et al. 2001; Milano, Pace et al. 2002; Sutton, Vishnivetskiy et al. 2005; Zhan, Gimenez et al. 2011) revealed very similar basal conformation: an elongated molecule consisting of two cup-like domains connected by highly conserved intra-molecular interactions. Many groups using a variety of methods invariably mapped receptor-binding elements to the concave sides of both arrestin domains (Ohguro, Palczewski et al. 1994; Pulvermuller, Schroder et al. 2000; Vishnivetskiy, Hosey et al. 2004; Hanson, Francis et al. 2006; Hanson and Gurevich 2006; Vishnivetskiy, Francis et al. 2010; Vishnivetskiy, Gimenez et al. 2011). Receptor binding induces a significant conformational change, involving

the release of the arrestin C-tail and other rearrangements (reviewed in (Gurevich and Gurevich 2004; Hanson, Francis et al. 2006)). Interestingly, microtubule binding, mediated by the same concave sides of the two domains (Hanson, Cleghorn et al. 2007), induces a distinct conformational rearrangement (Hanson, Francis et al. 2006; Hanson, Cleghorn et al. 2007). Thus, in the cell arrestins exist in at least three distinct conformations, free, receptor-bound or microtubule-bound (Gurevich, Gurevich et al. 2008), and many signaling proteins differentially bind arrestins in these states (Song, Raman et al. 2006; Song, Gurevich et al. 2007; Ahmed, Zhan et al. 2011).

Specific mutants of both arrestin-2 and arrestin-3 mimicking microtubule-associated and receptor-bound conformations were constructed (Gurevich 1998; Vishnivetskiy, Schubert et al. 2000; Vishnivetskiy, Hirsch et al. 2002; Carter, Gurevich et al. 2005; Hanson, Francis et al. 2006; Song, Raman et al. 2006; Hanson, Cleghorn et al. 2007). Here we used wild type (WT) non-visual arrestins and their confromationally restricted mutants to determine the states that preferentially bind individual kinases of c-Raf1-MEK1-ERK2 cascade in the presence or absence of activated β2-adrenergic receptor (β_2AR). We found that that the affinity of arrestin-2/3 for ERK2 dramatically increases when arrestins are associated with β_2AR. Arrestin-2 interaction with c-Raf1 is enhanced by arrestin binding to the receptor, but arrestin-3-c-Raf1 interaction is not. MEK1 interaction also does not show clear preference for receptor-bound arrestin. Using pure proteins we present the first evidence that the interaction of arrestins with ERK2 is direct, and that it is differentially affected by receptor binding. These findings

improve our understanding of arrestin-mediated scaffolding of MAP kinase cascades and pave the way for targeted manipulation of this branch of GPCR signaling.

METHODS

Protein purification and *in vitro* interactions of purified proteins

Rhodopsin was purified from cow eyes, phosphorylated, and regenerated by 11-*cis*-retinal generously supplied by Dr. R. K. Crouch (Medical University of South Carolina, Charleston, SC), as described (Vishnivetskiy, Raman et al. 2007). Bovine arrestins were expressed in E. coli and purified, as described (Hanson, Francis et al. 2006; Vishnivetskiy, Gimenez et al. 2011; Zhan, Gimenez et al. 2011). Active MEK1, active and inactive ERK2 were expressed in E. coli and purified, as described (Vishnivetskiy, Gimenez et al. ; Zhan, Gimenez et al. ; Hanson, Francis et al. 2006).

ERK2 interaction with the receptor-bound arrestins

Active (phosphorylated at T183 and Y185 by MEK1) or inactive ERK2 (30 pmol) was preincubated with or without 30 pmol of purified arrestins for 20 min at 30^{o}C, then phosphorylated rhodopsin (50 pmol) was added and incubated in the light (to produce P-Rh*) for 5 min. Rhodopsin-containing membranes were pelleted through 0.2 M sucrose cushion, dissolved in SDS sample buffer, and pelleted ERK2 (1/300 of each sample) was quantified by Western blot using anti-ERK antibodies (Cell Signaling) and purified ERK2 as a standard.

ERK2 interaction with the free arrestins

CNBr-activated Sepharose beads (30 μl) containing 9 μg of covalently attached active phosphorylated (without or with 1 mM ATP) or inactive ERK2 were incubated with 3 mg of indicated purified arrestins in 60 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EGTA, 1 mM DTT) for 20 minutes. at 30^{o}C. The beads were washed twice with 1 ml of ice-cold binding buffer supplemented with 0.01 mg/ml BSA and bound arrestins were eluted with SDS sample buffer and quantified by Western blot using respective purified arrestins as standards.

ERK2 phosphorylation by purified MEK1

ERK2 (12 pmol) was incubated with MEK1 (2 pmol) in 0.1 ml of 50 mM Hepes-Na, pH 7.2, 100 mM NaCl, and 0.1 mM [γ-^{32}P]ATP in the absence (control) or presence of 44 pmol of arrestin-2 or arrestin-3 for 30 min at 30^{o}C. The reaction was stopped by MeOH-precipitation of the proteins. The pellet was dissolved in SDS sample buffer and subjected to SDS-PAGE. The gels were stained, dried, and exposed to X-ray film to visualize radiolabeled bands. ERK2 bands were cut out and ^{32}P incorporation was quantified by scintillation counting.

Co-immunoprecipitation and Western blotting

Monkey kidney COS-7 cells were transfected with the indicated plasmids using Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA), according to the manufacturers protocol (3 μL of Lipofectamine™ 2000 per 1 μg of DNA). 24

hours post-transfection, cells were serum-starved and lysed with lysis buffer (50mM Tris, 2mM EDTA, 250mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1mM NaVO3, 10mM N-ethylmaleimide, benzamidine and phenylmethylsulfonylfluoride) on ice for 20 min. Cell debris were pelleted by centrifugation for 10 min at 10,000 x g. Lysates were precleared with 30 μl of protein G agarose, followed by incubation with either rabbit anti FLAG or rat anti HA antibody for 2 hours and by the addition of 30 μl of protein G-agarose beads for 2 hours. The beads were then washed 3 times with lysis buffer, and bound proteins were eluted with Laemmli SDS buffer. In experiments involving ERK2, prior to lysis the cells were treated with 1 mM cross-linking reagent dithiobis(succinimidyl propionate) (DSP; Pierce) for 30 min followed by 2 mM Tris-HCl, pH 7.5, for 15 min at room temperature. The proteins were separated by SDS PAGE (10%) and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Blots were incubated with primary antibodies from Cell Signaling (mouse anti-HA (6E2) mAb #2367, or Sigma (mouse anti-FLAG M2, #F3165, 1:1500; rabbit anti-FLAG #F7425), followed by anti-mouse horseradish peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch. Protein bands were visualized by enhanced chemiluminescence (ECL, Pierce) followed by exposure to X-ray film. The bands were quantified using VersaDoc with QuantityOne software (Bio-Rad Laboratories).

Arrestin-dependent ERK activation

COS-7 cells

COS-7 cells were transfected using Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA), according to the manufacturer's protocol (3 μL of Lipofectamine™ 2000 per 1 μg of DNA) with Flag-tagged arrestin-2 together with ERK2-HA. 24-48 hours post-transfection, cells were serum starved for 24 hours and then treated for 10 min at 37°C with saturating concentrations of isoproterenol (10μM), epinephrine (10μM), propranolol (10μM), alprenolol (1μM), ICI118551 (1μM) or carazolol (100nM). COS-7 were then harvested and lysed in 50mM Tris, 2mM EDTA, 100mM NaCl, 1% Nonidet P-40, supplemented with protease (Roche, 04693124001) and phosphatase (Roche, 04906845001) inhibitors cocktails on ice for 20 min.

Mouse embryonic fibroblasts (MEFs)

For retrovirus production, human embryonic kidney (HEK) 293T cells were transfected using Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA), according to the manufacturer's protocol (3 μL of Lipofectamine™ 2000 per 1 μg of DNA) with the following constructs: pVPack-GP (Stratagene, 217566), pVack-VSV-G (Stratagene, 217567), together with pFB-arrestin-2, pFB-arrestin-2-Arg307Ala, pFB-arrestin-3, pFB arrestin-3-K308A, or pFB-GFP. 24-48 hours post-transfection, media containing the virus produced by HEK293T cells was collected and used to infect arrestin-2/3 double knockout MEFs (a generous gift of Dr. R. J. Lefkowitz, Duke University) (Kohout, Lin et al. 2001). Fresh virus-

containing media was used daily for 3 days. Then MEFs were serum starved for 2 hours and treated with 1 μM ICI118551, a biased ligand of β2-adrenergic receptor (β2AR), which is an inverse agonist of G protein signaling and an agonist of arrestin recruitment (Azzi, Charest et al. 2003), or 10 μM β2AR agonist isoproterenol for 10 min at 37^{o}C. MEFs were harvested and lysed in 50mM Tris, 2mM EDTA, 100mM NaCl, 1% Nonidet P-40, supplemented with protease (Roche, 04693124001) and phosphatase (Roche, 04906845001) inhibitors cocktails on ice for 20 minutes.

MATERIALS

[γ-^{32}P]ATP was from Perkin-Elmer. All restriction enzymes were from New England Biolabs. All other chemicals were from sources previously described (Gurevich and Benovic 2000; Coffa, Breitman et al. 2011).

RESULTS

ERK2 directly binds non-visual arrestins, and this interaction increases MEK1 phosphorylation of ERK2

Although ERK2 interaction with arrestins was reported a decade ago (Luttrell, Roudabush et al. 2001), this interaction was never shown to be direct. Experiments shown in Fig.15 were used to test the direct interaction of ERK2 with free arrestins. Active ERK (phosphorylated by MEK1) with or without ATP and inactive ERK2 was used to assess interaction with non-visual arrestins. As

expected, we did not detect ERK2 binding to WT visual arrestin-1 (Fig. 15), suggesting that either free arrestin-1 does not bind ERK2 or the affinity of this interaction is too low to keep it bound throughout the washing procedure. We observed that both non-visual arrestins bind with a comparably affinity to active ERK2. In addition, we noted that the presence of 1 mM ATP (ERK2 co-substrate) significantly reduced the binding of arrestin-2, but not that of arrestin-3 (Fig. 15), suggesting that inside the cell (where >2 mM ATP is always present) free arrestin-3 may bind ERK2 with higher affinity than arrestin-2. Despite the fact that both non-visual arrestins preferentially bind to active ERK2, arrestin-2 is significantly more selective (binding to inactive ERK2 is 33% of that to active form, whereas for arrestin-3 it is 67%).

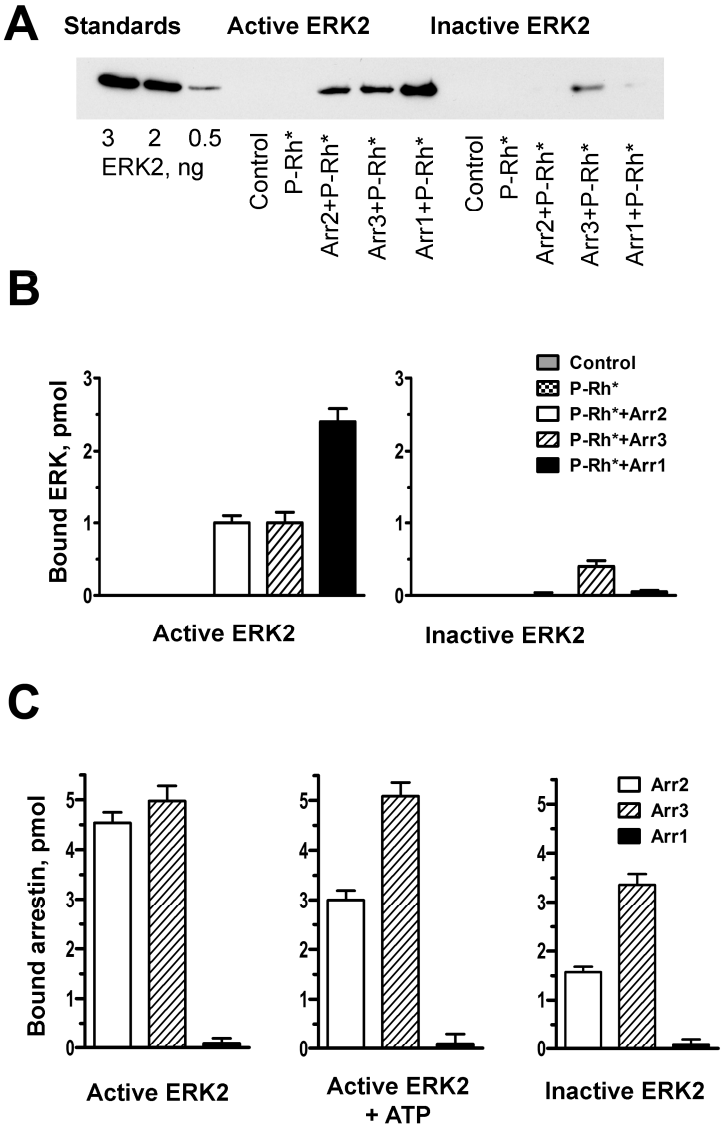

Figure 15. ERK2 binds free wild type non-visual arrestins.
A, Active (phosphorylated at T183 and Y185 by MEK1) or inactive ERK2 (30 pmol) was preincubated with or without 30 pmol of indicated arrestin for 20 minutes at 30^{o}C, then phosphorylated rhodopsin (50 pmol) was added and incubated in the light (to produce P-Rh*) for 5 min. Rhodopsin-containing membranes were pelleted through 0.2 M sucrose cushion, dissolved in SDS sample buffer, and pelleted ERK2 (1/300 of each sample) was quantified by Western blot using anti-ERK antibodies (Cell Signaling) and purified ERK2 as a

standard. Abbreviations: VA, visual arrestin-1, A2, arrestin-2, A3, arrestin-3. **B**. Quantification of data shown in panel **A**. **C**, CNBr-activated Sepharose (30 μl) containing 9 μg of covalently attached active phosphorylated (without or with 1 mM ATP) or inactive ERK2 were incubated with 3 μg of indicated purified arrestins in 60 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EGTA, 1 mM DTT) for 20 min at 30^{o}C. The beads were washed twice with 1 ml of ice-cold binding buffer supplemented with 0.01 mg/ml BSA and bound arrestins were eluted with SDS sample buffer and quantified by Western blot using respective arrestins as standards.

Next, we tested whether arrestin binding affects MEK1 phosphorylation of ERK2. Purified inactive (unphosphorylated) ERK2 and purified active MEK1 (which phosphorylates ERK2) were used to reconstruct this module of c-Raf1-MEK1-ERK1/2 cascade *in vitro* (Fig. 16). ERK2 phosphorylation by MEK1 was evaluated in the absence or presence of purified arrestins. We found that in the absence of arrestins MEK1 phosphorylates approximately 10 % of ERK2 present (Fig. 16). In the presence of arrestin-2 or arrestin-3 the extent of ERK2 phosphorylation was increased by 33 or 41%, respectively. Thus, free non-visual arrestins moderately facilitate the phosphorylation of ERK2 by MEK1.

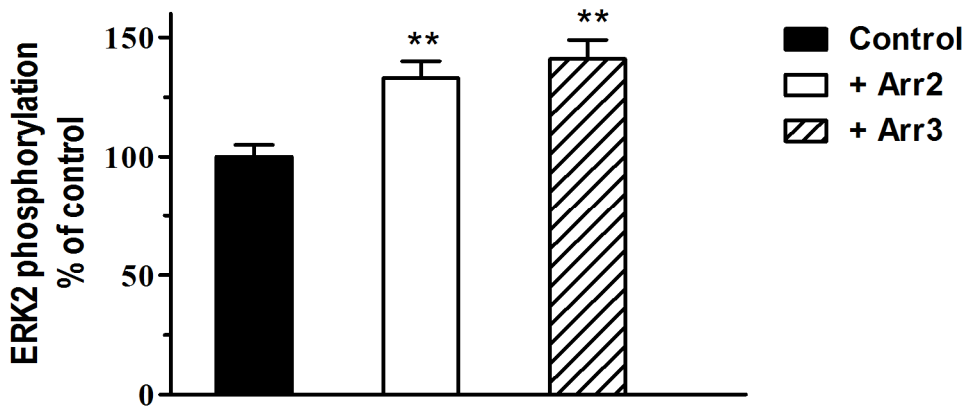

Figure 16. Free non-visual arrestins enhance ERK2 phosphorylation by MEK1. ERK2 (12 pmol) was incubated with MEK1 (2 pmol) in 0.1 ml of 50 mM Hepes-Na, pH 7.2, 100 mM NaCl, and 0.1 mM [γ-^{32}P]ATP in the absence (control) or presence of 44 pmol of arrestin-2 (Arr2) or arrestin-3 (Arr3) for 30 min at 30^{o}C. The reaction was stopped by MeOH-precipitation of the proteins. The pellet was dissolved in SDS sample buffer and subjected to SDS-PAGE. The gels were stained, dried, and exposed to X-ray film to visualize radiolabeled bands (upper panel). ERK2 bands were cut out and ^{32}P incorporation was quantified by scintillation counting (lower panel). (**) $p<0.01$, as compared to control.

The effect of arrestin-2 and arrestin-3 interactions with β2AR on the binding of the three kinases in c-Raf1-MEK1-ERK2 cascade

The original description of scaffolding of c-Raf1-MEK1-ERK1/2 cascade by arrestins in response to receptor activation suggested that only receptor-bound arrestins interact with c-Raf1 and ERK1/2, whereas MEK1 does not bind arrestins directly, but is recruited via c-Raf1 and ERK to the complex (Luttrell, Roudabush et al. 2001). However, subsequent studies suggested that all three kinases bind free non-visual arrestins, and that ERK demonstrates the lowest affinity of the three (Song, Coffa et al. 2009). MEK1 interaction with free arrestin-2 was independently confirmed by another group (Meng, Lynch et al. 2009). However, the effects of arrestin-2 and -3 conformation and receptor binding on their interaction with these kinases were never systematically investigated. Therefore, we used two known conformationally biased forms of arrestin-2 and -3, "pre-activated" 3A mutants (Kovoor, Celver et al. 1999; Celver, Vishnivetskiy et al. 2002; Pan, Gurevich et al. 2003) and and mutants "frozen" in the basal state by 7-residue deletion in the inter-domain hinge (Δ7) (Vishnivetskiy, Hirsch et al. 2002; Song, Raman et al. 2006; Hanson, Cleghorn et al. 2007; Song, Gurevich et al. 2007) to address this question in COS-7 cells expressing only endogenous β2AR, or additional plasmid-encoded β2AR at significantly greater level.

We found that the stimulation of endogenous β2AR by an agonist isoproterenol increased ERK2 binding to arrestin-2 and arrestin-3 (Fig. 17).

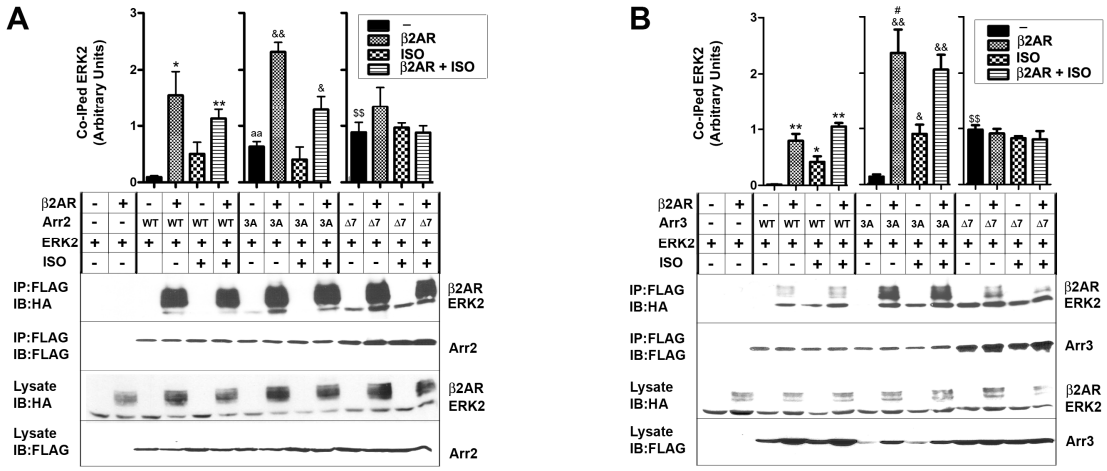

Figure 17. Conformational dependence of the interaction of non-visual arrestins with ERK2. COS-7 cells were transfected with WT, 3A, or Δ7 mutant forms of Flag-tagged arrestin-2 (**A**) or arestin-3 (**B**), along with ERK2-HA, with or without β2AR-HA. Cells were serum starved 24 hours after transfection, stimulated for 10 min at 37°C with 10 μM β2AR agonist isoproterenol. Arrestins were immunoprecipitated with anti-Flag antibody, and co-immunoprecipitated ERK2 and β2AR were detected with anti-HA antibody. Bar graphs show the ratio of co-immunoprecipitated ERK2 to immunoprecipitated arrestin. The data from 3 independent experiments were statistically analyzed by ANOVA, and the significance of the differences is indicated, as follows: * or $^{\&}$, $p<0.05$; ** or $^{\&\&}$, $p<0.01$, as compared to corresponding within group basal level of ERK2 co-immunoprecipitation (black bars); ^{a} or $^{\$}$ or $^{\#}$, $p<0.05$ compared to WT control (black bar in WT group).

Interestingly, overexpression of β2AR resulted in the formation of an arrestin-receptor complex independent of isoproterenol stimulation and further increased the binding of ERK2 to arrestins (Fig. 17). Apparently, due to the known constitutive activity of β2AR (Samama, Cotecchia et al. 1993), its over-expression induces arrestin/β2AR interaction that is not significantly enhanced by isoproterenol stimulation. Pre-activated 3A mutants bind ERK2 much better than corresponding wild type (WT) arrestins. Co-expression of β2AR with 3A mutants further enhanced arrestin-ERK2 interaction (Fig. 17). Unexpectedly, we found that Δ7 mutants of both arrestins also bind ERK2 significantly better than WT proteins or even 3A mutants (Fig. 17). This is consistent with the reported ability of Δ7 mutants of arrestin-2 and -3 to recruit ERK1/2 to microtubules, which they bind with high affinity (Hanson, Cleghorn et al. 2007). In agreement with impaired receptor binding of Δ7 mutants, we found that neither isoproterenol stimulation nor β2AR over-expression affected ERK2 binding to these mutants (Fig. 17). Thus, ERK2 preferentially interacts with arrestins in receptor-bound and microtubule-associated conformation, with free arrestins in the basal state having the lowest affinity for this kinase.

In contrast, we found that the isoproterenol, with or without β2AR over-expression, did not affect MEK1 binding to WT arrestin-2, arrestin-3, and their Δ7 mutants (Fig. 18). Interestingly, while the binding of the 3A mutants to MEK1 was not affected by isoproterenol stimulation of endogenous β2AR, receptor over-expression dramatically increased the amount of MEK1 co-immunoprecipitated with 3A mutants of both arrestins (Fig. 18). Thus, receptor binding apparently

increases arrestin interactions with MEK1, and this effect becomes more prominent when arrestins are rendered conformationally loose by 3A mutation.

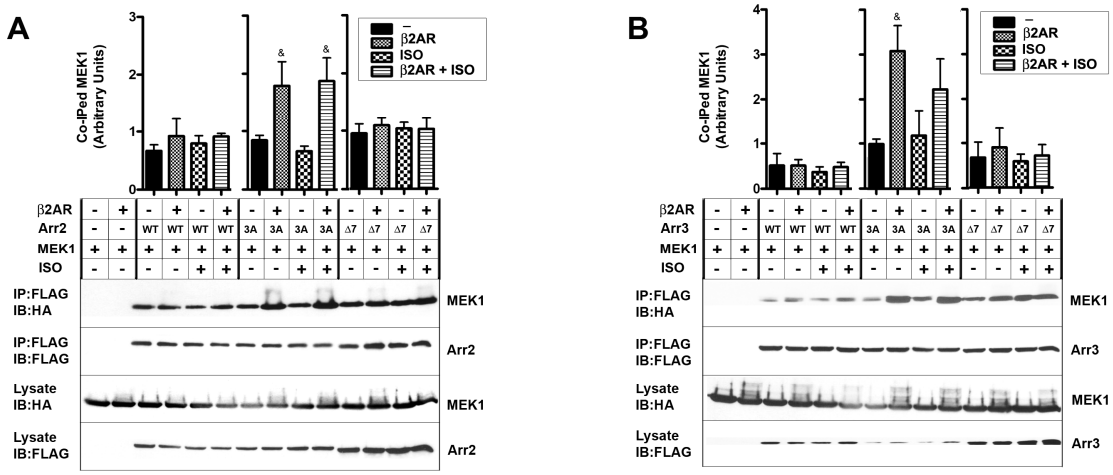

Figure 18. The binding of MEK1 is largely insensitive to arrestin conformation. COS-7 cells were transfected with WT, 3A, or Δ7 mutant forms of Flag-tagged arrestin-2 (**A**) or arestin-3 (**B**), along with MEK1-HA, with or without β2AR-HA. Cells were serum starved 24 hours after transfection, stimulated for 10 minutes at 37°C with 10 μM β2AR agonist isoproterenol. Arrestins were immunoprecipitated with anti-Flag antibody, and co-immunoprecipitated MEK1 and β2AR were detected with anti-HA antibody. Bar graphs show the ratio of co-immunoprecipitated MEK1 to immunoprecipitated arrestin. The analysis of the data from 3 independent experiments by ANOVA, revealed the following differences: **&**, $p<0.05$, as compared to corresponding within group basal level of MEK1 co-immunoprecipitation (black bars).

In contrast to ERK2 and MEK1, the binding of c-Raf1 to WT arrestin-2 and -3 is differentially affected by β2AR over-expression (Fig. 19). In the case of arrestin-2 the presence of extra β2AR resulted in a dramatic increase in c-Raf1 binding, whereas in the case of arrestin-3 receptor effect was only marginal (Fig. 19). When arrestins were rendered conformationally flexible by 3A mutation, β2AR over-expression increased c-Raf1 binding to both arrestins comparably (Fig. 19). Similar to ERK2 and MEK1, more c-Raf1 co-immunoprecipitated with Δ7 mutants than with WT forms of both arrestins. In the case of arrestin-2-Δ7, even the effect of β2AR over-expression was significant, likely reflecting residual ability arrestin-2-Δ7 to bind receptors (Hanson, Cleghorn et al. 2007).

To summarize, isoproterenol activation of the endogenous receptor present at relatively low levels resulted in a detectable increase of the interaction with WT arrestins only for ERK2 (Fig. 17), which was previously shown to have the lowest affinity for free arrestins (Song, Coffa et al. 2009). In contrast, massive expression of exogenous β2AR increased WT arrestin binding of ERK2 and c-Raf1, but not MEK1 (Figs. 17-19). As could be expected, in the case of 3A mutants that mimic receptor-bound conformation and bind GPCRs more readily, the interaction with all three kinases is increased by receptor over-expression, whereas Δ7 with impaired receptor binding are essentially unresponsive to β2AR (Figs. 17-19). Unexpectedly, Δ7 mutants of arrestin-2 and -3 bind ERK2 and c-Raf1 better than parental WT arrestins (Figs. 17, 19). The same tendency was observed with MEK1, although it did not reach statistical significance (Fig. 18).

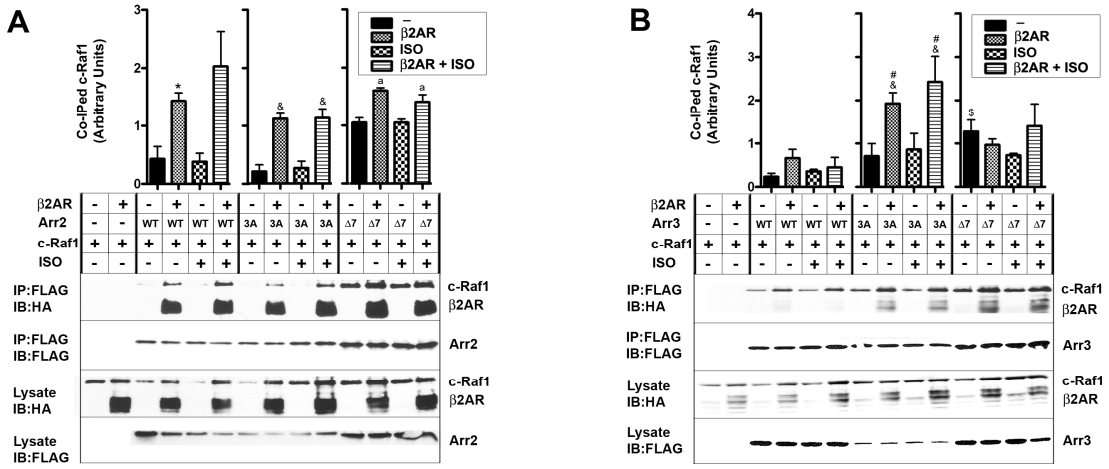

Figure 19. Conformational dependence of arrestin interactions with c-Raf1. COS-7 cells were transfected with WT, 3A, or Δ7 mutant forms of Flag-tagged arrestin-2 (**A**) or arrestin-3 (**B**), along with c-Raf1-HA, with or without β2AR-HA. Cells were serum starved 24 hours after transfection, stimulated for 10 minutes at 37°C with 10 μM β2AR agonist isoproterenol. Arrestins were immunoprecipitated with anti-Flag antibody, and co-immunoprecipitated c-Raf1 and β2AR were detected with anti-HA antibody. Bar graphs show the ratio of co-immunoprecipitated c-Raf1 to immunoprecipitated arrestin. The differences revealed by the analysis of the data from 3 independent experiments by ANOVA are indicated, as follows: * or $^{\&}$ or ^{a}, $p<0.05$, as compared to corresponding within group basal level of c-Raf1 co-immunoprecipitation (black bars); $^{\$}$ or $^{\#}$, $p<0.05$, compared to WT control (black bar in WT group).

Receptor-stimulated arrestin-dependent ERK activation

Next, we tested whether arrestin-ERK2 interaction correlates with receptor-dependent ERK2 activation. To this end, we expressed HA-ERK2 with arrestin-2-Flag (Fig. 20A) or arrestin-3-Flag (Fig. 20D) in COS-7 cells and stimulated endogenous β2AR with saturating concentrations of agonists (isoproterenol, epinephrine), antagonists (propranolol, alprenolol), or inverse agonists (ICI118551, carazolol). The amount of ERK2 co-immunoprecipitated with arrestin-2 (Fig. 20B) or arrestin-3 (Fig. 20E) was significantly increased by treatment with agonists, antagonists and inverse agonists. Importantly, the level of ERK2 phosphorylation was also increased by different ligands in cells expressing arrestin-2 (Fig. 20C) and arrestin-3 (Fig. 20F). Inverse agonists ICI118551 and carazolol induced the most dramatic increase in ERK2 association with arrestins and significant increase in ERK2 activation (Fig. 20), supporting the idea that these compounds are in fact arrestin-biased ligands (Azzi, Charest et al. 2003). Presumed antagonists propranolol and alprenolol (that actually have partial agonist activity (Samama, Cotecchia et al. 1993)) also promoted ERK2 binding to arrestins and phosphorylation, albeit to a lesser degree (Fig. 20). Agonists isoproterenol and epinephrine produced disproportinally larger ERK2 activation relative to arrestin association (Fig. 20), likely because in contrast to other compounds tested these ligands increase G protein activation, and ERK can be also activated by GPCRs via G-protein mediated pathways (Luo, Busillo et al. 2008).

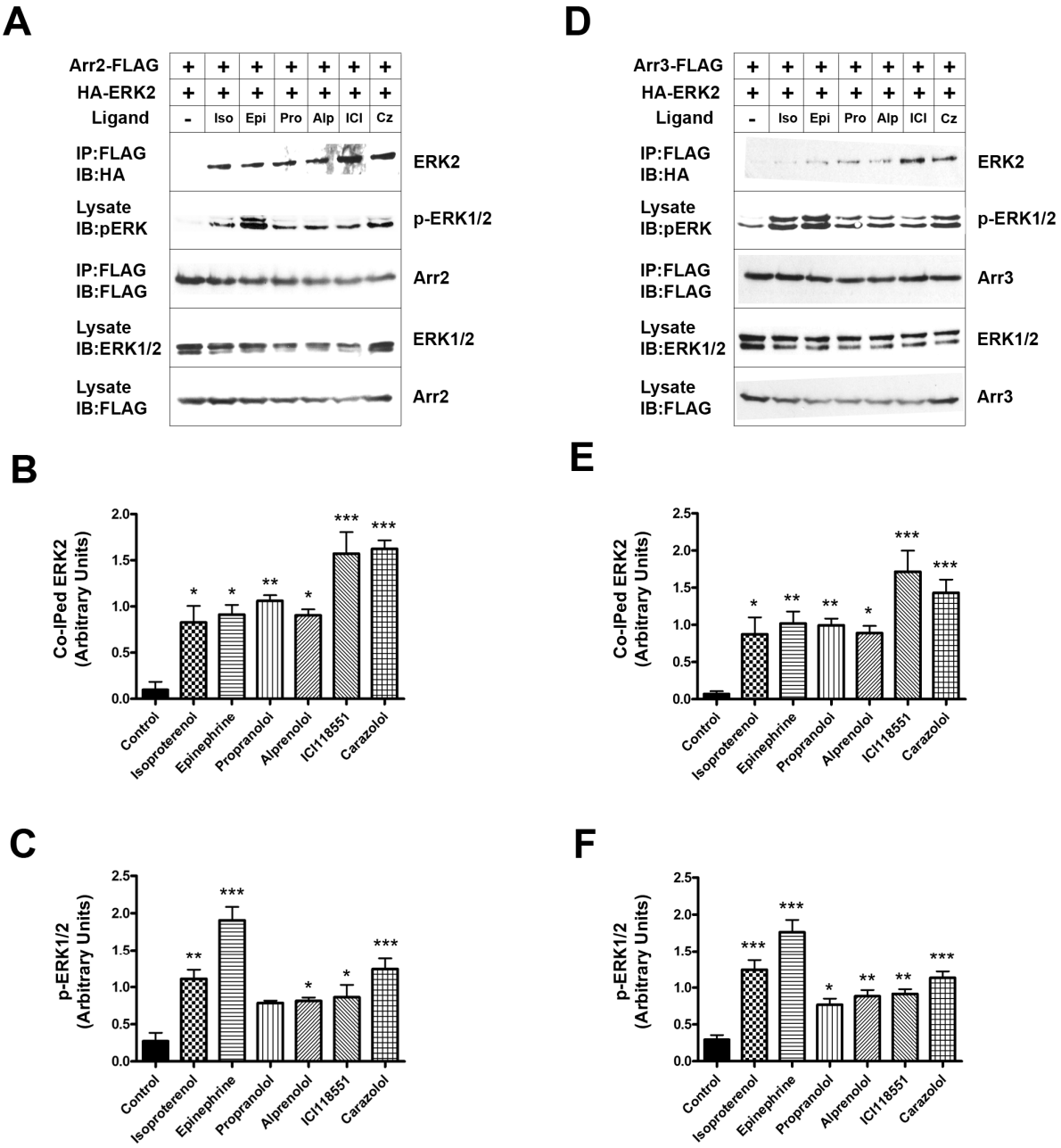

Figure 20. The effect of different β2AR ligands on the binding to arrestins and activation of ERK2. HA-tagged ERK2 was co-expressed with Flag-tagged WT arrestin-2 (**A,B,C**), or arrestin-3 (**D,E,F**). Cells were serum starved 24 hours after transfection and stimulated for 10 minutes at 37°C with 10 μM of indicated β2AR ligands. Arrestins were immunoprecipitated with anti-Flag antibody, and co-immunoprecipitated ERK2 was visualized with anti-HA antibody. The binding of ERK2 to arrestin-2 (**B**) or arrestin-3 (**E**) was significantly increased by

treatment with ligands. **C,D.** ERK1/2 activation in cell lysates was determined by Western blot with anti phospho-ERK1/2 antibody. Means ± SD of 3-4 independent experiments are shown in bar graphs; representative blots are shown in panels **A** and **D**. ANOVA with Bonferroni post-hoc test revealed the following differences: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, as compared to untreated cells.

Therefore, to exclude G protein-mediated mechanisms, we performed the next set of experiments in arrestin-2/3 double knockout (DKO) MEFs (Kohout, Lin et al. 2001), where ERK2 activation by β2AR inverse agonists is strictly arrestin-dependent (Coffa, Breitman et al. 2011). An inverse β2AR agonist ICI118551, was previously shown to act as an arrestin-biased agonist (Azzi, Charest et al. 2003). Indeed, we did not detect appreciable ERK1/2 activation by ICI118551 via endogenous β2AR in DKO MEFs (Fig. 21). We found that the expression of WT arrestin-2 rescues the ability of ICI118551 to stimulate ERK1/2 phosphorylation. Interestingly, arrestin-2-Δ7 was also effective, in contrast to arrestin-2-3A mutant (Fig. 21).

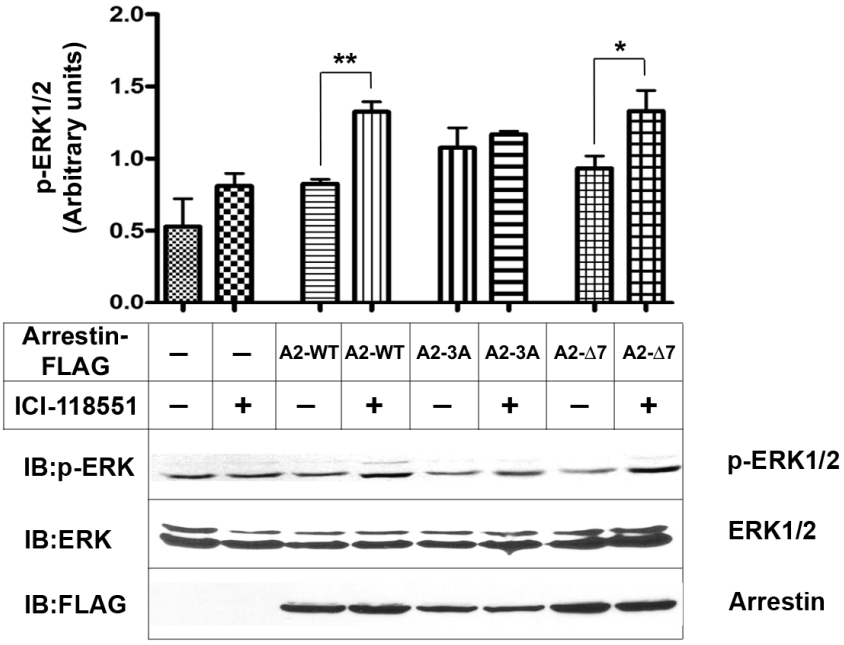

Figure 21. WT and Δ7 mutant of arrestin-2 rescue b2AR-mediated ERK activation in response to ICI118551 in arrestin-2/3 double knockout MEFs. DKO MEFs were infected with retrovirus encoding GFP (control, -), WT arrestin-2 (A2-WT), arrestin-2-3A (A2-3A), or arrestin-2-Δ7 (A2- Δ7). The cells were serum-starved 48 hours post-infection for 2 hours, stimulated with 1 μM ICI118551 for 10 minutes at 37^{o}C, lysed, and analyzed by Western blot. Means ± SD of 3-4 independent experiments are shown in bar graphs; representative blots are shown below. *, $p<0.05$; **, $p<0.01$.

To determine which β2AR ligands enhance ERK1/2 phosphorylation in arrestin-dependent fashion, we compared ERK1/2 activation in DKO MEFs expressing GFP (control), WT arrestin-2, as well as Δ7 or 3A mutants (Fig. 22A,B). In all cases we detected robust ERK1/2 activation in response to isoproterenol and epinephrine, further confirming that this effect is mediated by G protein, rather than arrestins. Only cells expressing WT arrestin-2 and Δ7 mutant showed ERK1/2 activation in response to ICI118551; however, we did not detect a statistically significant response to carazolol (Fig. 22A,B), which activated ERK1/2 in COS7 cells over-expressing arrestins (Fig. 20). To determine the possible reason for this difference, we compared the expression of arrestins in COS7 cells and DKO-MEFs, and found that the latter express all arrestins at much lower levels (Fig. 22C). Thus, ICI118551 appears to be more potent activator of arrestin-mediated signaling, effective even at fairly low arrestin expression levels.

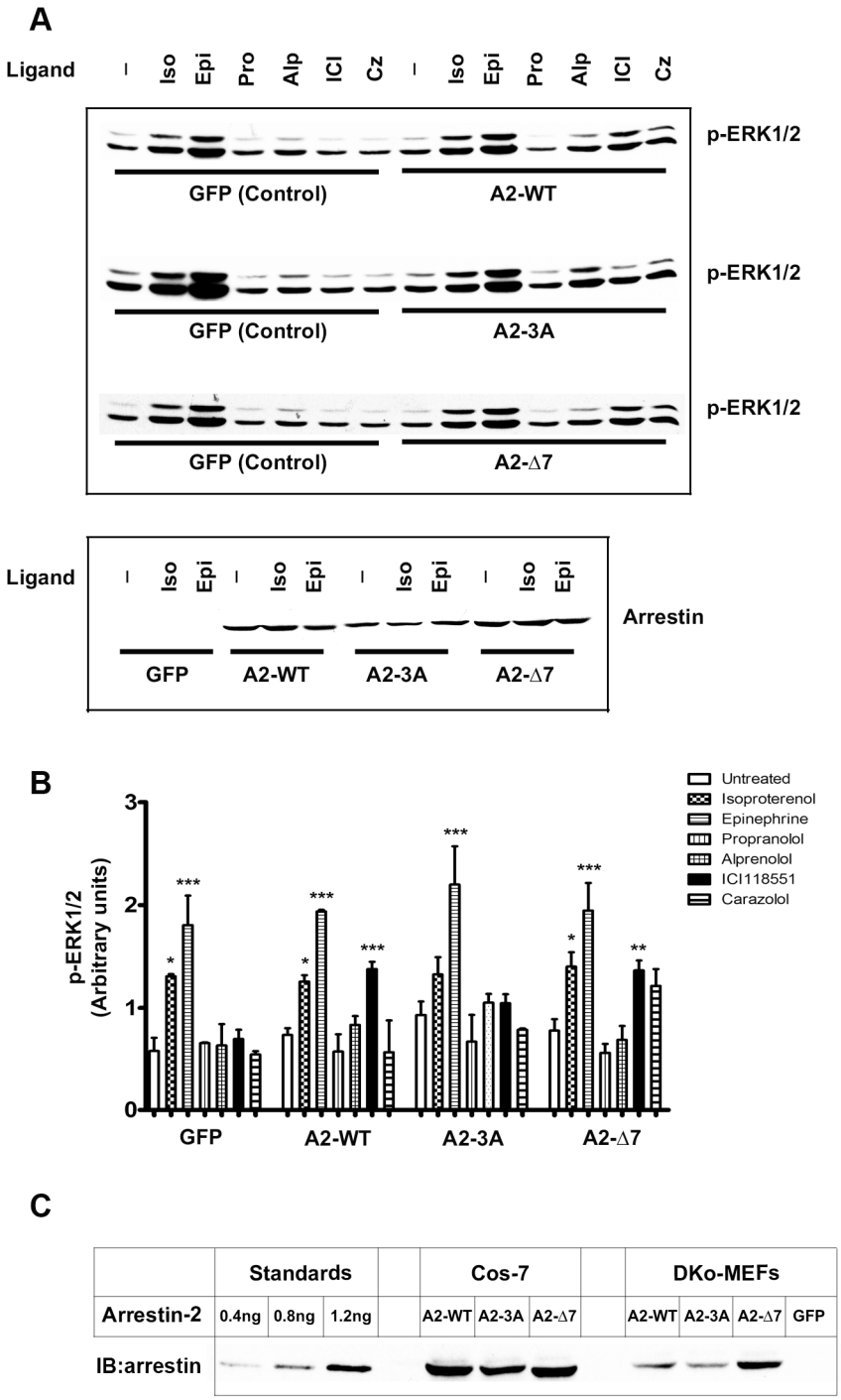

Figure 22. ERK2 activation by different β2AR ligands in DKO MEFs. A. DKO MEFs were infected with retrovirus encoding GFP, WT arrestin-2 (A2-WT), arrestin-2-3A (A2-3A), or arrestin-2-Δ7 (A2-Δ7). Serum-starved cells were stimulated with indicated β2AR ligands, lysed, and analyzed by Western blot. Representative blots are shown. The expression of different forms of arrestin-2 is compared in the blot below. **B.** Phospho-ERK1/2 bands were quantified. Means ±

SD of 3 independent experiments are shown. **C.** Comparison of arrestin expression levels in COS-7 cells (5 μg protein/lane) and DKO MEFs (10 μg protein/lane) was performed by Western blot with anti-arrestin antibody. Standards containing indicated amounts of purified arrestin-2 were run along with cell lysates to generate calibration curve. The following arrestin levels were detected in COS-7 cells: A2-WT, 100.1 pmol/mg; A2-3A, 81.1 pmol/mg; A2-Δ7, 92.8 pmol/mg. Arrestin expression in DKO MEFs was much lower: A2-WT, 13.2 pmol/mg; A2-3A, 12.3 pmol/mg; A2-Δ7, 21.7 pmol/mg.

DISCUSSION

In addition to classical G protein-mediated signaling, GPCRs were shown to initiate several signaling pathways via bound arrestins, which lead to the activation of ERK1/2 (Luttrell, Roudabush et al. 2001), c-Jun N-terminal kinase 3 (JNK3) (McDonald, Chow et al. 2000), and p38 (Bruchas, Macey et al. 2006). The ERK1/2 activating module consists of three kinases: c-Raf1, which phosphorylates MEK1, which in its turn phosphorylates ERK1/2 on both tyrosine and threonine residues (Boulton, Nye et al. 1991) within the activation loop. ERK1/2 activation by GPCRs can be mediated by the activation of Ras, PKC, tyrosine kinases (e.g., c-Src), trans-activation of receptor tyrosine kinases, or via arrestin scaffolds (DeWire, Ahn et al. 2007).

ERK1/2 activity controls many cellular functions, including proliferation, differentiation, and apoptosis. Arrestin-mediated ERK1/2 activation may result in different physiological responses than those achieved by G protein activation. G protein activation of ERK1/2 results in the accumulation of these kinases (ERKs) within the nucleus, where they can phosphorylate and activate various transcription factors (Pierce, Luttrell et al. 2001) In contrast, when ERK1/2 activation is promoted by arrestins, ERK1/2 is found mainly in cytoplasmic compartments of the cell where it can phosphorylate non-nuclear substrates (Tohgo, Pierce et al. 2002).

Previously we have shown, using immunoprecipitation assays, that the binding of ERK2 to free arrestin is quite low and undetectable without the use of cross-linking (Song, Coffa et al. 2009). Here, using *in vitro* binding of purified

proteins, we have shown for the first time that ERK2 directly interacts with non-visual arrestins (Fig. 15). Importantly, using purified protein we also observed an increase of phosphorylation of ERK2 by activated MEK1 *in vitro* in the presence of arrestin-2 and arrestin-3, as compared to control in the absence of arrestins (Fig. 16). This suggests two possible roles for arrestin: 1) binding of ERK2 to arrestin could change the conformation of ERK2, making it a better substrate for MEK1 (for example, it was recently shown using purified proteins that "scaffold" Ste5 in yeast acts by making MAPK Fus3 (but not related kinase Kss1) a better substrate for MAPKK Ste7, rather than by bringing Ste7 and Fus3 together (Good, Tang et al. 2009)); 2) both MEK1 and ERK2 can bind arrestin, and the binding facilitates the phosphorylation of ERK2 by bringing MEK1 to its substrate (true scaffolding; this mechanism was recently described for arrestin-3 dependent increase in JNK3α2 phosphorylation by MKK4 (Zhan, Kaoud et al. 2011)).

To determine whether ERK2 binding is dependent upon arrestin conformation, we co-expressed ERK2 with the following three forms of arrestins: a) WT; b) "pre-activated" 3A mutants partially mimicking receptor-bound conformation (Gurevich 1998; Pan, Gurevich et al. 2003; Carter, Gurevich et al. 2005); c) Δ7 mutants "frozen" in the basal conformation by the deletion of seven residues in the inter-domain hinge (Vishnivetskiy, Hirsch et al. 2002; Song, Raman et al. 2006; Hanson, Cleghorn et al. 2007). Our data suggest that β2AR binding-induced conformational change increases the affinity of ERK2 for both non-visual arrestins (Fig. 17). Unexpectedly, we found that ERK2 also binds Δ7

mutants with high affinity, demonstrating the lowest binding to free WT arrestins (Fig. 17). Both non-visual subtypes demonstrated the same conformational dependence of ERK2 binding (Fig. 17). Interestingly, we did not detect conformational dependence of MEK1 interactions with arrestin-2 or -3 (Fig. 18). In contrast, arrestin-2 binding to c-Raf1 is much more sensitive to its receptor interaction that that of arrestin-3 (Fig. 19). Since distinct structural features of arrestin-3 also result in lower selectivity for particular functional forms of the receptor than that of arrestin-2 (Zhan, Gimenez et al. 2011), these data suggest that higher conformational flexibility of arrestin-3 is responsible for more promiscuous interactions with GPCRs and other signaling proteins. Markedly different effects of receptor binding on arrestin-2 and -3 interaction with c-Raf1, is consistent with distinct ability of these subtypes to scaffold c-Raf1-MEK1-ERK1/2 cascade (Ahn, Wei et al. 2004).

To determine how receptor-dependent changes in arrestin interactions with these kinases translates into agonist-dependent ERK1/2 activation, we used β2AR that is endogenously expressed in most cultured cells at physiologically relevant levels, and took advantage of the availability of arrestin-biased agonists for this receptor (Azzi, Charest et al. 2003). Relatively low levels of endogenous arrestins in COS-7 cells ensure that exogenously expressed arrestin is the predominant species. We found that the expression of WT forms of arrestin-2 and -3, which are the most sensitive to receptor interaction (Figs. 17-19), enhanced the phosphorylation of endogenous ERK1/2 in response to β2AR stimulation by unbiased agonists adrenaline and isoproterenol, antagonists (with

low agonist activity (Samama, Cotecchia et al. 1993)) alprenolol and propranolol, as well as arrestin-biased agonists carazolol and ICI118551 (Fig. 20). ERK1/2 activation induced by carazolol and ICI118551, which are inverse agonists for G protein activation, is comparable to that induced by unbiased agonists that can act via G proteins and arrestins (Fig. 20), suggesting that a significant fraction of ERK1/2 is activated via arrestin-mediated scaffolding.

To conclusively dissect arrestin-dependent and –independent mechanisms, we compared WT MEFs, where ERK1/2 can be activated via both pathways and DKO MEFs lacking both non-visual arrestins (Kohout, Lin et al. 2001), where only G protein-mediated pathway is operative. Indeed, we found that while ERK1/2 phosphorylation in response to β2AR agonists that promote receptor coupling to G protein is essentially the same in WT and DKO MEFs, the response to ICI118551 is completely lost in DKO MEFs, indicating that it is mediated by non-visual arrestins absent in these cells (Fig. 21). The advantage of DKO MEFs is that one can be confident that the expressed form of arrestin is the only one present. For subsequent experiments we chose arrestin-2, which showed more pronounced changes in kinase interactions in response to receptor binding (Figs. 17-19). We found that both WT arrestin-2 and Δ7 mutant rescue ERK1/2 response to ICI118551 in DKO MEFs, whereas the 3A mutant does not (Fig. 21). Next we tested a wider range of β2AR ligands in DKO MEFs expressing GFP (control), WT arrestin-2, or 3A or Δ7 mutants (Fig. 22). We found that arrestin expression in DKO MEFs was 5-6 times lower than in COS7 cells (Fig. 22). In these conditions only ICI118551 induced robust ERK1/2 activation,

indicating that it is more potent stimulator of arrestin-mediated signaling than carazolol.

To summarize, we determined conformational dependence of arrestin-2 and -3 interactions with kinases c-Raf1, MEK1, and ERK2, which confirmed that ERK2 and c-Raf1 preferentially interact with the arrestin-receptor complex, and led to unexpected finding that the conformation of receptor binding-impaired Δ7 mutants is favored by ERK2 and c-Raf1. Moreover, we found that both WT arrestin-2 and Δ7 mutants rescue arrestin-dependent activation of ERK1/2 in response to receptor stimulation by arrestin-biased ligands. Since dramatically reduced binding of Δ7 forms of arrestin-1, -2, and -3 was described using light-activated rhodopsin, a form of receptor equally capable of coupling to G protein and arrestin, our data suggest that arrestin-2-Δ7 is likely more capable of binding receptors in a distinct conformation induced by arrestin-biased agonists. Further structural dissection of receptor conformations preferentially engaging G proteins and arrestins requires the solution of crystal structures of receptors in complex with these two types of partners.

CHAPTER V

CONCLUSIONS

We have showed that the individual arrestin N- and C-domains, which can be expressed separately while still remaining functional (Gurevich and Benovic 1992; Gurevich and Benovic 1993), interact with c-Raf1, MEK1 and ERK2 (Figs. 5A-C) (Song, Coffa et al. 2009). The individual arrestin N- and C- domains also interact with ASK1, MKK4 and JNK3 (Song, Coffa et al. 2009). Recent studies have shown that MEK1 binds non-visual arrestins directly (Meng, Lynch et al. 2009), contrary to what was previously believed (Pierce and Lefkowitz 2001). Taken together, these data suggest these kinases are arranged with their long axes parallel to the long axis of arrestin molecules (Figure 6).

Arrestins are relatively small proteins with a significant proportion of their surfaces occupied by bound receptor. It is unlikely that kinases such as c-Raf1, MEK1, ERK2, ASK1, MMK4 and JNK3 bind arrestin simultaneously. We hypothesized that these cascades must compete with each other for the limited binding space available, suggesting that arrestin must somehow favor one partner over another. We expect competition between kinases of differing signaling cascades and positive cooperativity among kinases of the same cascade. Without such cooperativity arrestins would scaffold non-functional cascades.

We have tested this hypothesis using co-immunoprecipitation in COS-7 cells (Fig. 23), but were not able to answer this question due to the presence of endogenous MAPK scaffolds such as endogenous arrestins and/or other scaffolds. A study using purified proteins, to compete for the *in vitro* binding of specific MAPK to arrestins will be necessary to show any cooperativity amongst members of the same MAPK cascades.

Our mutagenesis studies of arrestin-2 have shown that the Arg307Ala mutant has reduced binding to c-Raf-1 (Fig. 9A), while still being capable of binding phosphorylated receptor (Figs. 7, 8B) and the downstream kinases MEK1 (Fig. 9B) and ERK2 (Fig. 9C) (Coffa, Breitman et al.). Although WT arrestin-2 was able to rescue ERK1/2 activation (Fig. 11B) in arrestin 2/3 double knock-out mouse embryonic fibroblasts (DKO MEFs), the Arg307Ala mutant failed to do so (Fig. 11B), suggesting that Arg307 is necessary for the interaction of c-Raf1 with arrestin-2. Interestingly, the corresponding residue on arrestin-3, lysine (K308), when mutated to an alanine, was able to rescue ERK1/2 activation in DKO MEFs similarly to WT arrestin-3 (Fig. 13), indicating that this residue is not essential for the interaction between c-Raf-1 and arrestin-3 (Coffa, Breitman et al.). Additionally, we reported that the arrestin-2/c-Raf1 interaction is enhanced upon receptor binding (Fig. 19A), whereas the arrestin-3/c-Raf1 interaction is not (Fig. 19B), thus highlighting a major difference between the non-visual arrestins in the scaffolding of MAPKs.

Another major difference between the two non-visual arrestins is that Arrestin-2 C-terminus is known to be constitutively phosphorylated by ERK2, at

serine 412, whereas arrestin-3 is not (Lin, Krueger et al. 1997). This phosphorylation decreases the affinity for clathrin and thus dephosphorylation of Ser412 is necessary for receptor internalization to occur (Lin, Miller et al. 1999).

With this in mind, we could use the R307A mutant, deficient in c-Raf1 binding, and quantify levels of Ser412 phosphorylation and receptor internalization. Using this approach we could determine whether is necessary for arrestin-2 to scaffold the c-Raf1/MEK/ERK2 cascade in order for Ser412 to be phosphorylated, or whether other scaffolds can bring this cascade in close proximity to arrestin to mediate phosphorylation of this residue.

Receptor-bound arrestin-3, as well as free arrestin-2 and arrestin-3, were shown to scaffold the ASK1–MKK4–JNK3 cascade (McDonald, Chow et al. 2000; Song, Coffa et al. 2009), whereas only free arrestin-3 appeared able to promote JNK3 activation (Song, Raman et al. 2006; Song, Coffa et al. 2009). This is also supported by the finding that an arrestin-3 mutant 'frozen' in the basal conformation was shown to promote JNK3 activation as well as WT arrestin-3 (Song, Coffa et al. 2009). Moreover, our findings suggest that ERK2 does bind to free arrestin (Figs 5A, 9C, 15C), but this interaction is very weak and is significantly augmented by arrestin interaction with the β2AR (Figs. 17A, B).

Arrestin was recently shown to mediate the regulation of additional receptor types beyond GPCRs, including the IGF1 receptor tyrosine kinase (Lin, Daaka et al. 1998) and the TGFβRIII receptor (Chen, Kirkbride et al. 2003). It may be possible that while the mechanism of arrestin binding to GPCRs is conserved in all arrestins, different structural elements are ultimately engaged in

arrestin complexes with different receptors. Arrestin might be able to adopt multiple conformations depending upon the family, class and/or type of receptor involved. The ability of arrestin to scaffold and activate MAPK could be tested with such receptors. Immunoprecipitation and direct *in vitro* binding studies involving different receptors types, arrestins, and MAPKs could help answer this question.

Multiple GPCRs have been shown to activate ERK1/2 in an arrestin-dependent manner, including the β2AR (Coffa, Breitman et al. ; Shenoy, Drake et al. 2006), the angiotensin type 1A receptor (Ahn, Shenoy et al. 2004), the μ-opioid receptor (Macey, Lowe et al. 2006), and the protease-activated receptor-2 (DeFea, Zalevsky et al. 2000). Our studies suggested that arrestin association with the β2AR significantly enhances both ERK2 binding (Fig.17) and activation (Figs. 11, 13, 14). We also reported that the interactions between free arrestin-2 or arrestin-3 and ERK2 are very weak (Fig. 5), suggesting that the arrestin conformation change, upon receptor binding, is necessary for its interaction with ERK2. Arrestin-receptor interaction has been shown to induce the release of arrestin C-tail (Hanson, Francis et al. 2006), therefore studies involving mutagenesis within the arrestin C-tail will elucidate any residues comprising the C-tail that participate in ERK binding, increasing ERK affinity for the arrestin-receptor complex.

To determine how receptor-dependent changes in the arrestin interactions with MAPKs translate into agonist-dependent ERK1/2 activation, we used β2AR, endogenously expressed at physiologically relevant levels, and took advantage

of the available arrestin-biased agonists for this receptor (Azzi, Charest et al. 2003). We found that the expression of WT arrestin-2 and WT arrestin-3 enhanced the phosphorylation of endogenous ERK1/2 in response to β2AR stimulation by the unbiased agonists adrenaline and isoproterenol, antagonists (with low agonist activity (Samama, Cotecchia et al. 1993)) alprenolol and propranolol, as well as arrestin-biased agonists carazolol and ICI118551 (Fig. 20). ERK1/2 activation induced by carazolol and ICI118551, which are inverse agonists for G protein activation, is comparable to that induced by unbiased agonists which can act via G proteins and arrestins (Fig. 20), suggesting that a significant fraction of ERK1/2 is activated via arrestin-mediated scaffolding.

One final question that still remains unanswered is: How is ERK activation mediated by arrestins? One could speculate that the receptor serves as an anchor that brings arrestin close to the plasma membrane where c-Raf1 becomes activated. This would result in ERK2 activation via MEK1. To test this hypothesis, generation of membrane-bound arrestin mutants that do not bind the receptor will be necessary, as well as determination of levels of ERK activation by such mutant. A membrane-bound arrestinR307A mutant, which we know does not interact with c-Raf1, could serve as a control for the quantification of basal ERK2 activation. Another possibility, in terms of arrestin activation of the ERK1/2 signaling cascade, is that the conformation change in arrestin, due to receptor binding, may allow c-Raf1 homo/hetero-dimerization, which is known to be a necessary, yet to be fully understood, mode of activation.

The construction of arrestins capable of increasing ERK1/2 activity could have great therapeutic potential in neurodegenerative diseases, whereas arrestins specifically designed to activate JNK3 could be utilized to oppose the effects of excessive cell proliferation in cancer.

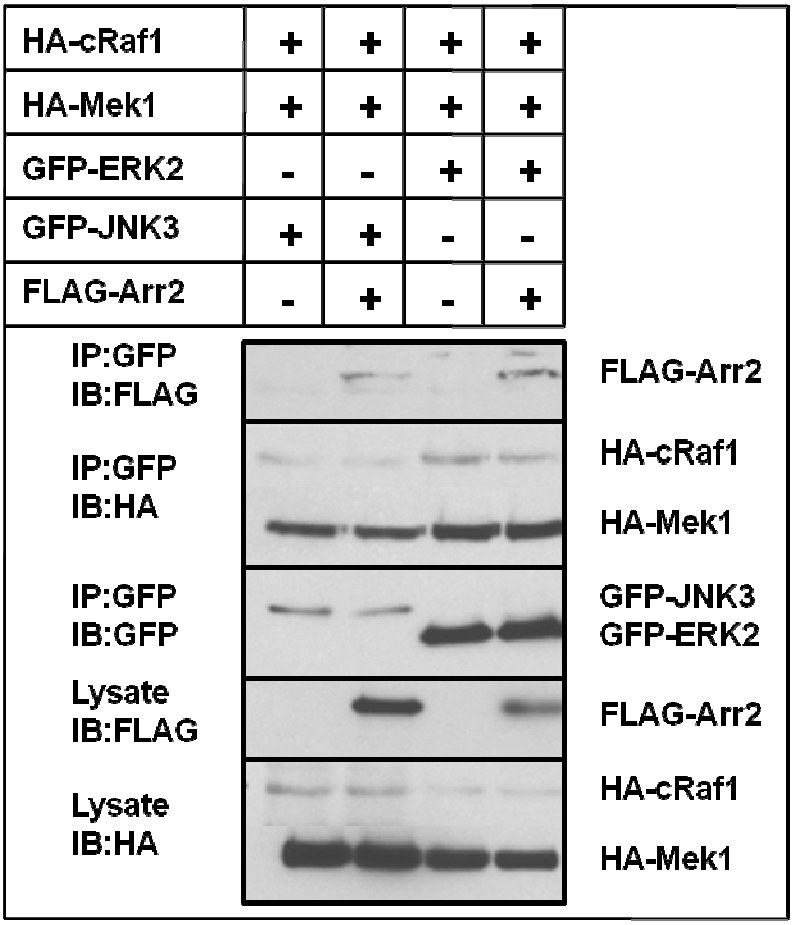

Figure 23. Arrestin-2 scaffolding MAPK cascades. COS-7 cells were transfected with plasmids encoding FLAG-tagged arrestin2, HA-tagged c-Raf1 and MEK1, and GFP-tagged ERK2 and JNK3. ERK2 or JNK3 were immunoprecipitated with anti-GFP antibody, and precipitates were probed for HA, FLAG and GFP antibodies as shown.

REFERENCES

Ahmed, M. R., X. Zhan, et al. (2011). "Ubiquitin ligase parkin promotes Mdm2-arrestin interaction but inhibits arrestin ubiquitination." Biochemistry **50**: 3749-63.

Ahn, S., S. K. Shenoy, et al. (2004). "Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor." J Biol Chem **279**(34): 35518-25.

Ahn, S., H. Wei, et al. (2004). "Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2." J Biol Chem **279**: 7807-7811.

Attramadal, H., J. L. Arriza, et al. (1992). "Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family." J Biol Chem **267**(25): 17882-90.

Azzi, M., P. G. Charest, et al. (2003). "Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors." Proc Natl Acad Sci U S A **100**(20): 11406-11.

Baldwin, J. M., G. F. Schertler, et al. (1997). "An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors." J Mol Biol **272**(1): 144-64.

Benovic, J. L., A. DeBlasi, et al. (1989). "Beta-adrenergic receptor kinase: primary structure delineates a multigene family." Science **246**(4927): 235-40.

Benovic, J. L., F. Mayor, Jr., et al. (1987). "Purification and characterization of the beta-adrenergic receptor kinase." J Biol Chem **262**(19): 9026-32.

Benovic, J. L., J. J. Onorato, et al. (1991). "Cloning, expression, and chromosomal localization of beta-adrenergic receptor kinase 2. A new member of the receptor kinase family." J Biol Chem **266**(23): 14939-46.

Benovic, J. L., R. H. Strasser, et al. (1986). "Beta-adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor." Proc Natl Acad Sci U S A **83**(9): 2797-801.

Bockaert, J. and J. P. Pin (1999). "Molecular tinkering of G protein-coupled receptors: an evolutionary success." EMBO J **18**(7): 1723-9.

Bohn, L. M., R. J. Lefkowitz, et al. (1999). "Enhanced morphine analgesia in mice lacking beta-arrestin 2." Science **286**(5449): 2495-8.

Boulton, T. G., S. H. Nye, et al. (1991). "ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF." Cell **65**(4): 663-75.

Bruchas, M. R., T. A. Macey, et al. (2006). "Kappa opioid receptor activation of p38 MAPK is GRK3- and arrestin-dependent in neurons and astrocytes." J Biol Chem **281**(26): 18081-9.

Carter, J. M., V. V. Gurevich, et al. (2005). "Conformational differences between arrestin2 and pre-activated mutants as revealed by hydrogen exchange mass spectrometry." J Mol Biol **351**: 865-878.

Catling, A. D., H. J. Schaeffer, et al. (1995). "A proline-rich sequence unique to MEK1 and MEK2 is required for raf binding and regulates MEK function." Mol Cell Biol **15**(10): 5214-25.

Celver, J., S. A. Vishnivetskiy, et al. (2002). "Conservation of the phosphate-sensitive elements in the arrestin family of proteins." J. Biol. Chem. **277**(11): 9043-9048.

Chen, W., K. C. Kirkbride, et al. (2003). "Beta-arrestin 2 mediates endocytosis of type III TGF-beta receptor and down-regulation of its signaling." Science **301**(5638): 1394-7.

Chien, E. Y., W. Liu, et al. "Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist." Science **330**(6007): 1091-5.

Choe, H. W., Y. J. Kim, et al. "Crystal structure of metarhodopsin II." Nature **471**(7340): 651-5.

Coffa, S., M. Breitman, et al. "A single mutation in arrestin-2 prevents ERK1/2 activation by reducing c-Raf1 binding." Biochemistry.

Conklin, B. R., Z. Farfel, et al. (1993). "Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha." Nature **363**(6426): 274-6.

Conner, D. A., M. A. Mathier, et al. (1997). "beta-Arrestin1 knockout mice appear normal but demonstrate altered cardiac responses to beta-adrenergic stimulation." Circ Res **81**(6): 1021-6.

DeFea, K. A., Z. D. Vaughn, et al. (2000). "The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex." Proc Natl Acad Sci U S A **97**(20): 11086-91.

DeFea, K. A., J. Zalevsky, et al. (2000). "beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2." J Cell Biol **148**(6): 1267-81.

DeWire, S. M., S. Ahn, et al. (2007). "Beta-arrestins and cell signaling." Annu Rev Physiol **69**: 483-510.

Gesty-Palmer, D., M. Chen, et al. (2006). "Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation." J Biol Chem **281**(16): 10856-64.

Gether, U. and B. K. Kobilka (1998). "G protein-coupled receptors. II. Mechanism of agonist activation." J Biol Chem **273**(29): 17979-82.

Good, M., G. Tang, et al. (2009). "The Ste5 scaffold directs mating signaling by catalytically unlocking the Fus3 MAP kinase for activation." Cell **136**: 1085-97.

Goodman, O. B., Jr., J. G. Krupnick, et al. (1996). "Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor." Nature **383**(6599): 447-50.

Gurevich, E. V. and V. V. Gurevich (2006). "Arrestins are ubiquitous regulators of cellular signaling pathways." Genome Biology **7**: 236.

Gurevich, V. V. (1996). "Use of bacteriophage RNA polymerase in RNA synthesis." Methods Enzymol **275**: 382-97.

Gurevich, V. V. (1998). "The selectivity of visual arrestin for light-activated phosphorhodopsin is controlled by multiple nonredundant mechanisms." J Biol Chem **273**: 15501-15506.

Gurevich, V. V. and J. L. Benovic (1992). "Cell-free expression of visual arrestin. Truncation mutagenesis identifies multiple domains involved in rhodopsin interaction." J Biol Chem **267**(30): 21919-23.

Gurevich, V. V. and J. L. Benovic (1993). "Visual arrestin interaction with rhodopsin. Sequential multisite binding ensures strict selectivity toward light-activated phosphorylated rhodopsin." J Biol Chem **268**(16): 11628-38.

Gurevich, V. V. and J. L. Benovic (1995). "Visual arrestin binding to rhodopsin: diverse functional roles of positively charged residues within the phosphorylation-recignition region of arrestin." J. Biol. Chem. **270**(11): 6010-6016.

Gurevich, V. V. and J. L. Benovic (2000). "Arrestin: mutagenesis, expression, purification, and functional characterization." Methods Enzymol **315**: 422-37.

Gurevich, V. V., S. B. Dion, et al. (1995). "Arrestin interaction with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, b2-adrenergic, and m2 muscarinic cholinergic receptors." J Biol Chem **270**: 720-731.

Gurevich, V. V. and E. V. Gurevich (2004). "The molecular acrobatics of arrestin activation." Trends Pharmacol Sci **25**: 59-112.

Gurevich, V. V. and E. V. Gurevich (2008). "GPCR monomers and oligomers: it takes all kinds." Trends Neurosci **31**(2): 74-81.

Gurevich, V. V. and E. V. Gurevich (2008). "Rich tapestry of G protein-coupled receptor signaling and regulatory mechanisms." Mol Pharmacol **74**(2): 312-6.

Gurevich, V. V. and E. V. Gurevich (2010). "Custom-designed proteins as novel therapeutic tools? The case of arrestins." Expert Rev Mol Med **12**: e13.

Gurevich, V. V., E. V. Gurevich, et al. (2008). "Arrestins as multi-functional signaling adaptors." Handb Exp Pharmacol **186**: 15-37.

Hall, R. A. and R. J. Lefkowitz (2002). "Regulation of G protein-coupled receptor signaling by scaffold proteins." Circ Res **91**(8): 672-80.

Han, M., V. V. Gurevich, et al. (2001). "Crystal structure of beta-arrestin at 1.9 A: possible mechanism of receptor binding and membrane Translocation." Structure **9**(9): 869-80.

Hanson, S. M., W. M. Cleghorn, et al. (2007). "Arrestin mobilizes signaling proteins to the cytoskeleton and redirects their activity." J Mol Biol: in press.

Hanson, S. M., D. J. Francis, et al. (2006). "Visual arrestin binding to microtubules involves a distinct conformational change." J Biol Chem **281**(14): 9765-72.

Hanson, S. M., D. J. Francis, et al. (2006). "Differential interaction of spin-labeled arrestin with inactive and active phosphorhodopsin." Proc Natl Acad Sci U S A **103**(13): 4900-5.

Hanson, S. M. and V. V. Gurevich (2006). "The differential engagement of arrestin surface charges by the various functional forms of the receptor." J Biol Chem **281**(6): 3458-62.

Hirsch, J. A., C. Schubert, et al. (1999). "The 2.8 A crystal structure of visual arrestin: a model for arrestin's regulation." Cell **97**(2): 257-69.

Hjorth, S. A., H. T. Schambye, et al. (1994). "Identification of peptide binding residues in the extracellular domains of the AT1 receptor." J Biol Chem **269**(49): 30953-9.

Ichijo, H. (1999). "From receptors to stress-activated MAP kinases." Oncogene **18**(45): 6087-93.

Jaakola, V. P., M. T. Griffith, et al. (2008). "The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist." Science **322**(5905): 1211-7.

Kang, D. S., R. C. Kern, et al. (2009). "Structure of an arrestin2/clathrin complex reveals a novel clathrin binding domain that modulates receptor trafficking." J Biol Chem **284**: 29860-72.

Kobilka, B. and G. F. Schertler (2008). "New G-protein-coupled receptor crystal structures: insights and limitations." Trends Pharmacol Sci **29**(2): 79-83.

Kohout, T. A., F. S. Lin, et al. (2001). "beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking." Proc Natl Acad Sci U S A **98**(4): 1601-6.

Kovoor, A., J. Celver, et al. (1999). "Targeted construction of phosphorylation-independent b-arrestin mutants with constitutive activity in cells." J. Biol. Chem. **274**: 6831-6834.

Krupnick, J. G., V. V. Gurevich, et al. (1997). "Mechanism of quenching of phototransduction. Binding competition between arrestin and transducin for phosphorhodopsin." J Biol Chem **272**: 18125-31.

Kunapuli, P. and J. L. Benovic (1993). "Cloning and expression of GRK5: a member of the G protein-coupled receptor kinase family." Proc Natl Acad Sci U S A **90**(12): 5588-92.

Lander, E. S., L. M. Linton, et al. (2001). "Initial sequencing and analysis of the human genome." Nature **409**(6822): 860-921.

Laporte, S. A., R. H. Oakley, et al. (1999). "The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis." Proc Natl Acad Sci U S A **96**(7): 3712-7.

Lefkowitz, R. J. (1998). "G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization." J Biol Chem **273**(30): 18677-80.

Lefkowitz, R. J. and S. K. Shenoy (2005). "Transduction of receptor signals by beta-arrestins." Science **308**(5721): 512-7.

Li, J., P. C. Edwards, et al. (2004). "Structure of bovine rhodopsin in a trigonal crystal form." J Mol Biol **343**(5): 1409-38.

Li, X., R. Macleod, et al. (2009). "A scanning peptide array approach uncovers association sites within the JNK/betaarrestin signalling complex." FEBS Lett **583**: 3310-6.

Lin, F. T., Y. Daaka, et al. (1998). "beta-arrestins regulate mitogenic signaling and clathrin-mediated endocytosis of the insulin-like growth factor I receptor." J Biol Chem **273**(48): 31640-3.

Lin, F. T., K. M. Krueger, et al. (1997). "Clathrin-mediated endocytosis of the beta-adrenergic receptor is regulated by phosphorylation/dephosphorylation of beta-arrestin1." J Biol Chem **272**(49): 31051-7.

Lin, F. T., W. E. Miller, et al. (1999). "Feedback regulation of beta-arrestin1 function by extracellular signal-regulated kinases." J Biol Chem **274**(23): 15971-4.

Lohse, M. J., J. L. Benovic, et al. (1990). "Multiple pathways of rapid beta 2-adrenergic receptor desensitization. Delineation with specific inhibitors." J Biol Chem **265**(6): 3202-11.

Lombardi, M. S., A. Kavelaars, et al. (2002). "Role and modulation of G protein-coupled receptor signaling in inflammatory processes." Crit Rev Immunol **22**(2): 141-63.

Lorenz, W., J. Inglese, et al. (1991). "The receptor kinase family: primary structure of rhodopsin kinase reveals similarities to the beta-adrenergic receptor kinase." Proc Natl Acad Sci U S A **88**(19): 8715-9.

Luo, J., J. M. Busillo, et al. (2008). "M3 muscarinic acetylcholine receptor-mediated signaling is regulated by distinct mechanisms." Mol Pharmacol **74**(2): 338-47.

Luttrell, L. M. (2003). "'Location, location, location': activation and targeting of MAP kinases by G protein-coupled receptors." J Mol Endocrinol **30**: 117-26.

Luttrell, L. M., S. S. Ferguson, et al. (1999). "Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes." Science **283**(5402): 655-61.

Luttrell, L. M. and R. J. Lefkowitz (2002). "The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals." J Cell Sci **115**(Pt 3): 455-65.

Luttrell, L. M., F. L. Roudabush, et al. (2001). "Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds." Proc Natl Acad Sci U S A **98**(5): 2449-54.

Macdonald, S. G., C. M. Crews, et al. (1993). "Reconstitution of the Raf-1-MEK-ERK signal transduction pathway in vitro." Mol Cell Biol **13**(11): 6615-20.

Macey, T. A., J. D. Lowe, et al. (2006). "Mu opioid receptor activation of ERK1/2 is GRK3 and arrestin dependent in striatal neurons." J Biol Chem **281**: 34515-24.

McDonald, P. H., C. W. Chow, et al. (2000). "Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3." Science **290**(5496): 1574-7.

Meng, D., M. J. Lynch, et al. (2009). "MEK1 binds directly to betaarrestin1, influencing both its phosphorylation by ERK and the timing of its isoprenaline-stimulated internalization." J Biol Chem **284**(17): 11425-35.

Metaye, T., H. Gibelin, et al. (2005). "Pathophysiological roles of G-protein-coupled receptor kinases." Cell Signal **17**(8): 917-28.

Milano, S. K., Y. M. Kim, et al. (2006). "Nonvisual arrestin oligomerization and cellular localization are regulated by inositol hexakisphosphate binding." J Biol Chem **281**: 9812-23.

Milano, S. K., H. C. Pace, et al. (2002). "Scaffolding functions of arrestin-2 revealed by crystal structure and mutagenesis." Biochemistry **41**(10): 3321-3328.

Miller, W. E. and R. J. Lefkowitz (2001). "Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking." Curr Opin Cell Biol **13**(2): 139-45.

Miller, W. E., S. Maudsley, et al. (2000). "beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis." J Biol Chem **275**(15): 11312-9.

Miller, W. E., P. H. McDonald, et al. (2001). "Identification of a motif in the carboxyl terminus of beta -arrestin2 responsible for activation of JNK3." Journal of Biological Chemistry **276**(30): 27770-7.

Moukhametzianov, R., T. Warne, et al. "Two distinct conformations of helix 6 observed in antagonist-bound structures of a beta1-adrenergic receptor." Proc Natl Acad Sci U S A **108**(20): 8228-32.

Muller, A., B. Homey, et al. (2001). "Involvement of chemokine receptors in breast cancer metastasis." Nature **410**(6824): 50-6.

Ohguro, H., K. Palczewski, et al. (1994). "Topographic study of arrestin using differential chemical modifications and hydrogen/deuterium exchange." **Protein Sci**(3): 2428-2434.

Ohyama, K., Y. Yamano, et al. (1995). "Disulfide bridges in extracellular domains of angiotensin II receptor type IA." Regul Pept **57**(2): 141-7.

Overington, J. P., B. Al-Lazikani, et al. (2006). "How many drug targets are there?" Nat Rev Drug Discov **5**(12): 993-6.

Palczewski, K., T. Kumasaka, et al. (2000). "Crystal structure of rhodopsin: A G protein-coupled receptor." Science **289**(5480): 739-45.

Pan, L., E. V. Gurevich, et al. (2003). "The nature of the arrestin x receptor complex determines the ultimate fate of the internalized receptor." J Biol Chem **278**(13): 11623-32.

Papasaikas, P. K., P. G. Bagos, et al. (2003). "A novel method for GPCR recognition and family classification from sequence alone using signatures derived from profile hidden Markov models." SAR QSAR Environ Res **14**(5-6): 413-20.

Park, J. H., P. Scheerer, et al. (2008). "Crystal structure of the ligand-free G-protein-coupled receptor opsin." Nature **454**(7201): 183-7.

Perry, S. J. and R. J. Lefkowitz (2002). "Arresting developments in heptahelical receptor signaling and regulation." Trends Cell Biol **12**(3): 130-8.

Pierce, K. L. and R. J. Lefkowitz (2001). "Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors." Nat Rev Neurosci **2**(10): 727-33.

Pierce, K. L., L. M. Luttrell, et al. (2001). "New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades." Oncogene **20**(13): 1532-9.

Premont, R. T. (2005). "Once and future signaling: G protein-coupled receptor kinase control of neuronal sensitivity." Neuromolecular Med **7**(1-2): 129-47.

Premont, R. T., W. J. Koch, et al. (1994). "Identification, purification, and characterization of GRK5, a member of the family of G protein-coupled receptor kinases." J Biol Chem **269**(9): 6832-41.

Pulvermuller, A., K. Schroder, et al. (2000). "Interactions of metarhodopsin II. Arrestin peptides compete with arrestin and transducin." J. Biol. Chem. **275**: 37679-37685.

Rasmussen, S. G., B. T. Devree, et al. "Crystal structure of the beta(2) adrenergic receptor-Gs protein complex." Nature.

Rockman, H. A., W. J. Koch, et al. (2002). "Seven-transmembrane-spanning receptors and heart function." Nature **415**(6868): 206-12.

Samama, P., S. Cotecchia, et al. (1993). "A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model." J Biol Chem **268**: 4625-4636.

Schaeffer, H. J. and M. J. Weber (1999). "Mitogen-activated protein kinases: specific messages from ubiquitous messengers." Mol Cell Biol **19**(4): 2435-44.

Scheerer, P., J. H. Park, et al. (2008). "Crystal structure of opsin in its G-protein-interacting conformation." Nature **455**(7212): 497-502.

Scott, M. G., E. Le Rouzic, et al. (2002). "Differential nucleocytoplasmic shuttling of beta-arrestins. Characterization of a leucine-rich nuclear export signal in beta-arrestin2." J Biol Chem **277**(40): 37693-701.

Shenoy, S. K., M. T. Drake, et al. (2006). "beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor." J Biol Chem **281**: 1261-1273.

Shenoy, S. K. and R. J. Lefkowitz (2003). "Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination." J Biol Chem **278**(16): 14498-506.

Shenoy, S. K. and R. J. Lefkowitz (2005). "Angiotensin II-stimulated signaling through G proteins and beta-arrestin." Sci STKE **2005**(311): cm14.

Shimamura, T., M. Shiroishi, et al. "Structure of the human histamine H1 receptor complex with doxepin." Nature **475**(7354): 65-70.

Song, X., S. Coffa, et al. (2009). "How does arrestin assemble MAPKs into a signaling complex?" J Biol Chem **284**(1): 685-95.

Song, X., E. V. Gurevich, et al. (2007). "Cone arrestin binding to JNK3 and Mdm2: conformational preference and localization of interaction sites." J Neurochem **103**: 1053-62.

Song, X., D. Raman, et al. (2006). "Visual and both non-visual arrestins in their "inactive" conformation bind JNK3 and Mdm2 and relocalize them from the nucleus to the cytoplasm." J Biol Chem **281**(30): 21491-9.

Standfuss, J., P. C. Edwards, et al. "The structural basis of agonist-induced activation in constitutively active rhodopsin." Nature **471**(7340): 656-60.

Standfuss, J., G. Xie, et al. (2007). "Crystal structure of a thermally stable rhodopsin mutant." J Mol Biol **372**(5): 1179-88.

Sterne-Marr, R., V. V. Gurevich, et al. (1993). "Polypeptide variants of beta-arrestin and arrestin3." J Biol Chem **268**(21): 15640-8.

Strasser, R. H., D. R. Sibley, et al. (1986). "A novel catecholamine-activated adenosine cyclic 3',5'-phosphate independent pathway for beta-adrenergic receptor phosphorylation in wild-type and mutant S49 lymphoma cells: mechanism of homologous desensitization of adenylate cyclase." Biochemistry **25**(6): 1371-7.

Sun, Y., Z. Cheng, et al. (2002). "Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation." J Biol Chem **277**(51): 49212-9.

Sutton, R. B., S. A. Vishnivetskiy, et al. (2005). "Crystal Structure of Cone Arrestin at 2.3Å: Evolution of Receptor Specificity." J Mol Biol **354**: 1069-1080.

Tohgo, A., K. L. Pierce, et al. (2002). "beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation." J Biol Chem **277**(11): 9429-36.

van Koppen, C. J. (2001). "Multiple pathways for the dynamin-regulated internalization of muscarinic acetylcholine receptors." Biochem Soc Trans **29**(Pt 4): 505-8.

Venter, J. C., M. D. Adams, et al. (2001). "The sequence of the human genome." Science **291**(5507): 1304-51.

Vishnivetskiy, S. A., D. J. Francis, et al. (2010). "The role of arrestin alpha-helix I in receptor binding." J. Mol. Biol. **395**: 42-54.

Vishnivetskiy, S. A., L. E. Gimenez, et al. "Few Residues within an Extensive Binding Interface Drive Receptor Interaction and Determine the Specificity of Arrestin Proteins." J Biol Chem **286**(27): 24288-99.

Vishnivetskiy, S. A., J. A. Hirsch, et al. (2002). "Transition of arrestin in the active receptor-binding state requires an extended interdomain hinge." J. Biol. Chem. **277**(46): 43961-43968.

Vishnivetskiy, S. A., M. M. Hosey, et al. (2004). "Mapping the arrestin-receptor interface: structural elements responsible for receptor specificity of arrestin proteins." J. Biol. Chem. **279**(2): 1262-1268.

Vishnivetskiy, S. A., C. L. Paz, et al. (1999). "How does arrestin respond to the phosphorylated state of rhodopsin?" J Biol Chem **274**(17): 11451-4.

Vishnivetskiy, S. A., D. Raman, et al. (2007). "Regulation of arrestin binding by rhodopsin phosphorylation level." J Biol Chem **282**: 32075-83.

Vishnivetskiy, S. A., C. Schubert, et al. (2000). "An additional phosphate-binding element in arrestin molecule: implications for the mechanism of arrestin activation." J. Biol. Chem. **275**(52): 41049-41057.

Wang, P., Y. Wu, et al. (2003). "Subcellular localization of beta-arrestins is determined by their intact N domain and the nuclear export signal at the C terminus." J Biol Chem **278**(13): 11648-53.

Warne, T., R. Moukhametzianov, et al. "The structural basis for agonist and partial agonist action on a beta(1)-adrenergic receptor." Nature **469**(7329): 241-4.

Warne, T., M. J. Serrano-Vega, et al. (2008). "Structure of a beta1-adrenergic G-protein-coupled receptor." Nature **454**(7203): 486-91.

Wess, J. (1998). "Molecular basis of receptor/G-protein-coupling selectivity." Pharmacol Ther **80**(3): 231-64.

Wilden, U. (1995). "Duration and amplitude of the light-induced cGMP hydrolysis in vertebrate photoreceptors are regulated by multiple phosphorylation of rhodopsin and by arrestin binding." Biochemistry **34**: 1446-54.

Wilden, U., S. W. Hall, et al. (1986). "Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments." Proc Natl Acad Sci USA **83**: 1174-1178.

Willars, G. B. (2006). "Mammalian RGS proteins: multifunctional regulators of cellular signalling." Semin Cell Dev Biol **17**(3): 363-76.

Wu, B., E. Y. Chien, et al. "Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists." Science **330**(6007): 1066-71.

Wu, N., S. M. Hanson, et al. (2006). "Arrestin binding to calmodulin: a direct interaction between two ubiquitous signaling proteins." J Mol Biol **364**(5): 955-63.

Wu, X., S. J. Noh, et al. (1996). "Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated Hela cells." J Biol Chem **271**(6): 3265-71.

Xu, F., H. Wu, et al. "Structure of an agonist-bound human A2A adenosine receptor." Science **332**(6027): 322-7.

Zhan, X., L. E. Gimenez, et al. "Crystal structure of arrestin-3 reveals the basis of the difference in receptor binding between two non-visual subtypes." J Mol Biol **406**(3): 467-78.