Identification of a Nuclear Localization Sequence in β-Arrestin-1 and Its Functional Implications

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Background: β-Arrestin-1 is present in the nucleus but lacks an identifiable nuclear localization signal.
Results: Using sequence comparison and mutagenesis, we found Lys157 is critical to β-arrestin-1 nuclear localization and its regulation of NF-κB activation.
Conclusion: β-Arrestin-1 uses a novel basic sequence for its entry into the nucleus.
Significance: This work provides a structural basis for the nuclear function of β-arrestin-1.

A mounting body of evidence suggests that β-arrestin-1 plays important roles in the nucleus, but how β-arrestin-1 enters the nucleus remains unclear because no nuclear import signal has been identified in the β-arrestins. We sought to characterize the cellular localization of wild type β-arrestin-1 and a series of N domain mutants to determine the structural basis and functional implications of β-arrestin-1 nuclear localization. A seven-residue candidate nuclear localization sequence (NLS) was identified based on sequence analysis. Mutation of the NLS led to a loss of β-arrestin-1 nuclear localization in transfected cells. Exogenous expression of wild type β-arrestin-1 enhanced the transcriptional activity of nuclear factor κB (NF-κB) induced by bradykinin, whereas mutation of the NLS reduced this effect by two-thirds relative to wild type controls. Loss of β-arrestin-1 nuclear localization was accompanied by reduced translational modification profile of p65/RelA. Further mutational analysis identified Lys157 within the putative NLS as being critical to nuclear localization of β-arrestin-1. Substitution of Lys157 to Ala led to reduced nuclear localization, decreased promoter binding by p65/RelA and decreased IL-1β gene transcription. These results demonstrate a critical role for β-arrestin-1 nuclear localization in scaffolding and transcriptional regulation.

The β-arrestin family is comprised of β-arrestin-1 (also known as arrestin-2) and β-arrestin-2 (arrestin-3), which act to regulate a diverse array of cell functions. Initially identified for their roles in desensitization of G protein-coupled receptors (GPCRs)1 (1, 2), the β-arrestins are now appreciated as critical scaffolds in a variety of cellular signaling cascades. One or both of the β-arrestins have been shown to interact with the Src family tyrosine kinases (3, 4), PI3K (5), Akt (6, 7), and other proteins including heat shock proteins (8, 9). β-Arrestins direct specific cellular functions by scaffolding the MAP kinases including JNK3 and ERK (10, 11). Of specific interest to this work, β-arrestins interact with several members of the nuclear factor κB (NF-κB) signaling pathway, including TRAF6 (12), p105 (13), and the inhibitory protein IκBα (14). β-Arrestins also interact with the upstream activating kinases, IκB kinase α, β, and NF-κB inducing kinase (15). It is reported that both β-arrestins work in the cytosol to stabilize IκBα at resting state, thus minimizing NF-κB activation. However, other published data have shown an enhancement of NF-κB activity in cells stimulated by GPCR ligands (7, 16, 17), suggesting that the scaffolding functions of the β-arrestins are able to overcome the IκBα stabilizing effect and promote NF-κB activation.

In addition to its role in scaffolding signaling pathways within the cytosol, emerging evidence suggests that β-arrestin-1 mediates signaling within the nucleus. In studies conducted by different groups, β-arrestin-1 was found to serve as an activator of CREB-mediated transcription following δ opioid receptor activation (18), but act as an inhibitor of STAT1-mediated transcription following IFN-γ stimulation (19). These data indicate that β-arrestin-1 can facilitate both activation and repression depending on cell type and the nature of stimulation. In all reported cases, β-arrestin-1 acts essentially by a scaffolding mechanism in the nucleus and the cytosol, recruiting specific proteins for complex formation. In these examples it is by the recruitment of the histone acetyltransferase p300 (18) or the tyrosine phosphatase TC45 (19) that β-arrestin-1 serves as an activator or inhibitor, respectively. Work on the nuclear function of the β-arrestins has been primarily limited to β-arrestin-1, because β-arrestin-2 has a strong nuclear export signal (NES) in its C terminus, which excludes it from the sustained presence in the nucleus (20).

Although mounting evidence demonstrates the nuclear functions of β-arrestin-1, the regulatory mechanism for its nuclear localization is still unknown. At ~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.
nuclear localization. Using DNA mutagenesis, we identified a NF-
 receptor (B2BKR) and the NF-
p65/RelA. This loss of promoter binding ultimately led to a
tion, and
which brings them to the transport protein importin
Importin 1 is responsible for shuttling NLS-containing pro-
teins through the nuclear pore complex in an energy-depen-
dent process involving GTP hydrolysis (23).

To further explore the nuclear functions of β-arrestin-1, we
analyzed the structural determinants required for β-arrestin nuclear localization. Using DNA mutagenesis, we identified a
region of seven amino acids (Lys157–Arg161/Arg169 and Lys170)
as being critical to nuclear localization of β-arrestin-1. We
identified these residues as necessary to mediate nuclear import
of β-arrestin-1 via interaction with importin β1. Further char-
cacterization of this region led to the identification of Lys157 as
being required for β-arrestin-1 nuclear localization. Function-
ally, nuclear localization of β-arrestin-1 is important for
enhanced NF-κB activation following bradykinin (BK) stimula-
tion, and β-arrestin-1 mutants are not capable of potentiating
NF-κB activation. The loss of function by the β-arrestin-1
mutants is due to a lack of post-translational modification to
the DNA-binding subunit p65/RelA. These modifications seem
to influence DNA binding, as there is a decrease in overall pro-
tein-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-κB targets, specifically IL-1β.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—All constructs were generated using previ-
ously described human β-arrestin-1 cDNA (NM_0020251) as a
template (16). β-Arrestin-1 Δ107–191 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 restric-
tion enzyme HindIII that cut at an endogenous site, were then
ligated into the pCI expression vector. The β-arrestin-1 mNLS
and NES + constructs were generated using the QuikChangeTM
mutagenesis kit (Stratagene, La Jolla, CA). 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 QuikChange mutations introduced substi-
tutions of Ala for the corresponding residues and for Lys157
substitution in β-arrestin-1. The NES + mutant was generated
with a primer converting Gln386 to Leu. Both constructs used
the previously generated β-arrestin-1-FLAG as a template.
β-Arrestin-1-GFP-tagged constructs were generated by PCR
amplification of WT, Δ107–191, mNLS, and NES + plasmids.
The PCR products were then subcloned into pEGFP-N1 (Clon-
tech, Mountain View, CA). HA-CBP was a kind gift from Dr.
J. R. Lundblad (Vollum Institute, Oregon Health Sciences Uni-
versity, Portland, OR). The constructs for the B2 bradykinin
receptor (B2BKR) and the NF-κB luciferase reporter were
described previously (16).

**Antibodies**—Antibodies against p65/RelA, p50, GFP, and
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were
from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG
was from Sigma. Phospho-p65 Ser276 was from Cell Signaling
Technology (Beverly, MA). Anti-acetyl-lysine, HDAC1, and
histone H1 were from Millipore (Temecula, CA). Anti-importin
β1 was from Calbiochem (La Jolla, CA).

**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 of penicil-
in, and 50 μg/ml of streptomycin. Cells were transfected with
dNA constructs listed above using Lipofectamine 2000 (Invit-
rogen) at a 3:1 reagent:DNA ratio. The DNA input was adjusted
to prevent overexpression 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
(24). Briefly, 5 × 106 cells were harvested by scraping in Hanks’
balanced salt solution and then pelleted by centrifugation at
1,000 × 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 mixture set III (Calbio-
chem) and to which 10% IGEPAE ca630 was added). Cell lysate
was incubated on ice for 10 min, 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, 1 mM EDTA) and incubated on ice for 30
min with occasional vortexing. Nuclear debris was pelleted by
centrifugation at 12,000 × g for 30 s. The supernatant was
recovered as the nuclear fraction. Fractions were then directly
analyzed by Western blotting. Protein concentration of sam-
ples was determined using the DC protein assay system (Bio-
Rad). Equivalent protein concentrations were separated on
4–12% BisTris precast SDS-acrylamide gels (Bio-Rad) at 60
mA. Proteins were then transferred to nitrocellulose mem-
branes at 100 V for 1 h. Membranes were then incubated with
5% nonfat 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.
Afterward, 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 incu-
bated with HRP-conjugated secondary antibodies for 1 h at
room temperature. Bound antibodies were detected using the
SuperSignal West Pico™ chemiluminescence substrate
(Pierce).

**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%
Triton X-100, 1 mM PMSF) containing protease/phosphatase
inhibitor mixture III (Calbiochem). 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) for 30 min at 4 °C. Samples were
centrifuged to pellet the beads, and the supernatant was trans-
ferrered to a new tube and incubated overnight with primary
antibodies. Protein complexes were then precipitated with the
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addition of 25 μl of 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 a 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 BK for the indicated time. To prepare samples for imaging, cells were first washed with Hanks’ balanced salt solution and then fixed by incubation in 4% paraformaldehyde for 20 min at room temperature. Free paraformaldehyde was quenched by the addition of 100 mM glycine. Samples were washed with Hanks’ balanced salt solution and then mounted onto glass coverslips using ProLong Gold Anti-fade Reagent with DAPI (Molecular Probes, Eugene, OR). 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 ImageJ software (National Institutes of Health, Bethesda, MD).

Luciferase Assay—HeLa cells in 12-well plates were transfected with vectors for the β-arrestin-1 constructs, B2BK, a 3× kB 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 BK for 4 h. Following stimulation, cells were washed and harvested in reporter lysis buffer (Promega). Luciferase expression was analyzed by incubating samples with luciferase substrate (Stratagene) and reading luminescence in a Femtometer FB12 luminometer (Zylux, Huntsville, AL). The β-galactosidase activity resulting from a co-transfected construct in the same cells was used for normalization of the NF-κB reporter data. All assays were performed three times with triplicate samples. Data were analyzed using GraphPad Prism software (version 4, GraphPad, La Jolla, CA).

Electromobility Shift and Supershift Assays—Nuclear extracts from transfected HeLa cells were generated as described under the “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 [γ-32P]ATP-labeled NF-κB consensus oligonucleotide probe (Promega). Supershift samples were preincubated 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 phosphor imager (GE Healthcare).

Quantitative Real Time PCR—HeLa cells (5 × 10^5) transfected with WT or mNLS β-arrestin-1 and the B2 receptor were stimulated with 500 μM BK for different periods of time. Total RNA was isolated with a RNasy isolation kit obtained from Qiagen. RNA was then reverse transcribed to create complimentary DNA (cDNA) using the SuperScript III RT Kit (Invitrogen). Real time quantitative PCR was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). 1 μg of cDNA from stimulated HeLa cells was analyzed using the GoTag qPCR Master Mix (Promega) containing 20 pmol of 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 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-1β were determined by using the C_t values and the formula: fold-increase = [(1 + ΔΔC_t)ΔC target (unstimulated – stimulated)]/(1 + ΔΔC_t)ΔC GAPDH (unstimulated – stimulated).

The sequences of the oligonucleotides used in this study are as follows: 5′-CTTGGTCCAGTGCCCTTCTC-3′ and 5′-TTCTGCCAGTGCCCTTTTG-3′ for IL-1; and 5′-ACCACAGTCCAGCATCAC-3′ and 5′-TCCACACCTTGTTGCCGTGTA-3′ for GAPDH.

Statistical Analysis—Quantification of Western blot data were performed using ImageJ software (National Institutes of Health). Statistical analysis was conducted using the GraphPad Prism software.

RESULTS

β-Arrestin-1 Translocates to Nucleus and Complexes with p65/RelA following Bradykinin Stimulation—We have previously shown that β-arrestin-1 enhances the activation of the transcription factor NF-κB following bradykinin stimulation via a Gβγ- and Akt-dependent mechanism (7). Recent reports have shown that in addition to scaffolding signaling events in the cytosolic compartment, β-arrestin-1 is able to act as a scaffold within the nucleus and has a direct effect on transcriptional responses (18, 25). Based on these reports we sought to determine whether β-arrestin-1 might influence NF-κB-mediated transcription directly in the nucleus in addition to regulating components in the cytosolic compartment, such as IkBα (14, 15), IKK, and p65/RelA (15). To test this we first determined if β-arrestin-1 moved into the nucleus following GPCR activation by agonists such as bradykinin, which activates NF-κB (26). The HeLa cell line was chosen because it has been widely used in studies of NF-κB and, unlike HEK293 and COS-7, it expresses modest levels of exogenous proteins upon DNA transfection. In β-arrestin-1- and B2BK-transfected HeLa cells there was an appearance of β-arrestin-1 in the nuclear fraction (Fig. 1A, bottom panel). Although some β-arrestin-1 was present in the nucleus before BK stimulation, its concentration increased in the nucleus within 15 min after stimulation, and the increase continued for at least 60 min. Because β-arrestins have been shown to interact with members of the NF-κB family in the cytosolic compartment (7, 14), we sought to determine whether β-arrestin-1 also complexes with p65/RelA in the nucleus. Nuclear fractions were made from transfected and BK-stimulated HeLa cells, incubated with an anti-p65 antibody for IP, and analyzed by Western blot for the co-IP of the FLAG-tagged β-arrestin-1. Following stimulation there was a time-dependent increase in the association between β-arrestin-1 and p65/RelA (Fig. 1A), along with increased β-arrestin-1 nuclear accumulation. These results suggest that β-arrestin-1 forms a
complex with p65/RelA in the nucleus following BK stimulation.

Identification of Novel Nuclear Localization Signal in β-Arrestin-1—Based on our observation that β-Arrestin-1 is able to complex with p65/RelA in the nucleus, we determined the structural basis for nuclear localization of β-Arrestin-1. A previously published report indicated that the region between residues 1 and 185 of β-Arrestin-1, known as the N domain, is required for its nuclear localization (27). To further examine this region, we created a series of N-terminal truncation mutants and their impact on nuclear localization was determined using confocal microscopy and Western blotting. Although truncation of the first 90 residues of β-Arrestin-1 had no impact on nuclear localization, truncating to residue 180 led to a predominantly cytosolic distribution of β-Arrestin-1 (data not shown). Based on this observation, we generated a β-Arrestin-1 construct in which amino acids 107 and 191 were removed (βH004107–191, Fig. 1B). The available restriction sites in the β-Arrestin-1 cDNA favors the choice of this region. Visualization of this construct revealed absence of the βH004107–191 β-Arrestin-1 from nuclei (Fig. 1C, second row). Attempts to utilize available predictive algorithms to identify consensus protein localization domains within this region were unsuccessful. Manual analysis of the sequence, however, revealed two candidate regions of interest, one which bore similarity to the recently identified PY-NLS, and a second with similarity to the classical bipartite NLS (reviewed in Ref. 28). 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-Arrestin-1 is found between amino acids 86 and 114, with the critical PY (YP) residues located at 113–114 (Table 1). Although a portion of the PY-NLS is outside of our original βH004107–191 mutant, the critical PY (YP) residues are within the N-terminal portion of this construct with p65/RelA in the nucleus following BK stimulation.

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**TABLE 1**

Common motifs found in the nuclear localization signals

The table lists examples of various families of previously identified nuclear localization signals. Monopartite NLSs such as that from SV40 and v-Jun are characterized by a cluster of basic residues. Bipartite NLSs such as those in nucleoplasmin, NIN2, Plk1, and parafibromin are characterized by 2 clusters of basic residues separated by a 7–10-residue linker. Finally, PY NLSs like those in M9 and TAP 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. Candidate NLS in β-arrestin-1 are also listed.

| NLS protein | Sequence | Source |
|-------------|----------|--------|
| Simple NLSs |          |        |
| SV40        | PKKKRKV  | 45     |
| v-Jun       | KSKRKL   | 46     |
| Bipartite NLSs |        |        |
| Nucleoplasmin |     |        |
| KRPATKKAGQAKKKKLDK | 36     |
| NIN2        | RKKRTTEESPLKDAAKKSK | 40     |
| Plk1        | RRRSLLEHKRRK | 47     |
| Parafibromin | KRAADELAEAKKPR | 48     |
| PY NLSs     |          |        |
| M9          | FGPMKGGNGFGRSGPY | 49     |
| TAP         | YAMDSQPDPRVNY | 50     |
| Candidate NLSs |       |        |
| β-Arrestin-1 | 157KIHKRNSVRLVIRK | 170    |
| β-Arrestin-1 | 106LQERLKKKGEHAYP | 174    |

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. Images representative of 5 independent experiments are shown.
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-arrestin-1, this NLS is located between residues 157–161 and 169–170, separated by 7 amino acids. To investigate the roles of these regions in β-arrestin-1 nuclear localization, alanine substitutions were made to generate two mutants, YP (YP to AA at amino acids 113–114) and mNLS (KIHKR/RR 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. 1B). As can be seen in Fig. 1C, substitution of PY to AA had no impact on subcellular distribution of β-arrestin-1 (Fig. 1C, fourth row). Mutation of the candidate bipartite NLS (mNLS), however, caused a profound shift of β-arrestin-1 distribution from the nucleus to the cytosol (Fig. 1C, third row). A β-arrestin-1 mutant, NES−, which contains the NES found in β-arrestin-2, 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 (Fig. 1C, fifth row).

Loss of Inducible Nuclear Translocation of β-Arrestin-1 by Mutation of NLS—Mutation of the candidate NLS in β-arrestin-1 leads to a loss of its nuclear expression at resting state. However, this does not eliminate the possibility that activation-dependent changes to β-arrestin-1 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-arrestin-1 constructs were stimulated with BK and assayed for the appearance of β-arrestin-1 in the nucleus. Increased nuclear localization of the wild type β-arrestin-1 was observed following BK stimulation (Fig. 2A). Unlike wild type β-arrestin-1, there was no inducible nuclear translocation of the mNLS mutant following BK stimulation (Fig. 2A, lower panel). To ensure that the loss of function was specific and not caused by misfolding of the mutated protein, we examined whether the mNLS mutant was able to translocate to the membrane upon agonist stimulation. The results showed that both the wild type and mNLS β-arrestin-1 moved to the plasma membrane in BK-stimulated cells (Fig. 2B), suggesting that this function of mNLS was retained. This result also suggests that membrane and nuclear translocation of β-arrestin-1 might be independently regulated.

β-Arrestin-1 Interacts with 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 (29, 30). Although several import pathways have been identified, many proteins use the importin β carrier proteins for nuclear transport (28). To examine if β-arrestin-1 NLS mediates nuclear translocation through this mechanism, we performed co-immunoprecipitation experiments between WT and mNLS β-arrestin-1 and members of the import machinery. As can be seen in Fig. 3A, following BK stimulation there was a stimulus-dependent increase in the association between β-arrestin-1 and importin β1, indicating active transport into the nucleus. A reciprocal immunoprecipitation showed similar results (Fig. 3C). The β-arrestin-1 NLS mutant mNLS was unable to interact with the nuclear import machinery following BK stimulation (Fig. 3, B and D). This loss of function suggests the mutated residues in mNLS β-arrestin-1 are important for mediating interaction with the nuclear import machinery.
Nuclear β-Arrestin-1 Enhances NF-κB-dependent Transcriptional Activity—Previously, we have shown that β-arrestin-1 is able to influence the activity of NF-κB following various GPCR activation (7, 16). We sought to determine whether the nuclear localization of β-arrestin-1 affects NF-κB activation. To examine this we tested our nuclear transport-deficient mutants in an NF-κB luciferase assay. As shown in Fig. 4A, the wild type β-arrestin-1 is able to increase NF-κB-driven luciferase expression following BK stimulation. Both the Δ107–191 and mNLS β-arrestin-1 were significantly less capable of potentiating NF-κB compared with the WT β-arrestin-1 (p < 0.01), suggesting that nuclear localization of β-arrestin-1 is required for optimal enhancement of NF-κB activity (Fig. 4A) and cytosolic accumulation of β-arrestin-1 alone is insufficient for augmenting NF-κB activation. To exclude the possibility that mutation in this region of β-arrestin-1 alters its interaction with key components of the NF-κB activation pathway, we examined another β-arrestin-1 construct containing the NES from β-arrestin-2. The NES construct was minimally altered in its sequence (Fig. 1B), yet cells expressing this construct also showed a significant loss of enhancement in NF-κB activation (p < 0.01) (Fig. 4A). In control experiments, we found that the loss of enhancement in NF-κB activation was not due to inefficient expression of the β-arrestin-1 mutants (Fig. 4A, Western blot), nor was it the result of lack of association with p65/RelA (Fig. 4B), as this function was unaltered with the mutations. These results suggest that nuclear localization of β-arrestin-1 is critical for its ability to potentiate NF-κB transcriptional activity.

Nuclear β-Arrestin-1 Regulates Post-translational Modification of p65/RelA—Next we determined how β-arrestin-1 regulates NF-κB activation in the nucleus. Because β-arrestin-1 formed a complex with p65/RelA in the nucleus, we investigated if mutation of the NLS in β-arrestin-1 affects the nuclear localization sequence of β-arrestin-1.
translocation of p65/RelA. Following BK stimulation, there was no apparent difference in nuclear localization of p65/RelA in cells expressing the wild type or mutant β-arrestin-1 (Fig. 5, A and B). 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. Because p65/RelA is modified in the nucleus by acetylation, which regulates its efficiency of transcriptional control (31, 32), we examined whether the nuclear β-arrestin-1 influenced acetylation of p65/RelA. We observed a significant loss of acetylation of p65/RelA in cells expressing the mNLS mutant compared with the wild type (Fig. 5C). Consistent with the loss of p65/RelA acetylation, there was a loss of association between p65/RelA and the acetyltransferase CBP (Fig. 5D) and between the mNLS mutant and CBP (Fig. 5E), which likely resulted from failed entry of the β-arrestin-1 mutant into the nucleus.

Functionally, acetylation of p65/RelA has been reported to be dependent upon its phosphorylation (33), and it is known that p65/RelA is phosphorylated at Ser276 in the nucleus by MSK1 (34). Therefore, we next examined the effect that NLS mutation of β-arrestin-1 might have on these modifications. Following stimulation with BK, there is a time-dependent increase in the phosphorylation of p65/RelA at Ser276 in cells expressing wild type β-arrestin-1, whereas in cells expressing the mNLS mutant there was a significant reduction of this phosphorylation at Ser276 (Fig. 6A). Quantification of additional blots verified these results (Fig. 6B). Acetylation and phosphorylation of p65/RelA has been shown to be important for transactivation and stable binding to the promoter (reviewed in Ref. 35). To examine this we performed electrophoretic mobility supershift assays with a canonical NF-κB binding sequence, using HeLa cells expressing the WT or mNLS mutant β-arrestin-1. As can be seen in Fig. 6C, BK stimulated an increase in NF-κB-DNA complex formation (lane 2 compared with lane 1). Consistent with the luciferase assay results (Fig. 4A), there was a decrease of protein/DNA binding in the mNLS expressing cells (lane 7) compared with cells expressing the WT β-arrestin-1 (lane 2). To identify the composition of the NF-κB dimers that bound to the DNA probe, we performed supershift assays in which the mobility of the NF-κB-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 NF-κB-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-κB 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). Lys157 Is Critical to Nuclear Localization of β-Arrestin-1—Because 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. 2B) and to bind p65/RelA (Fig. 4B). 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. To address this, we mutated charged amino acids in the bipartite NLS to Ala in combination or individually (Fig. 7A). This work identified Lys157 as being potentially important to 

\[\text{H}9252\,-\text{arrestin-1}\] nuclear localization. The K157A mutant was absent from the nucleus in transfected HeLa cells, whereas Ala substitution of other charged residues in this region did not affect 

\[\text{H}9252\,-\text{arrestin-1}\] nuclear localization (Fig. 7B). The inability of this mutant 

\[\text{H}9252\,-\text{arrestin-1}\] to translocate to the nucleus might be attributed to a failure to interact with importin 

\[\text{H}9252\,1\] (compare Fig. 7, C with D). In functional assays, the ability of the K157A mutant to enhance p65/RelA phosphorylation at Ser276 was markedly reduced compared with wild type controls (Fig. 8A, quantified in B). Another nuclear event examined was DNA binding of the NF-\(\kappa\)B proteins. In cells transfected with the K157A mutant,
Nuclear Localization Sequence of β-Arrestin-1

BK-induced DNA binding was drastically decreased when compared with cells transfected with the wild type β-arrestin-1 (Fig. 8C). To examine a role for β-arrestin-1 in gene transcription, we measured the IL-1β mRNA level in BK-stimulated HeLa cells that were transfected with wild type β-arrestin-1, the K157A or mNLS mutant. The enhancement effect of β-arrestin-1 on IL-1β production was significantly reduced in cells expressing either of the mutants. 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-arrestin-1.

DISCUSSION

The presence of a nuclear localization sequence in the β-arrestins is a debated subject. However, previously published reports have clearly shown that β-arrestin-1 is actively imported into the nucleus (20), and that an intact N domain (amino acids 1–185) is required for this import (27). Due to this, we limited our exploration of the molecule to that region. Initially, loss of nuclear localization of β-arrestin-1 was obtained with a mutant where 83 residues had been deleted from the N domain (Δ107–191). 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 that might yield similar results. Manual analysis of the primary structure of β-arrestin-1 suggested that the residues between Lys157 and Lys170 might be important, as they bore resemblance to the canonical bipartite NLS of nucleoplasmin (36). Thus, we mutated just these residues comprising a “basic cluster” in β-arrestin-1 to alanine. The resulting mutant did indeed lose nuclear localization in both basal and stimulated conditions. Although this mutant contained a dramatic 7-residue substitution, we observed a significant loss of function with the much less dramatic single-point mutation of K157A. We feel this is an important observation as based on published reports of the structure of bovine β-arrestin-1 (37), residues Lys157, Lys160, and Arg161 are not exposed simultaneously with residues Arg169 and Lys170. In our functional assays, mutation at Lys157 produced a phenotype similar to that of the 7-residue substitution in mNLS. This is the first report of identification of a novel nuclear localization sequence in β-arrestin-1. Our data suggest that this sequence mediates active transport of β-arrestin-1 into the nucleus via interaction with importin β1, a member of the karyophorin family of nuclear import proteins. Defined importin β binding motifs include stretches of basic amino acids (38), Arg-Gly-rich sequences (39), and sequences similar to the M9 NLS containing the YP motif (40). 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-arrestin-1 follows the more classical importin β-mediated nuclear import or bind to importin β proteins directly. Alternatively, because our experiments measure the formation of a complex rather than individual protein interaction, it may be possible that β-arrestin-1 is transported into the nucleus via binding to an unknown protein carrier that is present in the complex with importin β1. However, this is less likely because it was previously shown that β-arrestin could be actively imported into the nucleus in an in vivo import assay (20), where the unknown carrier may not exist. An additional finding is that the ability of β-arrestin-1 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-arrestin-2 is also able to bind to the nuclear import machinery as it contains the same sequence at its corresponding residues. In addition, importin β1 has been identified as a potential binding partner for β-arrestin-2 by proteomic analysis (8). Although both β-arrestins are imported into the nucleus, the nuclear function is most likely β-arrestin-1-specific because β-arrestin-2 is rapidly exported from the nucleus due to a strong leucine-rich nuclear export signal in its C terminus.

An interesting finding of this study is that all residues within the identified NLS, including Lys157, are located within the highly mobile “hinge” region of β-arrestin-1 (37), which has proven to be a “hot spot” of residues required for important arrestin functions such as IP6 binding (41) and receptor binding (42). In fact, in their previous report Milano et al. (41) speculated about the possibility of a 8-residue basic nuclear localization sequence in the β-arrestins that is masked by IP6 binding and oligomerization. Signal masking of the NLS is a common mechanism used to regulate the nuclear import of many proteins (43). We would predict that these residues are both necessary and sufficient for nuclear localization of β-arrestins, as it has been shown that β-arrestin-2 is actively imported into the nucleus of cells in an in vivo nuclear import assay in yeast (20). In that assay, β-arrestin-2 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-arrestin-2 fusion protein was able to direct the expression of lacZ, indicating that β-arrestin-2 is actively imported into the nucleus (20). These findings are consistent with our results and together they indicate an important role of the NLS in β-arrestin-1 for its nuclear localization.

β-Arrestins are found to 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 (13) and the inhibitory protein IκBα (14, 15), 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 NF-κB 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 β-arrestin-1 promotes NF-κB activation via interaction with c-Src following D2 dopamine receptor activation (16). β-Arrestin-1 also enhances NF-κB activation involving Gβγ and Akt (7). Thus, activation of NF-κB downstream of B2BKR is mediated through both G protein-dependent and β-arrestin-1-dependent signaling pathways, and there is a switch of the role for β-arrestins in regulating NF-κB activation upon agonist stimulation. A part of this switch occurs in the cytosolic compartment, whereas results shown in the present study suggest that nuclear translocation of β-arrestin-1 also contribute to the regulation of NF-κB activation. Indeed, the observed loss of β-arrestin-1 nuclear localization in cells expressing the mNLS and NES+ mutants was accompanied by reduced NF-κB activation, which could result from stabilization of the inactive NF-κB complex due to accumulation of β-arrestin-1 in the cytosolic compartment (14). However, it is notable that cytosolic accumulation of mNLS and NES+ had minimal impact on the nuclear translocation of p65/RelA. Unlike what has been described for JNK3 (44), it does not appear that β-arrestin-1 is shutting the members of the NF-κB pathway into and out of the nucleus, and p65/RelA is able to translocate into the nucleus independent of the translocation of β-arrestin-1. Based on these observations, we believe that accumulation of β-arrestin-1 in the nucleus contributes significantly to NF-κB activation.

Our results demonstrate that one of the mechanisms by which β-arrestin-1 modulates NF-κB activation and transcriptional response involves regulating the recruitment of protein modifiers to p65/RelA in the nucleus and scaffolding them together. Specifically, β-arrestin-1 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-arrestin-1 diminishes p65/RelA phosphorylation at Ser276 and profoundly decreases acetylation of p65/RelA, thereby confirming the nuclear function of β-arrestin-1 in regulating NF-κB activation. Post-translational modifications of p65/RelA, specifically its acetylation, have been reported to regulate the duration of its occupancy on specific promoters (32). Indeed, our results from electrophoretic mobility shift assays show not only an overall decrease in NF-κB-DNA interaction but also a more pronounced loss of p65/RelA-specific binding to the DNA probe. Transcriptional regulation by β-arrestin-1 was also reported in other studies and contributes to the regulation of expression of p27 and c-fos (18) and IFN-γ (19). Taken together, these findings support an important function of β-arrestin-1 in transcriptional regulation in the nucleus.

In conclusion, our study has led to the identification of a novel, functional NLS in β-arrestin-1. We have also shown a role for nuclear-localized β-arrestin-1 in the regulation of NF-κB activation. Given the potential significance of β-arrestin-1-mediated transcriptional regulation in the nucleus, one of the future research directions is to understand the dynamic process of β-arrestin-1 nuclear translocation in activated cells as well as in primary cells. Understanding how GPCRs, including B2BKR and the δ-opioid receptor, induce β-arrestin-1 nuclear translocation is of interest in uncovering additional physiological functions of β-arrestin-1.

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