Identification of a Nuclear Localization Sequence in Beta-Arrestin1:

Implications in NF- kB Activation

BY

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THESIS

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LIST OF ABBREVIATIONS

B2BKR:	B2 Bradykinin Receptor
BK:	Bradykinin
CBP:	CREB-binding protein
Cir:	circumference
DAPI:	4',6-diamidino-2-phenylindole
DTT:	Dithiothreitol
ERK:	Extracellular signal-regulated Kinase
FBS:	Fetal Bovine Serum
GAPDH:	Glyceraldehyde 3-phosphate Dehydrogenase
GFP:	Green Fluorescent Protein
GPCR:	G-Protein-Coupled Receptor
GRK:	G Protein-Coupled Receptor Kinase
HBSS:	Hank's Balanced Salt Solution
HDAC:	Histone Deacetylase
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP:	Horseradish Peroxidase
ΙκΒα:	Inhibitor of kappa B alpha
IP:	Immunoprecipitation
JNK:	c-Jun N terminal Kinase

LIST OF ABBREVIATIONS (continued)

- NES: Nuclear Export Sequence
- NF-κB: Nuclear Factor-kappa B
- NLS: Nuclear Localization Sequence
- PFA: para-Formaldehyde
- PPARγ: Peroxisome Proliferator-activated Receptor gamma
- PTEN: Phosphatase and Tensin Homolog Protein
- STAT: Signal Transducer and Activator of Transcription
- TBS: Tris-buffered Saline
- TRAF6: TNF Receptor Associated Factor

SUMMARY

At roughly 4% of the protein coding genes expressed, G Protein-Coupled Receptors (GPCRs) are the largest family of proteins encoded in the human genome. Activation of GPCRs results in a variety of cellular responses, including regulation of transcription. Of specific interest is the regulation of Nuclear Factor Kappa B (NF- κ B), a transcription factor family intimately involved in mediation of immune and inflammatory responses. Several lines of research have provided detailed analysis of direct GPCR-heterotrimeric G protein involvement in NF- κ B regulation. The aim of this work was to elucidate the role of protein mediators of GPCR activity, such as the β arrestins, in NF- κ B regulation.

The β -arrestin family is comprised of β -arrestin1 and β -arrestin2, which regulate numerous cell functions. Initially identified for their roles in desensitization of GPCRs, the β -arrestins are now appreciated as critical scaffolds in a variety of cellular signaling cascades. While both members of the β -arrestin family share many traits, one key difference is their cellular localization. Specifically, β -Arrestin2 is excluded from the nucleus, whereas β -Arrestin1 is expressed ubiquitously throughout the cell.

Recent studies have identified β -Arrestin1 as a key regulator of numerous nuclear signaling events. Nonetheless, the mechanism(s) governing β -Arrestin1 nuclear localization remains unknown. The β -arrestin proteins are too large to passively diffuse into the nucleus and must be actively transported in an energy-dependent process. To further explore the nuclear functions of β -arrestin1, it is important to create a tool to allow the analysis of nuclear specific events. Using DNA mutagenesis of wild type β -Arrestin1, we identified a region of seven amino acids as being critical to nuclear localization of β -arrestin1. We identified these residues as necessary to

SUMMARY (continued)

mediate nuclear import of β-arrestin1 via interaction with importin β1. Further characterization of this region identified Lys157 as being essential for β-arrestin1 nuclear localization. Functionally, nuclear localization of β-arrestin1 is important for enhanced NF- κ B activation following bradykinin (BK) stimulation, and β-arrestin1 mutants that are not localized in the nucleus are incapable of potentiating NF- κ B activation. We determined that loss of function by β-arrestin1 mutants occurs due to a lack of post-translational modification to the DNA-binding subunit p65/RelA. These modifications are likely to influence DNA binding, as there is a decrease in overall protein-DNA interaction as well as a specific loss of binding of p65/RelA. This loss of promoter binding ultimately leads to a decrease in transcription of NF- κ B targets, specifically IL-1β.

Dysregulation of NF- κ B is known to cause a variety of inflammatory and other cellular diseases. This work provides insight into the mechanism by which NF- κ B is regulated through the orchestration of a series of modification events by the scaffold protein β -Arrestin1. Understanding these interactions is important for the management of disease and development of interventions. Furthermore, given the potential significance of β -Arrestin1 mediated nuclear events the description of the nuclear localization sequence (NLS) of β -Arrestin1 provides a tool that will prove invaluable for future analysis of this critical protein's role in nuclear signaling and transcriptional regulation.

CHAPTER I: INTRODUCTION

<u>A. Nuclear Factor kappa B</u>

A.1 NF-KB Composition and Function

Originally discovered bound to the immunoglobin kappa chain gene and named Nuclear Factor-(kappa) B, the NF-κB family of transcription factors contains a number of structurally related members which are ubiquitously expressed in all cell types (Sen and Baltimore 1986; Ghosh, May et al. 1998). Since their discovery, the NF-κB transcription factors have become recognized for their importance in cell death, proliferation, and as key mediators of immunological processes. Dysregulation of NF-κB has been linked to cancer, inflammatory diseases, sepsis, atherosclerosis and developmental delays, among others. Currently, there are five known members of the NF-κB family: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel. All NF-κB proteins contain a Rel homology domain (RHD) in their N terminus that contain a nuclear localization signal (NLS) and is responsible for DNA binding. In addition, the RHD is responsible for dimerization, which is required for NF-κB activation. The most common NF-κB dimer found in mammalian cells is the p50/p65 heterodimer, it is known as the 'classical' pathway (Chen, Castranova et al. 1999). This is also the form of NF-κB that we observe in our system, and only this classical NF-κB pathway will be discussed in the thesis.

A.2 Activation of NF-KB

In unstimulated cells, NF- κ B is retained in the cytoplasm via interaction with an inhibitory protein, I κ B α (Figure 1). Upon stimulation, the I κ B protein is serine phosphorylated by a kinase

in the IKK complex. This phosphorylation event marks the $I\kappa B$ protein for ubiquitin mediated proteasomal degradation and the release of NF- κB (Hoffmann, Natoli et al. 2006). The free NF- κB dimer can then go on to mediate a response in the nucleus . NF- κB can be activated by a wide array of stimulants, including cytokines, oxidants, viruses, chemokines, and UV radiation (Rothwarf and Karin 1999).

Once in the nucleus, NF- κ B binds to chromatin by recognition of gene promoter regions containing the κ B consensus sequence, gggRnYYYcc, where R represents a purine and Y is a pyrimidine (Rothwarf and Karin 1999). Once bound to DNA, NF- κ B drives the expression of genes mostly involved with inflammation and cell survival however the number of potential kB binding sites within the human genome is extensive at over 3000 possible sites representing genes in a wide variety of cellular processes. A schematic representation of NF- κ B activation is presented in Figure 1. Selective regulation of specific genes expressed after NF- κ B activation is controlled by several factors, including the proximity of supporting transcription factors, interaction with chromatin remodeling proteins, and DNA architecture at the promoter (Natoli, Saccani et al. 2005; Perkins and Gilmore 2006). In addition to these factors, post-translational modifications of the NF- κ B activity is tightly controlled, and includes many mechanisms to ensure rapid termination of signaling once the job is done. For example, activation of NF- κ B serves to regulate itself as the gene for I κ B α is under control of a κ B promoter (Pahl 1999).

Figure 1. Overview of NF-kB Activation

In most cells, NF- κ B is held inactive in the cytosol via interaction with the inhibitory $I\kappa B\alpha$ protein. Following agonist activation by any number of inducers of NF- κ B, the $I\kappa B\alpha$ protein is phosphorylated by $I\kappa$ Kinase (IKK). This phosphorylation event release the NF- κ B dimer and marks IkBa for proteosomal degradation. Once free, the NF- κ B molecule is able to translocate to the nucleus. Once there it recognizes its consensus binding sequence on DNA and binds, thereby initiating a transcriptional response.



A.3 Regulation of NF-KB by Post-Translational Modifications

The p65/RelA subunit of NF-kB is subject to a variety of post-translational modifications to regulate its function. Published reports indicate that p65/RelA is modified in the nucleus by acetylation, which regulates its efficiency of transcriptional control (Chen, Mu et al. 2002; Greene and Chen 2004). p65/RelA contains several potential acetylation sites including lysines 122,123,218,221,310, 314, and 315 (Chen, Fischle et al. 2001; Chen, Mu et al. 2002; Kiernan, Bres et al. 2003; Buerki, Rothgiesser et al. 2008). The exact function of acetylation of NF-κB seems to be to impart yet another level of specificity on NF-kB dependent gene expression, as it can serve as both an activating and inhibiting signal. For example, acetylation of lysine 218 inhibits the binding of p65/RelA to IkBa which would otherwise move NF-kB from the nucleus to the cytoplasm and mutation of lysine 310 leads to a significant impairment of transactivation of NF-kB (Chen, Mu et al. 2002). In contrast, acetylation of p65/RelA at lysines 122, 123, 314, and 315 led to an inhibition of NF-kB dependent gene expression (Kiernan, Bres et al. 2003; Rothgiesser, Fey et al. 2010). It should be noted that lysines 218, 221, and 310 were found to be the major acetylation sites (Chen, Mu et al. 2002), suggesting acetylation is predominatly an activating signal on NF-κB.

Functionally, acetylation of p65/RelA appears to be regulated by its inducible phosphorylation (Chen, Williams et al. 2005). Multiple p65/RelA phosphorylation sites have now been described and include serines 205, 267, 276, 281, 311, 468, 471, 529, and 536; as well as threonines 254, 435, and 505 (Perkins 2006). While phosphorylation at any site listed has an impact on the transcriptional activity of p65/RelA, phosphorylation of serine 276 and 536 appear to be the most critical in regulating interaction of coactivator and repressor proteins to mediate acetylation

(Chen, Williams et al. 2005). While they are both critical for further regulation, S276 and S536 are regulated differently. Phosphorylation of S276 occurs in the nucleus by MSK1 and PKA (Zhong, Voll et al. 1998; Vermeulen, De Wilde et al. 2003) and regulates the association of p65/RelA with the histone deactylase HDAC1 (Ashburner, Westerheide et al. 2001; Zhong, May et al. 2002). Release of HDAC1 from p65/RelA allows for the efficient recruitment of p300/CBP to mediate acetylation of lysine 310 (Chen, Williams et al. 2005). In contrast, S536 is phosphorylated by a variety of kinases including IKK, TBK1, RSK1, Akt, and PKA (Zhong, SuYang et al. 1997; Sakurai, Chiba et al. 1999; Sizemore, Leung et al. 1999; Sizemore, Lerner et al. 2002; Jiang, Takahashi et al. 2003; Buss, Dorrie et al. 2004). In general it is believed these phosphorylation events occur in the cytoplasm and regulate both the interaction of p65/RelA with IkBα (Sizemore, Lerner et al. 2002) and HDAC3 (Hoberg, Popko et al. 2006). In addition, S536 is reported to be dephosphorylated in the nucleus (Sakurai, Suzuki et al. 2003).

B. Activation of NF-KB by G Protein-Coupled Receptors

Many of the hundreds of diverse inducers of NF-κB signal through G Protein-Coupled Receptors. Examples include bacterial and fungal products, inflammatory cytokines, environmental hazards, drugs, hormones, and various chemical agents.

B.1 Introduction to G Protein-Coupled Receptor Signaling

G Protein-Coupled Receptors (GPCRs) are a large family of cell surface receptors responsible for sensing a diverse array of stimulants. GPCR activation is responsible for the sense of taste and smell, as well as intimately involved in the immune response, growth, development, and reproduction. Due to its size, GPCRs are grouped into a protein superfamily. This superfamily can be further broken into 3 broad classes, based on sequence homology between its members. There is no discernable homology between the classes. By far the largest class is the class A, or rhodopsin-like, GPCRs, comprising nearly 85% of all known GPCRs. The other classes of GPCRs include the secretin like, pheromone receptors, and various non-canonical types of GPCRs. (Bjarnadottir, Gloriam et al. 2006). All GPCRs possess seven transmembrane alpha helices. The receptor is oriented with it N terminal region exposed on the extracellular surface, and its C terminal tail in the cytoplasm. Structurally, the receptor orients within the membrane into somewhat of a barrel which provides a cavity for agonist binding (Palczewski, Kumasaka et al. 2000; Rasmussen, Choi et al. 2007). GPCRs are activated when an agonist binds to the extracellular face, inducing a conformational change in the receptor. The mechanical shape change of the receptor leads to activation of receptor bound heterotrimeric G proteins.

G proteins are essentially molecular switches. Exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) regulates the on and off of the switch. There are four classes of heterotrimeric G proteins, which are divided by sequence homology. Cellular function of the G proteins is determined by the composition of their component members. There are twenty different alpha subunits, five beta subunits, and twelve gamma subunits from which the trimer can be built. Signal amplification cascades are initiated by activated G proteins through interaction with their various effector molecules (Cabrera-Vera, Vanhauwe et al. 2003). An overview of G protein families and effectors is shown in Figure 2.

Figure 2. G Protein Families and Their Function

In (A), general grouping of G proteins. There are 4 families of heterotrimeric G proteins, grouped according to sequence homology. They are Gi, Gs, Gq, and G12/13. Within each family there are several submembers based on the specific composition of the alpha subunit. There are 20 alpha subunits in total. In addition, diversity in trimer formation can also be accomplished through the rearrangement of the 5 beta and 12 gamma members. Each G protein family couples to a different set of effector molecules to activate a cellular response, shown in (**B**).



B.2 β -Arrestins are GPCR signaling molecules

In addition to canonical signaling through heterotimeric G proteins, GPCRs are also able to modulate cellular responses through G protein independent methods. One such mechanism is by interaction with β -Arrestin molecules.

B.2.1 Identification and Initial Characterization of Arrestin Proteins

The arrestin family of proteins is comprised of four homologous proteins (Arrestin 1-4) involved in regulation of cellular signaling. The first arrestin protein, retinal arrestin (arrestin1, S-antigen), was first identified as a binding partner of light-activated rhodopsin in rod cells (Pfister, Chabre et al. 1985; Wilden, Wust et al. 1986), and found to be involved in terminating its G protein signaling in concert with rhodopsin kinase (GRK1) (Kuhn and Wilden 1987). Retinal arrestin together with its cone counterpart, arrestin-C (arrestin4, X-arrestin), comprise the visual arrestins (Murakami, Yajima et al. 1993). Based on the observation that the β 2-adrenergic receptor was also desensitized in a manner similar to rhodopsin the hunt began for additional arrestin-like proteins and shortly therafter, two non-visual homologues of retinal arrestin were also identified. Once identified these proteins were named β -arrestin1 (arrestin2) and β -arrrestin2 (arrestin3) due to their function in the desensitization of the β^2 adrenergic receptor (Benovic, Kuhn et al. 1987; Lohse, Benovic et al. 1990; Attramadal, Arriza et al. 1992; Sterne-Marr, Gurevich et al. 1993). While the visual arrestins are specifically expressed in ocular cells, the β -arrestins are found in almost all cell types, with highest expression in the brain, peripheral blood leukocytes, and the lung (Lohse, Benovic et al. 1990; Parruti, Peracchia et al. 1993). The β-arrestins are highly conserved through evolution, sharing 67% sequence homology from *c.elegans* to humans (Alvarez 2008). This high level of conservation of the arrestin protein supports the notion of its

fundamental importance to basic cellular function. This idea is supported by data indicating that knock-out of the single arrestin gene in D. melanogaster, kurtz, is lethal (Roman, He et al. 2000). In mice, knock-out of both β -arrestin proteins is lethal whereas single knock-outs are viable presumably due to compensatory effects of the remaining alternative arrestin isoform (Kohout, Lin et al. 2001). Phylogenetic analysis of β -arrestin is shown in Figure 3. Each β -arrestin molecule exists as two splice variants, short and long. B-arrestin1 long can be identified by the addition of a 24 base pair segment, leading to the generation of an 8-residue addition (Parruti, Peracchia et al. 1993). Similarly, β -arrestin2 long can be distinguished due to an 11-residue insertion. The short isoform predominates in most somatic tissues (Sterne-Marr, Gurevich et al. 1993). β -arrestin1 appears to expressed at higher levels than β -arrestin2 in most tissues (Dawson, Arriza et al. 1993). Structurally, the β-arrestins are comprised of a two domain structure, N and C, which each form a 7 stranded β-sandwich (Han, Gurevich et al. 2001). Motifs required for GPCR internalization are generally believed to be located in the C domain, while motifs responsible for protein interactions are believed to be in the N domain (Luttrell 2005). A cartoon diagram depicting the structural domains of the β -arrestins is shown in Figure 4.

B.2.2 β -Arrestin interaction with G Protein Coupled Receptors

 β -arrestins have been shown to have direct interaction with GPCRs (Sohlemann, Hekman et al. 1995; Oakley, Laporte et al. 2001; Marion, Oakley et al. 2006). β -arrestins are involved in the regulation of GPCR signaling in three ways: a) homologous desensitization of the agonist activated, phosphorylated GPCR, b) internalization of the desensitized receptor, and c) signaling, either directly and independently of G proteins, or as scaffolds for other signaling molecules downstream of the receptor. In homologous desensitization, β -arrestins bind to GPCRs that have



Figure 3. Phylogentic Analysis of β-Arrestin Proteins

Evolutionary conservation of the β -arrestin family of proteins. Multiple protein sequence alignment was performed on the following arrestin genes using MUSCLE. NP_004032.2 (Homo Sapiens) β Arrestin1, NP_004032.2 (Pan Troglodytes), XP_850043.1 (Canis *lupus familiaris*), NP_776668.1 (*Bos taurus*), NP_796205.1 (*Mus musculus*), NP_037042.1 (*Rattus norvegicus*), XP_001337184.1 (*Danio rerio*), NP_524988.1 (*Drosophila melanogaster*), XP_317960.1 (*Anopheles gambiae*), NP_508183.1 (*Caenorhabditis elegans*).

Phylogenetic analysis performed on multiple alignment using tree view function of Homologene. MUSCLE is available at http://www.drive5. com/muscle. Homologene database canbe found at http://www.ncbi.nlm.nih.gov/homologene/



Figure 4. Cartoon Diagram of β-arrestin Structure

In humans there are two β -arrestin isoforms, β -arrestin1 and β -arrestin2. Both isoforms exist as two splice variants, generating a long and short isoform of each. The β -arrestin proteins are comprised of 2 β -sandwich domains (termed N and C domain) separated by a linker region which also serves as a phosphate sensor. There are N-and C- terminal regulatory regions which are involved in conformational dependent changes induced by binding active, phosphorylated receptors. The N-domain of β -arrestins have been shown to be important for nuclear localization, while the C-domain of β -arrestin2 contains a functional nuclear export signal. Both domains also contain residues important for IP6 binding which is known to regulate the homo- and hetero- oligomerization of the β -arrestin proteins.

been activated. The β-arrestin molecules preferentially bind to GPCRs which have been phosphorylated on their C terminal tails by GRKs (Pippig, Andexinger et al. 1993; Vaughan, Millman et al. 2006; Violin, Ren et al. 2006). Because of this interaction, β -arrestins effectively desensitize GPCRs by physically limiting their ability to associate with heterotrimeric G proteins (Shenoy and Lefkowitz 2003). Arrestins also play a role in the internalization of GPCRs, which once endocytosed can then be degraded or recycled back to the plasma membrane during resensitization (Ahn, Nelson et al. 2003). *β*-arrestin's role in the desensitization and internalization of receptors is supported by their interaction with several members of the endocytic machinery, including AP-2, clathrin, and dynamin (Oakley, Laporte et al. 1999). The affinity of arrestin-GPCR interaction in the internalization process defines 2 sets of receptors. Class 'A' GPCRs (β_2 Adrenergic (B_2 -AR), Endothelin-1a (ET-1a), and α 1-Adrenergic (α 1-AR)), have a preference for ßarrestin2, and bind only transiently. Class 'B' receptors (Angiotensin_{1a} $(AT_{1a}R)$, Vasopressin 2 (V2R)), on the other hand, bind to both β -arrestins tightly and stay associated after internalization (Oakley, Laporte et al. 2000; Kohout, Lin et al. 2001). The ability of the arrestin to bind to the GPCR appears to be regulated at the arrestin level by many factors including, phosphorylation, ubiquitination, and nitrosylation (Lin, Krueger et al. 1997; Lin, Chen et al. 2002; Shenoy and Lefkowitz 2005; Ozawa, Whalen et al. 2008). These post-translational modifications to the β -arrestin molecule can act as both activating and inhibiting signals. For example, the addition of a ubiquitin moiety to β -arrestin has been shown to increase its affinity for GPCRs, whereas the phosphorylation of β -arrestin by casein kinase II, GRK5, and other kinases decreases its affinity for the receptor (Kim, Barak et al. 2002; Barthet, Carrat et al. 2009) (Shenoy, Modi et al. 2009).

B.2.3 β -Arrestins as Signal Integrators and Scaffolds

Initially identified for their roles in desensitization of GPCRs, the β -arrestins are now appreciated as critical scaffolds in a variety of cellular signaling cascades. The β -arrestins interact with a multitude of intracellular signaling proteins including Src family tyrosine kinases, PI3K, and Akt (Miller, Maudsley et al. 2000; Penela, Elorza et al. 2001; Povsic, Kohout et al. 2003; Yang, He et al. 2009). The most well studied pathway in which β -arrestins interact is the MAP Kinase pathway. Both arrestins have been implicated in scaffolding the MAP Kinases JNK3 and ERK (Song, Raman et al. 2006). Of specific interest to this work, , β -arrestins interact with several members of the nuclear factor kappa B (NF- κ B) signaling pathway, including TRAF6, p105, and the inhibitory protein I κ B α (Witherow, Garrison et al. 2004; Parameswaran, Pao et al. 2006; Wang, Tang et al. 2006). β -arrestins also interact with the upstream activating kinases, I κ B kinase α , β , and NF- κ B inducing kinase (Gao, Sun et al. 2004). The interactions of β -Arrestin and NF- κ B will be discussed more fully in later sections.

In addition to its role in scaffolding signaling pathways within the cytosol, β -arrestin1 is known to mediate signaling within the nucleus. β -arrestin1has been shown to mediate PPAR γ receptor (Zhuang, Hu et al. 2011), PTEN (Palmitessa and Benovic 2010), and Polycomb group proteins (Yue, Kang et al. 2009) interactions in the nucleus. Again, the role of β -arrestin1 in enhancing or inhibiting a response is mixed. For example, in studies conducted by different groups, β -arrestin1 was found to act as an inhibitor of STAT1-mediated transcription following IFN- γ stimulation (Mo, Zhang et al. 2008), but serve as an activator of CREB-mediated transcription following δ opioid receptor activation(Kang, Shi et al. 2005). These data indicate that β -arrestin1 can facilitate both activation and repression depending on cell type and stimulus. In all reported cases, β -arrestin1 acts essentially by a scaffolding mechanism in the nucleus and the cytosol, recruiting specific proteins for complex formation.

B.2.4 Differential Subcellular Localization of β -Arrestins

While mounting evidence demonstrates the nuclear functions of β -arrestin1, the regulatory mechanism for its nuclear localization is still unknown. At approximately 48 kDa the β-arrestin proteins are too large to passively diffuse into the nucleus and must be actively transported in an energy-dependent process (Terry, Shows et al. 2007). Work on the nuclear function of the β arrestins has been primarily limited to β -arrestin1, because β -arrestin2 has a strong and functional nuclear export signal (NES) in its C- terminus, not present in β-Arrestin1, which excludes it from sustained presence in the nucleus (Scott, Le Rouzic et al. 2002). While the presence of a nuclear localization sequence (NLS) in the β -Arrestins is a debated subject several published reports strongly suggest that such a motif exists. In an *in vivo* import assay in yeast it was shown that β -arrestin2 is actively imported into the nucleus (Scott, Le Rouzic et al. 2002). In addition, it has been noted that an intact N-domain (residues 1-186) is critical for nuclear localization of β -arrestins (Wang, Wu et al. 2003). In the case of visual arrestin, interaction with cytosolic binding partners, such as microtubules, also influences the redistribution of the arrestin (Nair, Hanson et al. 2004; Nair, Hanson et al. 2005). Furthermore, the β -arrestins both homoand hetero-oligomerize in an IP6 dependent manner and this oligomerization has been shown to influence β-arrestin localization. Mutations disrupting either of the oligomerization/IP6 binding sites of arrestin leads to a more nuclear distribution of the protein, suggesting monomeric arrestin is in the nucleus (Milano, Kim et al. 2006).

B.3 Regulation of NF- κ B by β -arrestin

As mentioned previously, the β -arrestins interact with several members of the NF- κ B signaling pathway. A general consensus for the function of β -arrestin in NF- κ B activation is elusive, as conclusions regarding β -arrestins role in NF- κ B regulation is mixed. Downstream of angiotensin activation, it is believed that both β -arrestin1 and β -arrestin2 work in the cytosol to stabilize I κ B α at resting state, leading to a reduction in NF- κ B activation (Witherow, Garrison et al. 2004; Parameswaran, Pao et al. 2006). Similar "cytosolic sequestration" models have been predicted for other NF-kB members and signaling cascades (Wang, Tang et al. 2006; Kizaki, Izawa et al. 2008; Delekta, Apel et al. 2010). It should be noted in all studies listed above, β -arrestin2 was the predominant arrestin isoform used. In contrast, we have observed an enhancement of NF- κ B activity in cells which overexpress β -Arrestin1 when stimulated by GPCR ligands, namely bradykinin and quinpirole (a D2 δ -opioid receptor agonist) (Yang, Zhang et al. 2003; Yang, He et al. 2009). Interestingly, it is reported by another group that β -arrestin2 is required for lysophosphatidic acid-induced NF-kB activation and IL-6 expression(Sun and Lin 2008). These findings strongly support different roles for β -arrestins to regulate NF- κ B activation in resting cells and under stimulated conditions.

Physiologically the modulation of the NF- κ B family by the β -arrestins may impact disease progression. β -Arrestin1 has been reported to increase pro-inflammatory cytokine production and disease progression in a rheumatoid arthritis model, while β -Arrestin2 inhibits their expression (Li, Cook et al. 2011). Elevated β -Arrestin1 expression has also been linked to autoimmune diseases, such as multiple sclerosis (Shi, Feng et al. 2007). Conversely, knockout of both β -arrestin proteins resulted in decreased inflammatory cytokine production, which produced positive survival outcomes following LPS challenge (Porter, Gonipeta et al. 2011). It should be noted that anti-inflammatory role of β -arrestin1 was limited to CD11b⁻ splenocytes, whereas β arrestin2 was anti-inflammatory in other cell types as well. Other reports have confirmed the anti-inflammatory role of β -arrestin2, as knockdown or loss of β -arrestin2 expression specifically leads to an overall increase in chemokine, cytokine, and growth factor expression (Raghuwanshi, Nasser et al. 2008; Zakrzewicz, Krasteva et al. 2011).

The seemingly conflicting reports of the role of the β -arrestin proteins in modulating NF- κ B activity suggest the scaffolding functions of the β -arrestins may potentiate signals leading to NF- κ B activation or repression in an isoform-, cell type- and stimulus-dependent manner.

CHAPTER II: MATERIALS AND METHODS

DNA Constructs. All constructs were generated using previously described human β -arrestin1 cDNA (NM 0020251) as a template. β -arrestin1 Δ 107-191 was generated by PCR amplification of fragments encoding amino acids 1-107 and 191-409, with a C-terminal FLAG tag incorporated in the sequence. The DNA fragments, after digestion with the restriction enzyme *HindIII* that cut at an endogenous site, were then ligated into the pCI expression vector. The β arrestin1 mNLS and NES+ constructs were generated using the QuickchangeTM mutagenesis kit (Stratagene, La Jolla, CA, USA). The mNLS mutant was generated using primers for region 1 (157-161) and region 2 (170-171) of the putative bipartite NLS in two consecutive rounds of PCR. The Quickchange mutations introduced substitutions of Ala for the corresponding residues and for Lys157, His159, Lys160, Arg161, and Arg169/Lys170 substitutions in β -arrestin1. The NES+ mutant was generated with a primer converting Gln386 to Leu. Both constructs used the previously generated β -arrestin1-FLAG as a template. β -arrestin1-GFP tagged constructs were generated by PCR amplification of WT, $\Delta 107$ -191, mNLS, K157A and NES+ plasmids. The PCR products were then subcloned into pEGFP-N1 (Life Technologies, Carlsbad, CA). NLS dominant β-Arrestin1 contructs were generated by PCR amplification of WT or mNLS β-Arrestin1 which was then cloned into a vector which expressed repeats of the SV40 NLS (pAcGFP-Nuc) (Clontech Labs, Mountain View, CA) using In Fusion cloning methodology (Clontech Labs, Mountain View, CA). HA-CBP was a kind gift from Dr. J.R. Lundblad (Vollum Institute, Oregon Health Sciences University, Portland, OR, USA). Human β-arrestin 1 cDNA was kindly provided by Dr. Basil Rapoport (University of California, San Francisco, CA). pMetLuc-NF-kB was purchased from Clontech. The constructs for the B2 bradykinin receptor (B2BKR), β -galactosidase and the 3x NF- κ B luciferase reporter were described previously(Yang, Zhang et al. 2003).

Antibodies. Antibodies against p65/RelA, p50, GFP, HA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG and anti- Au5 was from Sigma (St. Louis, MO, USA). Phospho-p65 Ser276 was from Cell Signaling Technology (Beverly, MA, USA). Anti-Acetyl-lysine, HDAC1 and Histone H1 were from Millipore EMD (Temecula, CA, USA). Anti-Importin β1 was from Calbiochem (La Jolla, CA, USA). Anti β-Arrestin was from BD Transduction Labs (San Diego, CA, USA). Anti-CBP was from Abcam (Cambridge, MA, USA). Goat anti-mouse HRP conjugate and goat anti-rabbit HRP conjugate were from Pierce (Rockford, IL, USA).

Cell Culture and Transfection. HeLa cells were purchased from ATCC. Cells were maintained in DMEM containing 10% heat inactivated FBS, 2 mM glutamine, 100 units/mL penicillin, and 50 μ g/mL streptomycin. Cells were transfected with DNA constructs listed above using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a 3:1 reagent:DNA ratio. The DNA input was adjusted to prevent over-expression of the protein of interest relative to endogenous β -arrestins.

Cell Fractionation and Western Blot Analysis. Cells were fractionated using an IGEPAL ca630 and high salt lysis method(Dignam, Lebovitz et al. 1983). Briefly, 5 x 10^6 cells were harvested by scraping in HBSS and then pelleted by centrifugation at 1,000 x g. Pellets were then lysed in nuclear extract buffer-A (NEBA; 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA,

0.1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) containing protease inhibitor cocktail set III (EMD Calbiochem, Billerica, MA, USA) and to which 10% IGEPAL ca630 was added). Cell lysate was incubated on ice for 10 minutes, vortexed, and then spun to pellet the nuclei. The supernatant was removed and saved as the cytosolic fraction. The nuclei pellets were then resuspended in nuclear extract buffer-B (NEBB; 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EGTA, 1mM EDTA) and incubated on ice for 30 min with occasional vortexing. Nuclear debris was pelleted by centrifugation at $12,000 \ge g$ for 30 sec. The supernatant was recovered as the nuclear fraction. Fractions were then directly analyzed by Western blotting. Protein concentration of samples was determined using the DC protein assay system (Bio-Rad, Hercules, CA, USA). Equivalent protein concentrations were separated on 4-12% Bis-Tris precast SDS/acrylamide gels (Bio-Rad, Hurcules, CA, USA) at 60 mA. Proteins were then transferred to nitrocellulose membranes at 100 V for 1 h. Membranes were then incubated with 5% non-fat milk in TBS-T (20 mM Tris-HCl pH 7.5, 120 mM NaCl, and 0.01% Tween-20) for 1 h at room temperature. Afterwards, membranes were incubated with primary antibodies against proteins of interest overnight at 4°C. Following removal of primary antibody and a washing step, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Bound antibodies were detected using the SuperSignal West PicoTM chemilumiscence substrate (Pierce, Rockford, IL, USA).

Immunoprecipitation. For whole cell immunoprecipitation (IP), cells were harvested in IP lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM DTT, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 1% TX-100, 1 mM PMSF) containing protease/phosphatase inhibitor cocktail III (EMD Calbiochem, Billerica, MA, USA). For nuclear IP, cells were fractionated as described above.

Following lysis, samples were pre-cleared by incubation with protein A/G Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at 4°C. Samples were centrifuged to pellet the beads and the supernatant was transferred to a new tube and incubated overnight with primary antibodies. Protein complexes were then precipitated with the addition of 25 μ l protein A/G beads and incubated for 1 h. Following five washes, the samples were analyzed by Western blotting as described above.

Microscopy. Cells were plated on glass coverslip (18 mm diameter, 1 mm thickness) coated with 0.1% gelatin. Cells were transfected as above with β-arrestin-GFP constructs. For basal localization studies, samples were starved for 4 h and then prepared for imaging. For BK stimulated samples, cells were serum starved followed by stimulation with 500 nM of BK for the indicated time. To prepare samples for imaging, cells were first washed with HBSS and then fixed by incubation in 4% para-formaldehyde (PFA) for 20 min at room temperature. Free PFA was quenched by the addition of 100 mM glycine. Samples were washed with HBSS and then mounted onto glass coverslips using ProLong Gold Anti-fade Reagent with DAPI (Molecular Probes, Eugene, OR, USA). Samples were allowed to cure overnight at room temperature and then imaged on a Zeiss LSM 310 Meta Confocal Microscope. Images were analyzed using the LSM Imaging and Image J software (National Institutes of Health, Bethesda, MD, USA).

Luciferase Assay. HeLa cells in 12-well plates were transfected with vectors for the β -arrestin1 constructs, B2BKR, a 3x κ B luciferase reporter plasmid, and β -galactosidase as described above. Prior to being assayed, cells were serum starved for 4 h and then stimulated with 200 nM of BK for 4 h. Following stimulation, cells were washed and harvested in reporter

lysis buffer (Promega, Madison, WI, USA). Luciferase expression was analyzed by incubating samples with luciferase substrate (Stratagene) and reading luminescence in a Femtomaster FB12 luminometer (Zylux, Huntsville, AL, USA). The β -galactosidase activity resulting from a co-transfected construct in the same cells was used for normalization of the NF- κ B reporter data. For NLS dominant β -Arrestin1 samples, luciferase assay was performed using the 'Ready to Glow' Secreted Luciferase Kit (Clontech Labs, Mountain View, CA, USA), and samples were analyzed using the Wallac Viktor 2 (PerkinElmer, Waltham, MA, USA) All assays were performed three times with triplicate samples. Data was analyzed using GraphPad Prism software (Version 4, GraphPad, La Jolla, CA, USA).

Electromobility Shift and Supershift Assays. Nuclear extracts from transfected HeLa cells were generated as described in *Cell Fractionation* section except that supershift samples were lysed without dithiothreitol (DTT) in the buffer. Nuclear samples were then incubated for 10 min with a $[\gamma^{-32}P]$ ATP-labeled NF- κ B consensus oligo-nucleotide probe (Promega, Madison, WI, USA). Supershift samples were pre-incubated with specific antibodies to p50 or p65/RelA for 30 min prior to incubation with the probe. Samples were then separated on a 4% acrylamide gel for 3-4 h. The gels were dried and an autoradiograph image was taken using a phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA).

Quantitative Real-Time PCR. HeLa cells (5 x 10^6) transfected with WT or mNLS β -arrestin1 and the B2 receptor were stimulated with 500 uM BK for different periods of time. Total RNA was isolated with a RNeasy isolation kit obtained from Qiagen. RNA was then reverse transcribed to create complimentary DNA (cDNA) using the SuperScript III RT Kit (Invitrogen, Carlsbad, CA, USA) or the RNA to cDNA EcoDry Premix (double primed) (Clontech, Mountain View, CA, USA). Real-time quantitative PCR was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). 1 μ g of cDNA from stimulated HeLa cells was analyzed using the GoTag qPCR Master Mix (Promega, Madison, WI, USA) containing 20 pmol forward and reverse primers. The thermocycling program was 40 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 45 s, with an initial cycle of 95°C for 2 min. Accumulation of PCR products was detected by monitoring the increase in fluorescence of SYBR Green after each cycle. A dissociation curve was constructed in the range of 60°C to 95°C, to test for primer specificity. All data were analyzed with the ABI PRISM 7000 SDS software (version 1.1). Primers for the housekeeping gene GAPDH were used to normalize sample loading. Relative levels of mRNA for IL-1 β were determined by using the Ct value and the formula: fold increase = $((1 + \eta_{target})^{\Delta Ct target (unstimulated - stimulated)})/((1 + \eta_{GAPDH})^{\Delta Ct GAPDH (unstimulated - stimulated)}).$

The sequences of the oligonucleotides used in this study are as follows: 5'-CTTCGGTCC AGTTGCCTTCTC-3' and 5'-TTCTGCCAGTGC CTCTTTGC-3' for IL-1; and 5'-ACCACAGTCCA TGCCATCAC-3' and 5'-TCCACCACCCTGTTG CTGTA-3' for GAPDH.

Statistical Analysis. Quantification of Western blot data was performed using Image J software (National Institutes of Health, Bethesda, MY, USA). Statistical analysis was conducted using the GraphPad Prism software (GraphPad, La Jolla, CA, USA).

CHAPTER III: RESULTS

A. β -arrestin1, and not β -arrestin2, is localized in the nucleus of HeLa cells

To examine the subcellular distribution of the β -arrestins, plasmids encoding GFP tagged β -arrestin1 or 2 were transfected into HeLa cells. Subcellular distribution of the constructs was then visualized by confocal microscopy. As can be seen in Figure 5A, β -arrestin1 is distributed ubiquitously throughout the cell while β -arrestin2 is excluded from the nucleus. These data are consistent with previous published reports regarding the subcellular localization of the arrestins, and were confirmed through quantitation across several experiments (Figure 5B). The exclusion of β -arrestin2 from the nuclear compartment is caused by a single residue difference between the two arrestin proteins, as β -Arrestin2 has an LRL motif at residue 395 which appears as QRL in β -Arrestin1. This motif serves to act a nuclear export signal (Scott, Le Rouzic et al. 2002).

B. β -arrestin1 enhances NF- κ B following bradykinin stimulation

We have previously shown that β -arrestin1 enhances the activation of the transcription factor NF- κ B following bradykinin stimulation via a G $\beta\gamma$ - and Akt-dependent mechanism (Yang, He et al. 2009). To confirm these results we performed NF- κ B luciferase assays in BK stimulated cells (Figure 6A). In these assys, HeLa cells were transfected with a reporter gene containing 3 tandem κ B consensus binding sites fused to a Renilla luciferase gene. Cells were also transfected with β -arrestin1 and the B2 bradykinin receptor (B2BKR). To reduce background, cells were serum starved overnight prior to being assayed for luciferase expression. Cells were stimulated for 4 hours with 200 nM bradykinin (BK). In all assays samples were normalized to the expression of a co-transfected β -galactosidase gene. Although β -arrestin1 alone did not induce


Figure 5. β -arrestin1, and not β -arrestin2, is localized in the nucleus of HeLa cells.

In (**A**), HeLa cells were grown on 22cir glass coverslips and transfected with 0.5 μ g of either β Arr1-GFP, or β Arr2-GFP. Cells were allowed to grow for 48 hours following transfection and then were fixed with 4% PFA for 20 minutes at room temperature, washed in HBSS and subsequently mounted on glass slides using Prolong Gold Antifade Reagent + DAPI (Molecular Probes). Slides were visualized using a LSM 510 META Zeiss scanning laser confocal microscope. Scale bar is equal to 5 μ m in each image. In (**B**), nuclear intensity was quantified by calculating β -arrestin nuclear intensity (GFP fluorescence intensity) over total nuclear intensity (DAPI intensity). The ratio was then normalized to cells expressing no β -arrestin. n= No Arrestin (8 cells analyzed), β -arrestin1 (14 cells), β -arrestin2 (7 cells).

any increase in luciferase expression, it enhanced luciferase expression when combined with BK stimulation, indicating activation of NF- κ B.

C. *β*-arrestin1 interacts with p65/RelA in the nucleus following bradykinin stimulation Recent reports show that in addition to scaffolding signaling events in the cytosolic compartment, β -arrestin1 is able to act as a scaffold within the nucleus and mediate transcriptional responses (Kang, Shi et al. 2005; Shi, Feng et al. 2007). These reports coupled with our observation of β -arrestin1s' expression in the nucleus, led to the hypothesis that β arrestin1 might influence NF-kB activation directly in the nucleus. To test this we first determined if β -arrestin1 moved into the nucleus following bradykinin activation. In β -arrestin1 and B2BKR transfected HeLa cells there was an appearance of β -arrestin1 in the nuclear fraction following stimulation with 500 nM bradykinin (Figure 6B). While β-arrestin1 was present in the nucleus before BK stimulation, its concentration increased in the nucleus within 5 minutes after stimulation, and the increase continued until 60 minutes. The time course for p65/RelA translocation to the nucleus followed a similar pattern with maximal p65/RelA translocation at 60 minutes. A graphical representation of these results can be seen in Figure 6C. Since β arrestins have been shown to interact with members of the NF-κB family in the cytosolic compartment (Gao, Sun et al. 2004; Witherow, Garrison et al. 2004; Yang, He et al. 2009) we sought to determine if β-arrestin1 also interacts with p65/RelA in the nucleus. Nuclear fractions of transfected, bradykinin-stimulated cells were incubated with an anti-p65 antibody overnight and then analyzed by Western blot for the co-immunoprecipitation of FLAG-tagged β -arrestin1. Following stimulation there is a time-dependent increase in the association between β -arrestin1 and p65/RelA (Figure 6D). These results suggest a direct interaction between p65/RelA and β-

Figure 6. β-Arrestin1 interacts with p65/RelA in the nucleus and enhances NF-κB activation following bradykinin stimulation.

NF-kB luciferase assays (A) were conducted using HeLa cells transfected with a 3x- κ B reporter, a β -galactosidase (β -gal) reporter, the B2BKR and β -arrestin1 constucts. Forty-eight hours after transfection, the cells were serum starved and then stimulated with BK or vehicle for 4 h. Samples were then harvested and assayed for luciferase expression. The results were normalized against the activity of a co-transfected β -gal construct. Data is representative of at least 3 independent experiments. In (B), HeLa cells were transfected with expression construct of FLAG-tagged wild type β-arrestin1 together with the B2BKR expression construct. Forty-eight hours after transfection, cells were serum starved for 12 h and then stimulated with 500 nM of BK for the indicated time points. Western blot analysis of nuclear fractions was performed to determine the translocation of β -arrestin1 and p65/RelA to the nucleus. Histone H1 was used as a nuclear loading control. Quantification from several experiments of the p65/RelA and β-arrestin1 (βArr1) bands (C) were conducted against histone H1 from the same time points, showing time-dependent increase in the nuclear contents of β arrestin1 and p65/RelA. In (D), nuclear fractions collected from different stimulation were immunoprecipitated with an anti-p65 time points antibody. Coimmunoprecipitation of β-arrestin1-FLAG was determined by Western blotting with an anti-FLAG antibody. The expression of total β -arrestin1-FLAG in the nuclear lysate was determined (lower gel image). Data shown in this figure are representative of at least 3 independent experiments.





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arrestin1 in the nucleus following BK stimulation. Also, these results suggest the two proteins may translocate to the nucleus independently of each other, or different pools of arrestin interact with p65/RelA in the different subcellular compartments, as their interaction occurs after each has arrived in the nucleus.

D. The N-domain of β -arrestin1 is Critical for its Nuclear Localization

 β -Arrestin1 has the ability to interact with a variety of members of the NF- κ B pathway, presumably in both the nuclear and cytosolic compartments. As such, it is important to design a tool that would allow for the specific analysis of the function of β -Arrestin1 in the nucleus. Due to its size, β -Arrestin1 is too large to passively diffuse into the nucleus. It is likely that β -Arrestin1 contains a nuclear localization sequence which mediates its recognition by nuclear import proteins. Identification of this sequence, and subsequent mutation, would provide the tool necessary to study nuclear specific functions of β -Arrestin1. Previously published reports have indicated that the region between residues 1-186 of β -arrestin1, known as the N domain, is required for its nuclear localization (Wang, Wu et al. 2003). To further examine this region we first created a series of N-terminal truncation mutants and their impact on nuclear localization was determined using confocal microscopy and Western blot. As can be seen in Figure 7, truncation of the first 90 residues of β -arrestin1 had no impact on its nuclear localization, resulting in a subcellular distribution similar to that of wild type. In fact, statistical analysis across several cells reveals that truncation of the first 90 residues of β -Arrestin1 causes a slight increase in the nuclear expression of the protein (Figure 7B). In contrast, truncation of the first 180 residues if β -arrestin1 lead to a pronounced redistribution of expression from predominantly nuclear to a more diffuse cytosolic expression. This data confirms the requirement of the N-







In (**A**), HeLa cells were grown and transfected with either full length β Arr1-Au5, Δ 90N β Arr1-Au5, or Δ 180N β Arr1-Au5. Cells were allowed to grow for 48 hours post transfection and then were prepared for imaging as described in *methods*. Samples were incubated with an anti-AU5 antibody to detect arrestin proteins. Visualization was enabled by incubation with FITC conjugated goat anti-mouse and samples were mounted on glass slides. Slides were visualized using a LSM 510 META Zeiss scanning laser confocal microscope. Scale bar is equal to 5µm in each image. In (**B**), nuclear intensity was quantified by calculating β -arrestin nuclear intensity (FITC intensity)/ total nuclear intensity (DAPI intensity). The ratio was then normalized to cells expressing no β -arrestin. n= No arrestin (8 cells), β -arrestin1 (14 cells), Δ 90N (6 cells), Δ 180N (9 cells).

domain and shows for the first time that the residues between 90 and 180 comprise the critical region within the N-domain for β -arrestin nuclear localization. It should be noted that this data also suggests size of epitope tag may influence the localization of β -arrestin1, as GFP tagged constructs are expressed more diffusely throughout the cell as compared to Au5 tagged constructs (compare Figs. 5A to 7A).

E. Identification of a novel nuclear localization signal in β -arrestin1.

As the loss of the first 90 residues had no impact on localization we then generated a construct in which only the amino acids between 107 and 191 were deleted. The available restriction sites in β-arrestin1 cDNA also influenced the choice of this region. Visualization of this construct revealed absence of the $\Delta 107-191 \beta$ -arrestin1 from nuclei (Fig 8B, 2nd row). Attempts to utilize available predictive algorithms to identify consensus protein localization domains within this region were unsuccessful. Manual analysis of the primary sequence, however, revealed two candidate regions of interest, one which bore similarity to the recently identified PY-nuclear localization signal (NLS), and a second with similarity to the classical bipartite NLS (reviewed in (Stewart 2007). These sequences are summarized in Table 1. A PY-NLS is characterized by a hydrophobic core of residues followed by a linker leading to the critical PY residues. The candidate PY-NLS in β -arrestin1 is found between amino acids 86-114, with the critical PY (YP) residues located at 113-114 (Table 1). While a portion of the PY-NLS is outside of our original $\Delta 107-191$ mutant, the critical PY (YP) residues are within the N-terminal portion of this region. The second candidate NLS has similarity to a classical bipartite NLS, which is characterized by the presence of contiguous arginine and lysine residues separated by a ~10-residue linker followed by a cluster of 3-5 basic residues. In β-arrestin1, this NLS is located between residues

NLS Protein	Sequence	Source
<i>Simple NLSs</i> SV40 v-Jun	P <u>KKKRK</u> V KS <u>RKRK</u> L	(Kalderon, 1984) (Chida,1992)
Bipartite NLSs		
Nucleoplasmin	<u>KR</u> PAATKKAGQAKK <u>KKLDK</u>	(Robbins, 1991)
NIN2 Plk1	<u>RK</u> KRKTEEESPLKDKA <u>KKSK</u> <u>RR</u> RSLLEL <u>HKRRK</u>	(Kleinschmidt, 1988) (Taniguchi, 2002)
Parafibromin	<u>KR</u> AADEVLAEA <u>KKPR</u>	(Hahn 2005)
PY NLSs M9 TAP	<u>FGPM</u> KGGNFGGRSSG <u>PY</u> <u>VAMS</u> DAQDGP <u>R</u> VRYN <u>PY</u>	(Lee, 2006) (Truant, 1999)
Candidate NLS in β -Arrestin1		
¹⁵⁷ - KIHKR NSVRLVI RK - ¹⁷⁰		

¹⁰⁰-LQERLIKKLGEHAYP-¹¹⁴

Table 1. Common motifs found in nuclear localization signals.

The table lists examples of various families of previously identified nuclear localization signals. Monopartite NLSs such as that from SV40 (Kalderon, 1984) and v-Jun (Chida, K, 1992) are characterized by a cluster of basic residues. Bi-partite NLSs such as nucleoplasmin (Robbins, J, 1991), NIN2 (Kleinschmidt, 1988)), Plk1 (Taniguchi 2002), and parafibromin (Hahn, 2005) are characterized by 2 clusters of basic residues separated by a 7-10 residue linker. Finally, PY NLSs like those in M9 (Lee, BJ, 2006) and TAP (Truant, 1999) have a string of hydrophobic or basic residues followed by a R/H/K and then a PY motif, separated by a 3-5 residue linker Candidiate stretches within β Arrestin1 primary sequence are listed, and candidate residues are underlined..

Figure 8. Identification of a novel nuclear localizatin sequence in β-arrestin1.

Shown in (A) is a schematic representation of β -arrestin1-GFP and its deletion and substitution mutants, $\Delta 107$ -179, mNLS, YP and NES+, as detailed in the text and in *Materials and Methods*. In (B), confocal microscopy images of β -arrestin1 localization are shown. HeLa cells were transfected with GFP-tagged wild type or mutant β -arrestin1 constructs shown in (A). Forty-eight hours after transfection, cells were serum starved for 4 h and then fixed and images were taken. DAPI was used to visualize nuclei. In (C), nuclear intensity was quantified by calculating β -arrestin nuclear intensity (GFP intensity)/ total nuclear intensity (DAPI intensity). The ratio was then normalized to cells expressing no β -arrestin.. n= No Arrestin (8 cells), WT(14 cells), $\Delta 107$ -191 (14 cells), mNLS (12 cells), YP (8 cells), NES (14 cells).

Α



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157-161 and 169-170, separated by 7 amino acids. To investigate the roles of these regions in βarrestin1 nuclear localization, alanine substitutions were made to generate two mutants, YP (YP to AA at amino acids 113-114) and mNLS (KIHKR/RK to AAAAA/AA at amino acids 157-161/169-170). Only those residues that are critical to nuclear localization in their homologous NLSs were mutated, with all other residues left intact (Fig. 8A). As can be seen in Figure 8B, substitution of PY to AA had minimal impact on subcellular distribution of β-arrestin1 (Fig. 8B, 4th row). Mutation of the candidate bipartite NLS (mNLS), however, caused a profound shift of β-arrestin1 distribution from predominantly nuclear to the more diffusely cytosolic (Figure 8B, 3rd row). A β-arrestin1 mutant, NES+, that contains the nuclear export signal (NES) found in βarrestin2, was also prepared. As expected, NES+ was localized in the cytosolic compartment and was used as a control for visualization of non-nuclear β-arrestin protein (Figure 8B, 5th row). Graphical representation of the nuclear intensities if the various mutants is provided in Figure 8C.

F. Loss of inducible nuclear translocation of β -arrestin1 by mutation of the NLS.

Mutation of the candidate NLS in β -arrestin1 leads to a loss of nuclear expression at resting state. However, this does not eliminate the possibility that activation-dependent changes to β arrestin1 could impact its nuclear translocation. To test this possibility, HeLa cells transfected to express the BK receptor B2BKR and either wild type or mutant β -arrestin1 constructs were stimulated with BK and assayed for the appearance of β -arrestin1 in the nucleus. Increased nuclear localization of the wild type β -arrestin1 was observed following BK stimulation (Fig. 9A). Unlike wild type β -arrestin1, there was no inducible nuclear translocation of the mNLS mutant following BK stimulation (Fig. 9B). To ensure that the loss of function was specific and

Figure 9. Effects of NLS mutation on nuclear translocation of β -arrestin1.

HeLa cells were transfected with expression construct of FLAG-tagged wild type (A) or NLS mutant (mNLS, B) β -arrestin1 together with the B2BKR expression construct. Forty-eight hours after transfection, cells were serum starved for 4 h and then stimulated with 500 nM of BK for the indicated time points. Western blot analysis of cytosolic and nuclear fractions was performed to determine the translocation of β -arrestin1 from cytosolic compartment to nucleus. The protein content of nuclear fraction was approximately 4-fold higher than that of cytosolic fraction in the gel. GAPDH and HDAC1 were used as cytosolic and nuclear loading controls, respectively. In (C), HeLa cells were transfected and stimulated for 5 min as described above and then fixed, stained with DAPI to visualize nuclei. Confocal images were taken for β -arrestin1 translocation to the plasma membrane.





mNLS



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not caused by misfolding of the mutated protein, we examined whether the mNLS mutant was able to translocate to the plasma membrane upon agonist stimulation. The results showed that both the wild type and mNLS β -arrestin1 moved to the plasma membrane in BK-stimulated cells (Fig. 9C), suggesting that this function of mNLS β -arrestin1 is retained. A 5 minute time point was chosen as translocation of β -arrestins to the plasma membrane occurs rapidly following receptor activation. This result also suggests that membrane and nuclear translocation of β -arrestin1 might be independently regulated.

G. β -arrestin1 interacts with the nuclear import machinery.

In live cells proteins below ~40 kDa can passively diffuse into the nucleus, whereas larger proteins must be transported into the nucleus in an energy-dependent process (Davis 1995; Pante and Aebi 1996). While several import pathways have been identified, many proteins use the importin β carrier proteins for nuclear transport (Stewart 2007). To examine if β -arrestin1 NLS mediates nuclear translocation through this mechanism, we performed co-immunoprecipitation experiments between WT and mNLS β -arrestin1 and members of the import machinery. As can be seen in Figure 10A, following BK stimulation there was a stimulus-dependent increase in the association between β -arrestin1 and importin β 1 (Imp β 1), indicating active transport into the nucleus. A reciprocal immunoprecipitation showed similar results (Fig. 10C). The β -arrestin1 NLS mutant mNLS was unable to interact with the nuclear import machinery following BK stimulation (Fig. 10B, 10D). Likewise, stimulus dependent translocation through the nuclear pore was also absent for the mNLS β -Arrestin1 construct (data not shown). This loss of function suggests the mutated residues in mNLS β -arrestin1 are important for mediating interaction with the nuclear import machinery.



Figure 10. Interaction of β -arrestin1 with the nuclear import machinery.

HeLa cells were transfected with the B2BKR and FLAG-tagged WT (**A**) or mNLS (**B**) β -arrestin1 constructs. After 48 h, cells were serum starved and then stimulated with 500 nM of BK for the indicated time points. FLAG-tagged β -arrestin1 was immunoprecipitated from total cell lysate, and samples were analyzed by Western blotting for detection of the co-immunoprecipitated importin β 1. Samples were also analyzed for detection of FLAG as an IP control. Lysate samples were analyzed to determine equivalent expression of importin β 1 in WT and mNLS samples. Reciprocal immunoprecipitation with importin β 1 and either WT (**C**) or mNLS β -arrestin1 (**D**) were also performed. Western blots shown are representative of at least 4 independent experiments.

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H. Nuclear β -arrestin1 enhances NF- κ B DNA binding and transcriptional activity.

Previously, we have shown that β -arrestin1 is able to influence the activity of NF- κ B following various GPCR activation(Yang, Zhang et al. 2003; Yang, He et al. 2009). We sought to determine if the nuclear localization of β -arrestin1 affects NF- κ B activation. To examine this we tested our nuclear transport-deficient mutants in an NF-kB luciferase assay. As shown in Figure 11A, the wild type β -arrestin1 is able to increase NF- κ B-driven luciferase expression following BK stimulation. Both the $\Delta 107-191$ and mNLS β -arrestin1 were significantly less capable of potentiating NF- κ B compared to the WT β -arrestin1 (p<0.01), suggesting that nuclear localization of β -arrestin1 is required for optimal enhancement of NF- κ B activity (Fig. 11A) and the cytosolic function of β -arrestin1 alone is insufficient for augmenting NF- κ B activation. To exclude the possibility that mutation in this region of β -arrestin1 alters its interaction with key components of the NF- κ B activation pathway, we examined another β -arrestin1 construct containing the NES+ from β -arrestin2. The NES+ construct was minimally altered in its sequence (Fig. 8A), yet cells expressing this construct also showed a significant loss of enhancement in NF-κB activation (p<0.01) (Fig. 11A). In control experiments, we found that the loss of enhancement in NF-κB activation was not due to inefficient expression of the β-arrestin1 mutants (Fig. 11A, Western blot), nor was it the result of lack of association with p65/RelA (Fig. 11B), as this function was unaltered with the mutations. These results suggest that nuclear localization of β -arrestin1 is critical for its ability to potentiate NF- κ B transcriptional activity.

Figure 11. Effect of nucleus-localized β-arrestin1 on NF-κB activity.

NF- κ B luciferase assays (**A**) were conducted using HeLa cells transfected with a 3x- κ B reporter, a β -galactosidase (β -gal) reporter, the B2BKR and various β -arrestin1 constructs. Forty-eight hours after transfection, the cells were serum starved and then stimulated with BK or vehicle for 4 h. Samples were then harvested and assayed for luciferase expression. The results were normalized against the activity of a co-transfected β -gal construct. **, P<0.01. n=5. Proper protein expression in the luciferase assay was verified by Western blotting and shown in panel below. In (**B**), p65/RelA binding to the mutant forms of β -arrestin1 was analyzed by immunoprecipitation of FLAG-tagged β -arrestin1 from unstimulated HeLa cell lysates, followed by analysis of co-immunoprecipitation of p65/RelA by Western blotting. Blots shown are representative of at least 3 independent experiments.



I. Nuclear β -arrestin1 regulates the post-translational modification of p65/RelA.

Next we determined how β -arrestin1 regulates NF- κ B activation in the nucleus. Because β arrestin1 formed a complex with p65/RelA in the nucleus, we investigated if mutation of the NLS in β -arrestin1 affects the nuclear translocation of p65/RelA. Following BK stimulation, there was no significant difference in nuclear localization of p65/RelA in cells expressing the wild type or mutant β -arrestin1 (Fig. 12A, 12B). Interestingly, there was a time-dependent accumulation of p65/RelA in the nucleus whereas reduction in the cytosolic fraction was less obvious. This may be due to only a fraction of total p65/RelA translocating to the nucleus upon agonist stimulation. Since p65/RelA is modified in the nucleus by acetylation, which regulates its efficiency of transcriptional control (Chen, Fischle et al. 2001) (Chen, Mu et al. 2002), we examined whether the nuclear β -arrestin1 influenced acetylation of p65/RelA. We observed a significant loss of acetylation of p65/RelA in cells expressing the mNLS mutant compared to the wild type (Fig. 13A). Consistent with the loss of p65/RelA acetylation, there was a loss of association between p65/RelA and the acetyltransferase CBP (Fig. 13B) and between the mNLS mutant and CBP (Fig. 13C), which likely resulted from failed entry of the β -arrestin1 mutant into the nucleus. These data suggests that β -arrestin1 may be scaffolding CBP and p65/RelA together to facilitate appropriate acetylation.

Functionally, acetylation of p65/RelA has been reported to be dependent upon its phosphorylation (Chen, Williams et al. 2005), and it is known that p65/RelA is phosphorylated at Ser276 in the nucleus by MSK1 (Vermeulen, De Wilde et al. 2003). Therefore, we next examined the effect that NLS mutation of β -arrestin1 might have on these modifications. Following stimulation with BK, there is a time-dependent increase in the phosphorylation of



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Figure 12. Effect of β-Arrestin1 on p65/RelA nuclear translocation.

HeLa cells transfected with either WT (A) or mNLS (B) β -arrestin1 along with the B2BKR were stimulated with 500 nM of BK and fractionated. Translocation of p65/RelA into the nucleus was analyzed by Western blotting. GAPDH and HDAC1 were used as cytosolic and nuclear controls, respectively. Western blot results are representative of 3 independent experiments.

Figure 13. Effect of β -arrestin1 in regulating the post-translational profile of **p65/RelA in nucleus.** In (A), cell lysate from WT β-arrestin1 or mNLS-expressing cells stimulated with BK were immunoprecipitated with anti-p65/RelA. Acetylation of p65/RelA was then analyzed by probing p65/RelA immunoprecipitates with an antiacetyl lysine antibody by Western blotting. In (B), HeLa cells were transfected with FLAG-tagged WT or mNLS β-arrestin1 and the B2BKR construct. After transfection, cells were starved for 4 h and then stimulated with 500 nM of BK for the indicated time points. Following stimulation, whole cell lysate was immunoprecipitated for p65/RelA. IP samples were then analyzed by Western blot for the coimmunoprecipitation of CBP. The blot for p65 serves as a loading control. Representative blots from at least 3 experiments are shown. In (C), HeLa cells were transfected with FLAG-tagged WT or mNLS β -arrestin1, the B2BKR, and HA-tagged CBP constructs. Cells were starved for 4 h and then stimulated with 500 nM of BK for the indicated time points. Following stimulation samples were immunoprecipitated for HA-CBP. IP samples were then analyzed by Western blot for the coimmunoprecipitation of FLAG-β-arrestin1. The blot for HA-CBP serves as a loading control. Representative blots from at least 3 experiments are shown.





С



p65/RelA at Ser276 in cells expressing wild type β -arrestin1, whereas in cells expressing the mNLS mutant there was a significant reduction of this phosphorylation at Ser276 (Fig. 14A). Quantification of additional blots verified these results (Fig. 14B). Phosphorylation of Ser536 of P65/RelA was also examined, however the loss of this modification in mutant β-Arrestin1 expressing cells was less pronounced, likely due to its occurance in both the nuclear and cytosolic compartment (data not shown). Acetylation and phosphorylation of p65/RelA has been shown to be important for transactivation and stable binding to the promoter (reviewed in (Perkins 2006)). To examine this we performed electrophoretic mobility super-shift assays with a canonical NF- κ B binding sequence, using HeLa cells expressing the WT or mNLS mutant β arrestin1. As can be seen in Fig. 14C, BK stimulated an increase in NF-kB:DNA complex formation (Lane 2 compared to Lane 1). Consistent with the luciferase assay results (Fig. 11A), there was a decrease of protein:DNA binding in the mNLS expressing cells (Lane 7) compared to cells expressing the WT β -arrestin1 (Lane 2). To identify the composition of the NF- κ B dimers that bound to the DNA probe, we performed super-shift assays in which the mobility of the NF- κ B:DNA complex was shifted upwards with the addition of specific antibodies to p65/RelA or p50. With the addition of the individual antibody, there was a loss of the NFκB:DNA complex corresponding to the relative size of p50 and p65/RelA. These results demonstrate the presence of a p50/RelA heterodimer, although the experiment does not rule out the presence of other NF- κ B proteins. In cells expressing the mNLS mutant, there was not only decreased complex formation but also reduced complex shift. The change was more prominent in the sample containing the anti-p65/RelA antibody (Lane 9), suggesting reduced p65/RelA binding to the NF-κB probe. In comparison, the shift with anti-p50 antibody (Lane 8) was proportional to the NF-κB:DNA complex (Lane 7).

Figure 14. β-arrestin1 regulation of p65/RelA phosphorylation and DNA binding. In (A), HeLa cells were transfected with WT or mNLS β-arrestin1 and the B2BKR construct. Following serum starvation for 4 h, the cells were stimulated with 500 nM of BK. Total cell lysate was analyzed by Western blotting using an antibody against phospho-p65/RelA at Ser276. Total p65/RelA was used as a loading control. Quantification and statistical analysis of phosphorylation of Ser276 was performed and data are shown in (B). **, p < 0.01 compared WT samples at same time points. n =4. In (C), electrophoresis mobility super-shift assay for NF-κB was conducted using HeLa cells expressing WT or mNLS β-arrestin1 and the B2 BK receptor. After serum starvation for 4 h, the cells were stimulated with either vehicle or 500 nM of BK for 60 min. Nuclear fractions were obtained and incubated with a radiolabeled NF-κB consensus probe. The specificity of probe binding was verified by competition with a cold (unlabeled) probe, used in 100-fold excess. Supershift with an anti-p50 or anti-p65/RelA antibody was performed to identify which NF-κB proteins were responsible for DNA binding. NS, non-specific species. SS, super-shifted bands.



Α



С



Lane:1 2 3 4 5 6 7 8 9

J. Lys157 is critical to nuclear localization of β -arrestin1.

Since the construction of mNLS involves substitution of a total of 7 amino acids, potential alteration of the overall protein structure was a concern, although the mNLS mutant retained its ability to translocate to the plasma membrane upon BK stimulation (Fig. 9B) and to bind p65/RelA (Fig. 11B). Moreover, based on the crystal structures of β -arrestins, the 5 charged residues in the bipartite NLS (157-161/169,170) are located on different sides of the protein, suggesting that only one of the bipartite NLS is actually responsible for nuclear localization. The structure of β -Arrestin1, and mutated residues can be seen in figure 15. To address this, we mutated charged amino acids in the bipartite NLS to Ala in combination or individually (Fig. 16A). The constructs created included either single, or double point mutations within the identified NLS region and are: Arg169Ala/Lys170Ala, Lys160Ala/Arg161Ala, Lys157Ala, and Lys160Ala. Observation of the GFP tagged mutants revealed that the majority of the single or double point mutations were present in the nucleus (data not shown and figure 16B, lower 2 panels). Only the mutation of Lys157 created a β-Arrestin1 which was absent from the nucleus in transfected HeLa cells (Fig 16B, 2nd panel). This data does not exclude the possibility that these residues are involved in nuclear import, but it does highlight the critical importance of Lys157 for this function.

K. Mutation of Lys157 alone is sufficient for loss of nuclear function of β -arrestin1.

Since the imaging data of the single/double point mutants suggested Lys157 as the principal residue of the NLS we sought to confirm its importance in mediating import of β -Arrestin1 and subsequent functions in NF- κ B regulation. As expected, K157A β -Arrestin1 showed a significant reduction in binding to Importin β 1 as compared to wild type β -Arrestin1 (Figure 17),



Figure 15. Ribbon Diagram of Crystal Structure of bovine β -Arrestin1. Structure of β -Arrestin1 at 1.9 Angstrom. Residues identified as critical in mNLS mutant β -Arrestin1 are labeled and noted (red arrows). Their location may not be surface exposed at the same time based on order of β -strand within the protein conformation. N and C domains are labeled, and appear as megenta and cyan, respectively. The highly flexible inter-domain hinge is yellow. Modified from Han, M et al. 2001. Structure (9):869-880.



Α

В

Figure 16. Identification of Lys157 as critical determinant for β -Arrestin1 nuclear localization. In (A), a schematic representation of β -arrestin1 and the identified NLS region. (B), confocal microscopy images showing the localization of β -arrestin1-GFP, K157A-GFP, K160A/R161A-GFP, and R170A-GFP in HeLa cells. Forty-eight hours after transfection, cells were serum starved for 4 h and then fixed for imaging. DAPI was used to visualize nuclei. Images are representative of at least 2 independent experiments..



Figure 17. Mutation of Lys157 abrogates β -arrestin1 binding to importin β 1. HeLa cells were transfected with WT or K157A β -arrestin1 and the B2BKR construct. Following serum starvation for 4 h, the cells were stimulated with 500 nM of BK The interaction of wild type β -arrestin1 (**A**) and the K157A mutant (**B**) with importin β 1 was evaluated using procedures described in Fig. 6. Representative blots from a total of 3 independent experiments are shown.

similar to those results obtained from mNLS β -Arrestin1 (Figure 10). In functional assays, the ability of the K157A mutant to enhance p65/RelA acetylation was markedly reduced compared to wild type controls (Figure 18A, quantified in 18B). Likewise, phosphorylation of p65/RelA at Ser276 was also reduced (Figure 18C, quantified in 18D). Co-immunoprecipitation experiments confirm that both the K157A and mNLS mutants are still capable of interacting with p65/RelA (Figure 18E). Another nuclear event examined was DNA binding of the NF- κ B proteins. In cells transfected with the K157A mutant, BK-induced NF- κ B DNA binding was drastically decreased when compared to cells transfected with the wild type β -arrestin1 (Fig. 19A). Finally, to examine a role for β -arrestin1 in gene transcription, we measured IL-1 β mRNA level in BK-stimulated HeLa cells that were transfected with wild type β -arrestin1, the K157A or mNLS mutant. The enhancement effect of β -arrestin1 on IL-1 β production was significantly reduced in cells expressing either of the mutants (Figure 19B). These results show that the phenotypical changes of the K157A and mNLS mutants are very similar, suggesting that amino acids such as Lys157 are critical to nuclear localization of β -arrestin1.

L. NLS dominant β -Arrestin1 is incapable of potentiating NF- κ B activation

Given the observation that absence of β -Arrestin1 from the nuclear compartment due to mutation of the NLS leads to a loss of function in the activation of NF- κ B, it would stand to reason that fusion of a strong NLS to β -Arrestin1 would lead to a more nuclear-localized protein and hence produce a potentiation of NF- κ B activation. We created so called NLS dominant forms of β -Arrestin1 by cloning either wild type or mNLS β -Arrestin1 into an expression vector carrying tandem repeats of the SV40 NLS fused to a GFP protein. Imaging of the mutants confirms that the strong NLS of SV40 is able to drive the localization of the protein, as both the WT-

Figure 18.. β -arrestin1 mutation at Lys157 alters p65/RelA post-translational modification and NF- κ B activation.

In (**A**), acetylation of p65/RelA was detected using procedures described in Fig. 9A. In (**C**), phosphorylation of p65/RelA at Ser276 was detected using procedures described in Fig. 10A. Quantification and statistical analysis of acetylation and phosphorylation was performed and results are shown in (**B**) and (**D**), respectively. *, p < 0.05 and **, p < 0.01, compared to WT samples at the same time points, based on at least 3 experiments. In (**E**), p65/RelA binding to the WT, mNLS and K157A forms of β -arrestin1 was analyzed by immunoprecipitation of GFP-tagged β -arrestin1 from HeLa cells, followed by analysis of co-immunoprecipitation of p65/RelA by Western blotting. WCL= whole cell lysate. *Data in panel E was generated by Ni Cheng*.

**

60







D



Ε

0

**

30

K157A

**

60



В

Acetlyation Fold Change (acetyl-K/total p65)

Ò

30

WT

60

Α



Figure 19. β -arrestin1 mutation at Lys157 alters NF-kB:DNA interaction and transcriptional response.

In (**A**), electrophoresis mobility shift assay for NF- κ B was conducted using HeLa cells expressing WT or K157A β -arrestin1 and the B2 BK receptor. After serum starvation for 4 h, the cells were stimulated with either vehicle or 500 nM of BK for 60 min. Nuclear fractions were obtained and incubated with a radiolabeled NF- κ B consensus probe. The specificity of probe binding was verified by competition with a cold (unlabeled) probe, used in 100-fold excess. In (**B**), quantitative RT-PCR was performed on RNA from HeLa cells expressing WT, mNLS, or K157A β -arrestin1 and the B2BKR. After serum starvation overnight, the cells were stimulated with either vehicle or 500 nM of BK for the indicated time points. Total RNA was isolated as described in methods and the transcript level of IL-1 β was quantified by qPCR. Results are representative of 3 independent experiments. *Data in panel B was generated by Ni Cheng*.

βArrestin1-NLS and the mNLS-βArrestin1-NLS proteins are expressed exclusively in the nucleus of transfected HeLa cells (Figure 20A). We then sought to assay for gain of function of the mutants using an NF- κ B luciferase reporter assay. As expected, WT β-Arrestin1 and mNLS β-Arrestin1 provided results consistent with those previously obtained. Unfortunately, it appears exclusive nuclear expression of β-Arrestin1 is unable to potentiate NF- κ B activation, and in fact NLS dominant mutants of β-Arrestin1 are incapable of activating NF- κ B over basal stimulation (Figure 20B). This data suggests the mechanism of β-arrestin1 involvement in NF- κ B activation may be more complicated than simple scaffolding of required components within the nucleus.







In (A), Confocal microscopy images showing the localization of WT β -arrestin1-GFP, β -Arrestin1+NLS, and mNLS β -Arrestin1+NLS in HeLa cells. Forty-eight hours after transfection, cells were serum starved for 4 h and then fixed for imaging. DAPI was used to visualize nuclei. Images are representative of at least 2 independent experiments. In (B), NF- κ B luciferase assays were performed in cells transfected with a 3x- κ B reporter, a β -gal reporter, the B2BKR, as well as either WT β -Arrestin1, β -Arrestin1 mNLS, β -Arrestin1 +NLS, or mNLS β -Arrestin1 + NLS. Forty-eight hours after transfection, the cells were serum starved and then stimulated with BK or vehicle for 4 h. Samples were then harvested and assayed for luciferase expression. The results were normalized against the activity of a co-transfected β -gal construct.

CHAPTER IV: DISCUSSSION

The presence of a nuclear localization sequence in the β -arrestins is a debated subject. However previous published reports have clearly shown that β -arrestin1 is actively imported into the nucleus (Scott, Le Rouzic et al. 2002), and that an intact N domain (amino acids 1-185) is required for this import (Wang, Wu et al. 2003). Due to this we limited our exploration of the molecule to that region. Initially, loss of nuclear localization of β -arrestin1 was obtained with a mutant where 83 residues had been deleted from the N domain ($\Delta 107-191$). Presumably this level of truncation of the molecule would lead to a severe disruption of its functions due to alteration of the global structure. As such, we sought to identify a smaller region within this domain which might yield similar results. Manual analysis of the primary structure of β -arrestin1 suggested that the residues between K157 and K170 might be important, as they bore resemblance to the canonical bipartite NLS of nucleoplasmin (Robbins, Dilworth et al. 1991). Thus, we mutated just those residues comprising a "basic cluster" in β -arrestin1 to alanine. While our mutant contains a bipartitite basic motif, it is not similar to the classical bipartitite NLS in proteins such as nucleoplasmin. Some key differences include the relatively short linker between the basic residues (7 amino acids), as well as the relative spacing of the basic clusters within the primary sequence of the protein. The resulting mutant did indeed lose nuclear localization in both basal and stimulated conditions. While this mutant contained a dramatic 7residue substitution, we observed a significant loss of function with the much less severe singlepoint mutation of K157A. We feel this is an important observation as based on published reports of the structure of bovine β -arrestin1 (Han, Gurevich et al. 2001), residues K157,K160, and R161 are not exposed simultaneously with residues R169 and K170. In our functional assays,
mutation at Lys157 produced a phenotype similar to that of the 7-residue substitution in mNLS. A similar phenomenon was observed with mutation of Arg307, which alone reduced the ability of β -arrestin1 to activate ERK via Raf-1(Coffa, Breitman et al. 2011). Due to the high flexibility of the β -arrestin molecule and the variety of intermediate conformations it may take, it appears alteration of single residue of β -arrestin has a profound impact on its conformation and subsequent ability to bind to effectors.

This is the first report of identification of a novel nuclear localization sequence in β -arrestin1. Our data suggest that this sequence mediates active transport of β -arrestin1 into the nucleus via interaction with importin β 1, a member of the karyophorin family of nuclear import proteins. Defined importin β binding motifs include stretches of basic amino acids (Muhlhausser, Muller et al. 2001), Arg-Gly rich sequences (Leslie, Zhang et al. 2004), and sequences similar to the M9 NLS containing the YP motif (Kleinschmidt and Seiter 1988; Weis 2003). The variety of NLS in this category shows how difficult it is to define a consensus motif for importin β binding. At this time it is unclear if β -arrestin1 follows the more classical importin α/β - mediated nuclear import or bind to importin β proteins directly (Mattaj and Englmeier 1998; Weis 2003). Alternatively, since our experiments measure the formation of a complex rather than individual protein interaction, it may be possible that β -arrestin1 is transported into the nucleus via binding to an unknown protein carrier which is present in the complex with importin β 1. However, this is less likely because it was previously shown that β -arrestin2 could be actively imported into the nucleus in an nuclear import assay (Scott, Le Rouzic et al. 2002), where the unknown carrier may not exist. An additional finding is that the ability of β-arrestin1 to translocate and function in the nucleus appears to be independent of its membrane and cytosolic functions, as our results

show that mNLS is fully capable of translocating to the plasma membrane upon BK stimulation and to bind p65/RelA. We would predict that β -arrestin2 is also able to bind to the nuclear import machinery as it contains the same sequence at its corresponding residues. In addition, importin β 1 has been identified as a potential binding partner for β -arrestin2 by proteomic analysis (Xiao, McClatchy et al. 2007). Although both β -arrestins are imported into the nucleus, the nuclear function is most likely β -arrestin1-specific because β -arrestin2 is rapidly exported from the nucleus due to a strong leucine-rich nuclear export signal in its C-terminus. This strong NES in β -arrestin2 likely limits the protein's duration in the nucleus and ability to function there.

In addition to the nuclear localization sequence identified in this report, nuclear translocation of β -arrestin is also regulated through its homo- and hetero- oligomerization, which is coordinated by IP6 binding (Milano, Kim et al. 2006). Monomeric arrestin is more prevalent in the nucleus, whereas oligomeric arrestin is more cytosolic. The impact of oligomerization of β -arrestin1 on its ability to interact with the nuclear import machinery has not been explored however it is hypothesized that oligomerization of β -arrestin sequesters it in the cytosplasm and regulates its ability to interact with both the plasma membrane and nuclear proteins. It is our prediction that dimers of β -Arrestin1 would not be able to interact with the nuclear import machinery, as Lys157 is a critical residue in both the NLS and oligomerization domains. In fact, all the residues in the identified NLS, including K157, are located within the highly mobile "hinge" region of β -arrestin1 (Han, Gurevich et al. 2001), which in addition to importin binding and oligomerization, are also important for receptor interactions (Zhan, Gimenez et al. 2010). The competition of the various interacting molecules for the residues of the hinge region may play a role in determining subcellular localization. This region is also present in β -arrestin2 and we would predict that these

residues of the NLS are both necessary and sufficient for nuclear localization of both β -arrestins, as it has been shown that β -arrestin2 is actively imported into the nucleus of cells in a nuclear import assay in yeast (Scott, Le Rouzic et al. 2002). In that assay, β -arrestin2 with its NES mutated was fused to a Gal4 activation domain that was attached to a LexA DNA binding domain lacking its NLS. The chimeric construct was introduced into cells containing a *lacz* reporter gene. The β -arrestin2 fusion protein was able to direct the expression of *lacz*, indicating that β -arrestin2 is actively imported into the nucleus (Scott, Le Rouzic et al. 2002). These findings are consistent with our results and together they indicate an important role of the NLS in β -arrestin1 for its nuclear localization.

β-arrestins regulate NF-κB activation, but the underlying mechanisms are not fully understood. Several possibilities are suggested based on published reports and data from the present study. In the cytosolic compartment, β-arrestins bind to several NF-κB proteins including p105 (Parameswaran, Pao et al. 2006) and the inhibitory protein IκBα(Gao, Sun et al. 2004; Witherow, Garrison et al. 2004; Kizaki, Izawa et al. 2008), thus stabilizing the NF-κB complex in resting cells. Stimulation of cells with various agonists for GPCRs and cytokine receptors leads to NF-κB activation, indicating that the activation signals are able to overcome the inhibitory function of endogenous β-arrestins. Exactly how β-arrestins dissociate from the NFκB proteins and allow nuclear translocation of the NF-κB proteins has yet to be determined. In agonist-stimulated cells, both β-arrestins serve as scaffolding proteins facilitating diverse signaling events, including those leading to NF-κB activation. We have previously shown that βarrestin1 promotes NF-κB activation via interaction with c-Src following D2 dopamine receptor activation (Yang, Zhang et al. 2003). β -arrestin1 also enhances NF- κ B activation involving G $\beta\gamma$ and Akt (Yang, He et al. 2009). Thus, activation of NF- κ B downstream of B2BKR is mediated through both G protein-dependent and β -arrestin1-dependent signaling pathways, similar to what is described for the angiotensin receptor AT₁ (Wei, Ahn et al. 2003).

There appear to be two mechanisms by which β -arrestins regulate NF- κ B activation upon agonist stimulation. A part of this mechansim occurs in the cytosolic compartment, whereas results shown in the present study suggest that nuclear translocation of β-arrestin1 also contributes to the regulation of NF- κ B activation. It is true the observed loss of β -arrestin1 nuclear localization in cells expressing the mNLS and NES+ mutants was accompanied by reduced NF- κ B activation, which could result from stabilization of the inactive NF- κ B complex due to expression of β -arrestin1 in the cytosolic compartment (Witherow, Garrison et al. 2004). However, it is notable that cytosolic accumulation of mNLS had minimal impact on the nuclear translocation of p65/RelA (see Figure 12). Additionally, based on our data it is unlikely cytosolic sequestration of NF-kB occurs following bradykinin stimulation because if so there would be relief of this inhibition in cells expressing the NLS dominant β -Arrestin1 constructs. As such, we believe that expression of β -arrestin1 in the nucleus contributes significantly to NF- κ B activation in addition to those functions carried out in the cytosol. These events may be independently regulated as at this time it is unclear whether the β -arrestin1 protein activated by the GPCR at the plasma membrane and the β -arrestin1 protein signaling in the nucleus are subjected to the same temporal and spatial regulation. As such it may be possible that different pools of arrestin are responsible for p65/RelA binding in different subcellular compartments.

Based on the findings of this and our previous studies, we speculate that both the nuclear and cytosolic functions of β -arrestin1 are required for maximal activation of NF- κ B as we have observed that small interfering RNA-mediated knockdown of β -arrestin1 lead to a complete loss of NF- κ B activation in BK-stimulated cells (Yang, He et al. 2009). Furthermore, the seemingly conflicting reports of β -arrestin-NF- κ B interaction suggest stimulus dependent activation of β -arrestin1 to act as both "on" and "off" switch for NF- κ B makes sense in context of its fundamental role as a molecular scaffold. New evidence provides insight into how conformational changes in β -arrestin induced by receptor binding affect its ability to interact with protein partners (Coffa, Breitman et al. 2011). Indeed, our data with NLS dominant β -arrestin1 molecules which are incapable of receptor binding or NF- κ B activation suggests a complexity in arrestin involvement which at this time is not understood.

Our results demonstrate that one of the mechanisms by which β -arrestin1 modulates NF- κ B activation and transcriptional response involves regulating the recruitment of protein modifiers to p65/RelA in the nucleus and scaffolding them together. A model summarizing results of the current study is in Figure 21. Specifically, β -arrestin1 is able to recruit CBP/p300 and a protein kinase to p65/RelA, which directly contributes to the phosphorylation and acetylation of p65/RelA in the nucleus and increases transcriptional responsiveness. Our results show that mutation of the NLS in β -arrestin1 diminishes p65/RelA phosphorylation at Ser276 and profoundly decreases acetylation of p65/RelA, thereby confirming the nuclear function of β -arrestin1 in regulating NF- κ B activation. Post-translational modifications of p65/RelA, specifically its acetylation, have been reported to regulate the duration of its occupancy on



Figure 21. Proposed Model

Following BK stimulation, β Arrestin1 binds to the import machinery and translocates into the nucleus. The NF- κ B heterodimer also translocates to the nucleus independently of β Arrestin1. Once in the nucleus, β arrestin1 binds to the acetyltransferase CBP and a protein kinase and recruits them to p65. p65 is then acetylated and phosphorylated which enhances promoter stability and gene transcription. specific promoters (Chen, Mu et al. 2002). Indeed, our results from electrophoretic mobility shift assay show not only an overall decrease in NF- κ B-DNA interaction but also a more pronounced loss of p65/RelA-specific binding to the DNA probe. In comparison, the loss of DNA binding is not obvious with p50, suggesting that the β -arrestin1-facilitated post-translational modification of NF- κ B proteins influences the binding of specific NF- κ B proteins to the promoter. Transcriptional regulation by β -arrestin1 was also reported in other studies and contributes to the regulation of expression of homeobox genes (Yue, Kang et al. 2009), p27 and c-fos (Kang, Shi et al. 2005), and IFN- γ (Mo, Zhang et al. 2008). Furthermore, β -arrestin1 has been shown to mediate transcriptional responsiveness through modulation of chromatin modifying proteins such as histone acetyltransferases (data from current work and (Kang, Shi et al. 2005; Porter, Gonipeta et al. 2011)). Taken together, these findings support an important function of β arrestin1 in transcriptional regulation in the nucleus, specifically through the modulation of chromatin modifying proteins.

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CHAPTER V: CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

In conclusion, our study has led to the identification of a novel, functional NLS in β -arrestin1. We have also shown a role for nuclear localized β -arrestin1 in the regulation of NF- κ B activation. Due to its importance as molecular scaffold in a variety of signaling cascades, our report of the NLS sequence in β -Arrestin1 provides a valuable tool for the study of its function in other pathways. Given the potential significance of β -arrestin1-mediated transcriptional regulation in the nucleus, one of the future research directions is to understand the dynamic process of β -arrestin1 nuclear translocation.

Functionally, the arrestin proteins appear to be regulated in part via post-translational modification. Ubiquitination (Shenoy and Lefkowitz 2005; Shenoy, Barak et al. 2007), phosphorylation (Kim, Barak et al. 2002; Tohgo, Pierce et al. 2002), and nitrosylation (Ozawa, Whalen et al. 2008; Wyatt, Malik et al. 2011) marks have all been reported on β -arrestin1 or β -arrestin2 following various cellular stimuli. The modifications have been shown to impact the ability of the arrestin molecule to interact with its signaling partners. It is interesting to speculate that the regulation of β -arrestin's interaction with nuclear import proteins, and subsequent nuclear localization, may also be regulated in some part by modifications to arrestin, and should be explored. To date, no modifications to the residues identified within the nuclear localization sequence of β -arrestin1 have been observed however the region contains several lysine and serine residues, both of which are capable of being modified.

Furthermore, A few reports have suggested that β -arrestin may function differently based upon the cell type in which they are expressed. For example, exogenous expression of wildtype β - Arrestin1 was able to overcome a β 2AR resensitization defect in HEK293 cells but not COS-7 cells, under equivalent experimental conditions (Zhang, Barak et al. 1997). Differences in cellular function may be partly due to stiochiometric differences in the level of β -arrestin protein expression (Menard, Ferguson et al. 1997), and should be examined between cell types and carefully controlled for in future analyses of β -arrestin function. Indeed, careful examination of the data presented in this work suggests that nuclear localization may be influenced by the level of protein concentration as nuclear intensity of β -Arrestin1 seems to vary across experiments and expression levels (see Figures 5, 7, 8, 9, and 16). Additionally, variations in relative concentration of the β -arrestin isoforms expressed between cells could also be playing a role, and would explain the observation of anti-inflammatory function of β -Arrestin1 or β -Arrestin2 in CD11^{b-} versus CD11^{b+} splenocytes, respectively (Porter, Gonipeta et al. 2011).

It is likely that not only the stochiometric concentration, but also isoform prevalence, and modification profile of the β -arrestin molecule within a given cell ultimately determines β arrestin's function and the cellular response. Given the large number of known interacting partners for β -Arrestin1(Xiao, McClatchy et al. 2007), the ability of the protein to induce various pathway responses based on location of interaction provides exciting possibilities for understanding the complexity of cell signaling cascades and ultimately cell fates. In fact, it is likely that only minor changes in expression of, modification of, or localization of β -arrestin proteins could have a profound impact on the functions of the cell.

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EDUCATION	
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AWARDS	
University of Illinois Alumni Association Leadership Award	2010
Chancellor's Student Service and Leadership Award	2009 - 2010
Graduate Student Council Travel Award	2009
American Heart Association PreDoctoral Research Fellowship	
Award- Greater Midwest Affiliate	2008 - 2010
Beta Beta Biological Honor Society Distinguished	
Graduate Award	2004
University of Tulsa Undergraduate Research Award	2003
Honorable Mention, Symposia presentation at the 78 th Annual	• • • •
AAAS-Southwest and Rocky Mountain Division meeting.	2003
University of Tulsa Presidential Scholarship	2000 – 2002
ACADEMIC SERVICE	
Member, Student Research Forum Planning Committee	2010
Member, Dean of the Graduate College Search Committee	2010
Member, Tuition Advisory Committee	2010
President, Graduate Student Council	2009 - 2010
Member, Campus Care Advisory Committee	2009
Member, Vice-Chancellor for Research Search Committee	2009
Vice-President, Graduate Student Council	2008-2010
Graduate Representative, Tuition Advisory Council	
Executive Board	2008 - 2009
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Pharmacology Representative, Graduate Student Council	2007 - 2008
Teaching Assistant, GCLS 515	2007
Coordinator, Pharmacology Student Mentoring Program	2006 - 2008
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PUBLICATIONS AND PROFFESSIONAL PRESENTATIONS

Hoeppner, **Z**., Cheng, N and Ye, RD. (2012). "Identification of a Nuclear Localization Sequence in β-Arrestin1 and its Functional Implications". J Biol Chem, in press. doi: 10.1074/jbc.M111.294058

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Hoeppner, CZ, Cheng, N, and Ye RD. (2009) "Identification of the Nuclear Localization Sequence in Beta Arrestin1: Functional Implications in NF-kB activation". Presented at the College of Medicine Research Forum, University of Illinois at Chicago. Chicago, Ill.

Hanson, CZ and Ye, Rd. (2008) "The Role of Beta Arrestin1 in NF-kB activation". Presented at the Sigma Xi Research Forum. Chicago, Ill.

Hanson, CZ and Wymore, R. (2003) "The Role of Ion Channel Accessory Subunits in Cancer". Presented at the 78th Annual Meeting of AAAS-Southwest and Rocky Mountain Division. Tulsa, Oklahoma.

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