**Ubiquination of β-Arrestin Links Seven-transmembrane Receptor Endocytosis and ERK Activation**

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β-Arrestin2 and its ubiquitination play crucial roles in both internalization and signaling of seven-transmembrane receptors (7TMRs). To understand the connection between ubiquitination and the endocytic and signaling functions of β-arrestin, we generated a β-arrestin2 mutant that is defective in ubiquitination (β-arrestin20K), by mutating all of the ubiquitin acceptor lysines to arginines and compared its properties with the wild type and a stably ubiquitinated β-arrestin2-ubiquitin (Ub) chimera. In vitro translated β-arrestin2 and β-arrestin20K displayed equivalent binding to recombinant β2-adrenergic receptor (β2AR) reconstituted in vesicles, whereas β-arrestin2-Ub bound ~4-fold more. In cellular coimmunoprecipitation assays, β-arrestin20K bound nonreceptor partners, such as AP-2 and c-Raf and scaffolded phosphorylated ERK robustly but displayed weak binding to clathrin. Moreover, β-arrestin20K was recruited only transiently to activated receptors at the membrane, did not enhance receptor internalization, and decreased the amount of phosphorylated ERK assimilated into isolated β2AR complexes. Although the wild type β-arrestin2 formed ERK signaling complexes with the β2AR at the membrane, a stably ubiquitinated β-arrestin2-Ub chimera not only stabilized the ERK signalosomes but also led to their endosomal targeting. Interestingly, in cellular fractionation assays, the ubiquitination state of β-arrestin2 favors its distribution in membrane fractions, suggesting that ubiquitination increases the propensity of β-arrestin for membrane association. Our findings suggest that although β-arrestin ubiquitination is dispensable for β-arrestin cytosol to membrane translocation and its “constitutive” interactions with some cytosolic proteins, it nevertheless is a prerequisite both for the formation of tight complexes with 7TMRs in vivo and for membrane compartment interactions that are crucial for downstream endocytic and signaling processes.

The multifunctional adaptor proteins β-arrestins (β-arrestin1 and -2) were originally identified as desensitizing molecules that prevent the coupling between seven-transmembrane receptors (7TMRs) and G proteins (1–3). More recently, however, it was found that β-arrestin binding to receptors not only stops G protein-mediated second messenger signaling but also engages several novel signaling pathways, including mitogen-activated protein kinase (MAPK) cascades (4, 5). Furthermore, β-arrestins have also been shown to bind and regulate cell surface receptors other than 7TMRs, and their signaling has been implicated in regulating the actin cytoskeleton, chemotaxis, antiapoptosis, and metastasis (6).

β-Arrestins serve as endocytic adaptors that bind clathrin and adaptin protein subunit 2 (AP-2) and facilitate receptor internalization via clathrin-coated vesicles (7–9). The differing affinity and trafficking patterns of GFP-β-arrestins induced by several 7TMRs have led to the classification of receptors into two groups, Class A and Class B (10). Class A receptors (e.g. β2-adrenergic, α1b-adrenergic, μ-opioid, endothelin 1A, and dopamine D1A receptors) show higher affinity for β-arrestin2 than β-arrestin1 and recruit GFP-β-arrestins only to the plasma membrane. Class B receptors (e.g. vasopressin V2, angiotensin AT1A, neurotensin1, thyrotropin-releasing hormone, and neurokinin NK-1 receptors) bind to both β-arrestin1 and -2 with equal affinity and colocalize with GFP-β-arrestin in endocytic vesicles. Thus, complexes formed between β-arrestin and Class A receptors are transient and exist only at the membrane, whereas those formed between β-arrestin and Class B receptors are stable and persist after receptor endocytosis (10). These differential patterns of β-arrestin2 recruitment correlate with the amplitude of β-arrestin-bound phosphorylated ERK1/2 (pERK). Class B receptors, such as the angiotensin 1A and the V2 vasopressin receptors activate a β-arrestin-bound pool of ERK more persistently than Class A receptors, such as the β2-adrenergic receptor (β2AR) and the α1b-adrenergic receptor (11).

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**Experimental Procedures**

**Supplemental Data**

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**References**

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**Footnotes**

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**Supplemental Material**

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**Supplemental References**

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**Cross-References**

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**Online Version**

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**Appendix**

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**Abbreviations**

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**β-Arrestin Ubiquitination and 7TMR Signalosomes**

β-Arrestins also become ubiquitinated (attachment of ubiquitin (Ub) on lysine residues) upon agonist stimulation of various 7TMRs. Upon β2AR stimulation, Mdm2 (mouse double minute2), a RING (really interesting new gene) type E3 ligase, ubiquitinitates β-arrestin2, and this modification is required for rapid internalization of the receptor (12). The pattern of β-arrestin ubiquitination correlates with the stability of receptor-β-arrestin interaction (*i.e.* transient interaction (Class A) is associated with transient ubiquitination, and persistent interaction (Class B) is associated with sustained ubiquitination) (13, 14). Exchanging the carboxyl-terminal amino acid residues of these two types of receptors reverses the patterns of β-arrestin trafficking as well as the time course of ubiquitination and the extent of β-arrestin-bound ERK activation (11, 13, 15). Additionally, translational fusion of ubiquitin to the C terminus of β-arrestin (β-arrestin2-Ub) leads to its cotrafficking and colocalization with the β2AR (Class A) in endocytic vesicles, thus mimicking a Class B trafficking pattern (13).

Interestingly, specific lysine residues are targeted for modification in response to agonist stimulation of a particular 7TMR. For example, angiotensin 1a receptor (AT1aR)-dependent sustained β-arrestin ubiquitination occurs primarily at lysines 11 and 12 in β-arrestin2 (16). Mutation of these lysines to arginines leads to the reversal of angiotensin II-stimulated β-arrestin ubiquitination from a sustained to a transient pattern, with a corresponding reversal of AT1aR-β-arrestin binding from stable endosome-localized complexes to transiently associated complexes seen only at the plasma membrane.

In an attempt to understand the role of ubiquitination in the regulation of the endocytic and signaling functions of β-arrestin, we generated a β-arrestin2 mutant (β-arrestin2AK) that is defective in ubiquitination by mutating all of the ubiquitin acceptor lysines in β-arrestin2 to arginines and compared it with the wild type and a stably ubiquitinated form in its ability to interact with 7TMRs and nonreceptor partners as well as its capability to facilitate receptor internalization and signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Reagents, and Plasmids—**COS-7 and HEK-293 cells were obtained from the American Type Culture Collection. COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and transiently transfected with pEGFP-C1 vector to obtain the expression plasmid for GFP-β-arrestin2-Ub. The lysine residue at position 48 in Ub was replaced with arginine using a QuikChange® site-directed mutagenesis kit (Stratagene). GFP-β-arrestin2-UbK48R was used in this work actually represents GFP-β-arrestin2-UbK48R.

Five rounds of mutations accomplished the construction of β-arrestin2AK, each mutagenesis step targeting 5–7 lysine residues. We used the QuikChange® multisite-directed mutagenesis kit (Stratagene) and followed the manufacturer’s instructions for the design of oligonucleotides and PCR protocols. The DNA fragment encoding β-arrestin2AK was later cloned into pEGFP-N1 to yield β-arrestin2AK-GFP. All DNA constructs were verified by sequencing. HA-β2AR plasmid was a gift from Dr. Neil Freedman (Duke University); HA-V2R plasmid was provided by Dr. Marc Caron (Duke University). Myc-c-Raf and RFP-ERK2 have been previously reported (18).

To achieve equivalent expression of β-arrestin2 WT and lysine mutants (Fig. 1), we transfected cells on a 100-mm dish with 1 μg of DNA for the WT and −7K, 3 μg of DNA for −14K, −19K, and −26K, and 2.5 μg for −31K. For the GFP-tagged plasmids (Fig. 5), 1 μg was used for the WT and −7K, and 2 μg was used for the rest.

**Immunoprecipitation and Immunodetection—**β-Arrestin2-FLAG/pCDNA3, β-arrestin2AK-FLAG/pCDNA3.1 or FLAG-β-arrestin2-Ub/pCDNA3.1 were used to immunoprecipitate β-arrestins. To detect active ERK in receptor immunoprecipitates, FLAG-β2AR was co-expressed with GFP-tagged β-arrestin constructs and RFP-ERK2. Cells were serum-starved for 2 h (COS-7) or 4 h (HEK-293) and then stimulated or not for the indicated times with the appropriate agonists. Cells were solubilized in a lysis buffer containing 50 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), aprotonin (5 μg/ml), pepstatin A (1 μg/ml), benzaminidine (100 μM), and 10 mM N-ethylmaleimide. The use of N-ethylmaleimide in lysis buffers in coimmunoprecipitation procedures is an important technical feature, since it stabilizes ubiquitinated species by preventing their deubiquitination. Soluble extracts were mixed with FLAG M2 affinity beads and rotated at 4 °C overnight. Nonspecific binding was eliminated by repeated washes with lysis buffer, and bound protein was eluted with sample buffer containing SDS. The proteins were separated on a gradient gel (4−20%; Invitrogen) and transferred to nitrocellulose membrane for Western blotting. Chemiluminescence detection was performed using SuperSignal® West Pico reagent (Pierce). pERK and β-arrestin signals were quantified by densitometry using GeneTools software.
In Vitro Translation of β-Arrestins and β-Arrestin-Recombinant β2AR Binding—[35S]β-arrestins were in vitro translated using a TNT® T7 quick coupled transcription/translation system (catalog number L1170; Invitrogen) according to the manufacturer’s recommended procedure. Briefly, reactions were assembled by mixing appropriate amounts of TNT® Quick Master Mix, [35S]methionine (catalog number AG1094; Amsham Biosciences), and pCDNA3.1-β-arrestin 2 wild type, β-arrestin20K, or β-arrestin2-Ub plasmids in 0.5-ml microcentrifuge tubes. The reactions were incubated at 30 °C for 90 min, and the in vitro translated [35S]β-arrestins were stored at −80 °C before performing binding experiments.

To study receptor binding, the in vitro translated [35S]β-arrestins were incubated in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA with 14.6 pmol (0.7 μg) of β2AR reconstituted in phospholipid vesicles at room temperature for 1 h. Purified GRK2 (0.5 μg), 80 μM ATP, 50 μM isoproterenol, or 50 μM propranolol were added to the reaction mixture where indicated. After the incubation period, an aliquot of the reaction was set aside to determine input levels of [35S]β-arrestins, and the remaining samples were diluted with ice-cold buffer and centrifuged at 85,000 rpm for 30 min with a bench top Optima TLX ultracentrifuge. After ultracentrifugation, the supernatant was removed, and the pellets were washed with 0.5 ml of PBS/HEPES to remove unreacted DTME and lysed in 40 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, and 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM EDTA. The samples were centrifuged again, and the wash was repeated five times. Finally, 30 μl of SDS-PAGE buffer were added to each sample, and proteins were separated by 4–20% gel. The gels were dried, and the amounts of β-arrestins bound to the β2AR were determined by autoradiography. Control experiments were performed by the same experimental procedure, except that empty vesicles were used in the place of receptor-containing vesicles. Bands were quantified by densitometry, and the amount of each β-arrestin was normalized to its input levels.

Cross-linking—COS-7 cells were transiently transfected with FLAG-β2AR along with pEGFP or β-arrestin20K-GFP. 30 h post-transfection, cells in 100-mm dishes were stimulated at 37 °C in phosphate-buffered saline (PBS) containing 10 mM HEPES (pH 7.4), with vehicle or agonist. Stimulations were terminated by the addition of diethio-bis-maleimidoethane (DTME; Pierce) to a final concentration of 2 mM, and plates were rocked for 40 min at room temperature. Cells were washed three times with PBS/HEPES to remove unreacted DTME and lysed in radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate), and receptors were immunoprecipitated.

Phospho-ERK Time Course—HEK-293 cells stably expressing the β,AR, transfected with vector, β-arrestin2-FLAG, FLAG-β-arrestin2-Ub, or β-arrestin20K-FLAG on 12-well plates were starved for at least 4 h in serum-free medium prior to stimulation. After stimulation, cells were solubilized by directly adding 2× SDS-sample buffer, followed by boiling at 100 °C for 5 min. For each transfection, an equal portion of the cells was set aside for protein determination (modified Bradford protocol). Equal amounts (μg) of cellular extracts were separated on 4–20% Tris-glycine polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes for immunoblotting. Phosphorylated ERK1/2, total ERK1/2, and β-arrestins were detected by immunoblotting with rabbit polyclonal anti-phospho-p44/42 MAPK (1:2000; Cell Signaling), anti-MAPK1/2 (1:10,000; Millipore), and anti-β-arrestin (1:3000; A1CT) antibodies, respectively. Chemiluminescence detection was performed using the SuperSignal West Pico reagent (Pierce), and phosphorylated ERK1/2 immunoblots were quantified using GeneTools software.

Confocal Microscopy—HEK-293 cells have a favorable morphology, such that sections of cytoplasm and nucleus can be simultaneously imaged; hence, they were used in these experiments. HEK-293 cells on 10-cm dishes were transiently transfected with HA-β2AR along with β-arrestin2-GFP, GFP-β-arrestin2-Ub, or βarrestin20K-GFP. Twenty-four hours post-transfection, cells were plated on collagen-coated 35-mm glass bottom plates. On the following day, cells were starved for at least 2 h in serum-free medium prior to stimulation. After stimulation, cells were fixed with 5% formaldehyde diluted in PBS containing calcium and magnesium. Fixed cells were permeabilized with 0.01% Triton in PBS containing 2% bovine serum albumin for 60 min and incubated at room temperature with appropriate primary antibody. The secondary antibody incubations were done for 1 h, followed by repeated washes using PBS. Confocal images were obtained on a Zeiss LSM510 laser-scanning microscope using multitrack sequential excitation (488, 568, and 633 nm) and emission (515–540 nm, GFP; 585–615 nm, Texas Red) filter sets. Live cell GFP images were acquired using a heated (37 °C) microscope stage and collected sequentially using single line excitation (488 nm).

Receptor Internalization—FLAG or HA epitope-tagged receptors expressed in HEK-293 cells in 12-well dishes were incubated with or without agonist for 30 min in serum-free medium at 37 °C. Cell surface receptors were labeled with M1 FLAG or 12CA5 monoclonal antibody and with fluorescein isothiocyanate-conjugated goat antibody to mouse IgG as a secondary antibody. Receptor internalization was quantified as loss of cell surface receptors as measured by fluorescence-assisted cell sorting (Duke University flow cytometry facility).

Subcellular Fractionation—Monolayers of COS-7 cells transfected with β-arrestin2 or β-arrestin2-Ub plasmids were gently scraped and collected in PBS containing protease inhibitors and 40 mM NaCl, subjected to two freeze-thaw cycles for lysis. Samples were centrifuged at 100,000 g for 5 min to precipitate unlysed cells. The resulting supernatant was centrifuged at 100,000 × g to separate soluble and membrane components. 40 μg of each fraction was separated on SDS gels and analyzed by Western blotting.

RESULTS

A Ubiquitin Minus β-Arrestin2 Mutant—To obtain a β-arrestin2 mutant that is not ubiquitinated upon 7TMR stimulation, we made conservative changes of groups of lysines to arginines, overexpressed FLAG-tagged mutants in COS-7 cells, and tested the β-arrestin precipitates for the ubiquitination signal induced by 1-min isoproterenol stimulation (Fig. 1, A and B). Surprisingly, elimination of such a signal required replacement of all 31 lysine residues of β-arrestin2.
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### Table 1

| Mutant   | Positions of lysine→arginine changes |
|----------|--------------------------------------|
| -7K      | 4, 50, 53, 158, 161, 272, 349         |
| -14K     | -7K and 11, 12, 78, 171, 298, 398, 401 |
| -19K     | -14K and 18, 107, 108, 207, 296       |
| -26K     | -19K and 25, 139, 228, 231, 232, 309, 314 |
| -31K     | -26K and 34, 153, 252, 326, 328       |

### Figure 1

**A** lysine-less β-arrestin2 (β-arrestin2\(^{\text{0K}}\)) is not ubiquitinated upon 7TMR stimulation. A, left column details the total number of lysine residues mutated to arginine. The right column depicts the position of the lysine in the primary sequence of rat β-arrestin2. B, COS-7 cells were transiently transfected with vector or the indicated β-arrestin2-FLAG plasmid and then stimulated with 1 μM isoproterenol (ISO) for 1 min. Anti-FLAG immunoprecipitates were probed for ubiquitin with FK2 antibody (top) and a polyclonal FLAG antibody (bottom). Serum-starved cells were stimulated with 1 μM isoproterenol for the indicated times, and FLAG immunoprecipitates were analyzed with FK2 ubiquitin antibody (top panel) and M2 FLAG antibody (bottom panel). The blots shown in B and C are from one of three separate experiments. IB, immunoblot.

To test whether the basic folding and binding properties of β-arrestin2\(^{\text{0K}}\) are retained, we compared the binding of *in vitro* translated β-arrestin2 and β-arrestin2\(^{\text{0K}}\) to purified recombinant B2AR reconstituted in vesicles. We also tested β-arrestin2-Ub for receptor binding under the same conditions. In these *in vitro* assays, both WT and β-arrestin2\(^{\text{0K}}\) represent nonubiquitinated forms, and only the β-arrestin2-Ub chimera constitutes the ubiquitinated form. As shown in Fig. 2, A and B, β-arrestin2\(^{\text{0K}}\) bound to the β2AR to the same extent as β-arrestin2. However, the presence of a single ubiquitin moiety increased the binding by 4-fold (Fig. 2, A and B). These experiments suggest that although both nonubiquitinated forms of β-arrestin2 (i.e. WT and 0K) are equipotent for β2AR binding, there is more binding between the β2AR and the ubiquitinated form (i.e. β-arrestin2-Ub). When binding was performed in the presence of isoproterenol, a small increase was observed for all three β-arrestin forms (data not shown). We hypothesized that reconstituted β-arrestin2 was already in an activated conformation due to the presence of zinterol in purification buffers. If so, inclusion of an antagonist could alter the observed binding. When β-arrestin-receptor complex formation was tested in the presence of propranolol, we found a dramatic decrease in binding for all three β-arrestin forms (Fig. 2, A and B), suggesting that propranolol destabilizes but does not eliminate receptor-β-arrestin binding in these experiments. Moreover, when reconstituted receptor samples were probed with a β2AR-specific phosphoserine antibody (serines 355 and 356), a small amount of phosphorylation was detected (Fig. 2C, top). The addition of GRK2 leads to an increase in the phosphorylation signal, and isoproterenol augments it further (Fig. 2C). We observed a comparable increase in binding above basal conditions for all three β-arrestin forms upon GRK2 phosphorylation and isoproterenol treatment of the reconstituted β2AR (Fig. 2D). Collectively, these *in vitro* binding assays confirm that, although ubiquitinated β-arrestin2 forms a tight complex with the β2AR, nonubiquitinated β-arrestin2 cannot bind reconstituted β2AR and that the protein-protein interaction domain(s) between the receptor and β-arrestin2\(^{\text{0K}}\) is mostly unperturbed.

To determine the isoproterenol-stimulated binding of β-arrestin2\(^{\text{0K}}\) to the β2AR in a cellular context, we employed immunoprecipitation assays utilizing chemical cross-linking with a sulphydryl-reactive compound, DTME (Fig. 3). We used COS-7 cells transiently transfected with FLAG-β2AR and β-arrestin2\(^{\text{0K}}\)-GFP, immunoprecipitated the receptors under nonstimulated or stimulated conditions (5 min, 1 μM isoproterenol), and detected β-arrestin2\(^{\text{0K}}\)-GFP by Western blotting (Fig. 3A). β-Arrestin2\(^{\text{0K}}\)-GFP binds to activated receptors with a 2–3-fold agonist-induced recruitment (Fig. 3B). In similar assays, the WT and β-arrestin2-Ub were recruited 10–12- and 12–15-fold, respectively (data not shown). These experiments further suggest that β-arrestin2\(^{\text{0K}}\)-GFP, albeit much weaker than the WT, nevertheless binds the β2AR upon agonist stimulation. Probably, the robust association of β-arrestin2\(^{\text{0K}}\) and
the β2AR does not occur in cells due to a lack of β-arrestin2 ubiquitination, which helps to stabilize the complex.

We next transfected HEK-293 cells stably expressing the β2AR with either β-arrestin2-GFP, GFP-β-arrestin2-Ub (stable ubiquitination), or β-arrestin20K-GFP (no ubiquitination) and examined the translocation patterns induced by isoproterenol. All three β-arrestin variants are uniformly distributed in the cytosol prior to agonist treatment (Fig. 4, A–C). Within 1 min of agonist stimulation, both WT and GFP-β-arrestin2-Ub are recruited to the cell membrane and form distinct puncta, and at 30 min, GFP-β-arrestin2-Ub is recruited to endosomal vesicles (Fig. 4B), whereas the WT remains at the membrane (Fig. 4A). As previously shown, with a Class A receptor, a stably ubiquitinated β-arrestin traffics into endosomes, whereas the transiently ubiquitinated WT β-arrestin dissociates and remains at the plasma membrane. On the other hand, agonist stimulation for 1 or 30 min does not lead to a major change in the intracellular distribution of β-arrestin2 0K-GFP (Fig. 4C, center panels).

To test whether the loss of translocation correlates with a loss of ubiquitination due to cumulative lysine mutations, we examined isoproterenol-induced recruitment of all of the mutants shown in Fig. 1, A and B. When the GFP-tagged version of each mutant was coexpressed with HA-β2AR in HEK-293 cells, we observed normal cytosolic expression under basal conditions for all of the β-arrestin2 variants (supplemental Fig. 1). However, upon 1 min of isoproterenol stimulation, a decrease in the level of recruitment was observed, correlating with the ubiquitination status of β-arrestin2 (Fig. 5, middle panels, and Fig. 1B). Some amount of recruitment remains even when 26 lysine residues are altered. The only mutant that is totally defective in translocation is
β-arrestin2<sup>0K</sup>, where no ubiquitination sites remain. These data suggest a strong correlation between β-arrestin ubiquitination status and its ability to bind activated receptors at the plasma membrane.

The above experiments also suggest that eliminating β-arrestin ubiquitination decreases its binding affinity for receptors in vivo, and hence only unstable receptor-β-arrestin2<sup>0K</sup>-GFP complexes arise at the cell membrane. In the case of Class A receptors, such as the β<sub>2</sub>AR, although receptor-β-arrestin complexes are initially formed at the plasma membrane, these complexes are not long lived. Thus, β-arrestin is rapidly deubiquitinated and dissociates from the receptor, and the receptor alone traffics into endosomes. This indicates that events occurring during pit/vesicle formation (e.g. β-arrestin deubiquitination) can influence the stability of β-arrestin-receptor complexes. Possibly, the lack of ubiquitin moieties on β-arrestin2<sup>0K</sup>-GFP leads to its rapid disengagement from the receptor complex.

We hypothesized that β-arrestin2<sup>0K</sup> binds activated receptor at the plasma membrane but that its deficiency in ubiquitination results in decreased stability of the complex as the receptor moves into pits. If this were true, then blocking the internalization of receptors should result in the retention of β-arrestin2<sup>0K</sup>-receptor complexes at the plasma membrane. Indeed, when we inhibited the internalization of either the β<sub>2</sub>AR (Fig. 6A) or the
Class B V2R (Fig. 6B) by co-expressing dynaminK44A (a classical inhibitor of endocytosis (19, 20)), we trapped activated receptors as well as β-arrestin20K-GFP at the membrane. These experiments clearly indicate that the deficiency in ubiquitination does not inhibit translocation of cytosolic β-arrestin20K to activated receptors at the cell membrane but rather decreases the stability of the receptor-β-arrestin complexes that are formed.

Role of β-Arrestin2 Ubiquitination in Receptor Internalization—A characteristic feature of β-arrestin2 is its ability to augment receptor internalization upon overexpression (9). This effect is particularly striking in COS-7 cells, which express very low levels of endogenous β-arrestin2 (21). To characterize the ability of β-arrestin20K to promote receptor internalization, we overexpressed it together with HA-tagged β2AR or V2R and measured the decrease in cell surface receptors after a 30-min agonist treatment. Overexpression of β-arrestin20K does not lead to any increase in receptor internalization, whereas WT β-arrestin2 leads to ~2.5-fold increase in both β2AR and V2R internalization (Fig. 7, A and B). Similarly, overexpression of β-arrestin20K results in no change in receptor internalization in HEK-293 cells (Fig. 7, C and D). Predictably, because of the unstable interaction of β-arrestin20K with activated receptors compared with the WT, the mutant does not have any inhibitory effect on receptor internalization in both cell types. We have previously demonstrated that the stably ubiquitinated form of β-arrestin2 (β-arrestin2-Ub) enhances receptor internalization compared with the WT (13). In contrast, β-arrestin20K, which is not ubiquitinated, forms unstable complexes with activated receptors and does not support internalization of either the β2AR or the V2R.

We further tested if the above lack of effect of β-arrestin20K to enhance receptor internalization was due to altered binding to endocytic proteins, such as clathrin and AP-2. Clathrin binds β-arrestin directly and stoichiometrically, and β-arrestin-clathrin binding is essential for receptor internalization via clathrin-coated vesicles (7). AP-2-β-arrestin interaction is required for the movement of receptors to clathrin-coated pits (8). When β-arrestin2, β-arrestin20K, or β-arrestin2-Ub were immunoprecipitated from COS-7 cells transfected with HA-β2AR after 0, 1, and 10 min of isoproterenol treatment, an agonist-dependent increase in clathrin binding was observed for both WT and β-arrestin2-Ub but not for β-arrestin20K (Fig. 8, A and B). β-Arrestin20K displayed only a weak interaction and a decrease in binding in the presence of isoproterenol (Fig. 8, A and B). For the wild type β-arrestin2, we found a 5-fold increase in AP-2 binding at 1 min of agonist treatment, and this binding decreased to basal levels at 10 min (Fig. 8, A and C). A
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![FIGURE 8.](image)

**FIGURE 8.** β-Arrestin2XK does not support receptor internalization. COS-7 (A and B) or HEK-293 (C and D) cells were transiently transfected with FLAG-β2AR (A and C) or HA-V2R (B and D). In each case, the receptor was cotransfected with vector plasmid (Mock), β-arrestin2(WT), or β-arrestin2XK (Mutant). After serum starvation, cells were treated with 10 μM isoproterenol (A and C) or 1 μM AVP (B and D) for 30 min at 37°C. Cell surface receptors before and after agonist treatment were determined by flow cytometry. Data in A represent the mean ± S.E. of four independent experiments done in triplicate. Data in B and C are the mean ± S.E. of three independent experiments done in triplicate. Data in D represent the average of two independent experiments done in triplicate. 25 μg of lysate protein from each transfection was tested for β-arrestin2 and β-arrestin2XK expression by Western blot as shown below each bar graph. A β-arrestin antibody (A1CT) was used for detection. It should be noted that unlike HEK-293 cells that express easily detectable amounts of both β-arrestin1 and β-arrestin2 (seen as a doublet), COS cells express less endogenous β-arrestin2 than HEK-293 cells. IB, Immunoblot.

![FIGURE 7.](image)

**FIGURE 7.** β-Arrestin2XK does not support receptor internalization. COS-7 (A and B) or HEK-293 (C and D) cells were transiently transfected with vector plasmid (Mock), β-arrestin2(WT), or β-arrestin2XK (Mutant). Isoproterenol-stimulated clathrin and AP-2 binding to β-arrestin2, β-arrestin2XK, and β-arrestin2XK. A, COS-7 cells were transiently transfected with vector or the respective FLAG-tagged β-arrestin2 plasmid and HA-β2AR. Cells were serum-deprived for 1 h and stimulated for the indicated times with 10 μM isoproterenol (Iso). Isolated anti-FLAG immunoprecipitates were simultaneously probed for clathrin heavy chain and the AP-2 subunit. The top panel represents the endogenous levels of both these proteins in COS-7 cells as detected by the antibodies. The top panel represents the amount of clathrin and AP-2 in β-arrestin IPs. The middle panel presents reprobing of the IP blot for β-arrestin2, β-arrestin2XK, and β-arrestin2XK with a β-arrestin antibody (A1CT). Shown are representative blots from one of four independent experiments. B, the bar graph depicts the quantification of clathrin associated with each type of β-arrestin2. Data were normalized to the amount of clathrin bound to WT under basal conditions. β-Arrestin2XK bound significantly less clathrin than the WT at 1 and 10 min of agonist treatment. *, p < 0.05; **, p < 0.01 versus the respective signal in the WT; two-way analysis of variance, Bonferroni post-tests. C, the bar graph shows quantification of AP-2 associated with each type of β-arrestin2 normalized to the levels detected in the WT immunoprecipitate under basal conditions. β-Arrestin2XK bound significantly higher AP2 than the WT basally and at 1 and 10 min of agonist treatment. *, p < 0.05; **, p < 0.01 versus respective signal in the WT; two-way analysis of variance, Bonferroni post-tests. IB, Immunoblot.

Similar time course of AP-2-β-arrestin2 interaction has been previously reported (8). Surprisingly, both β-arrestin2Ub and β-arrestin2XK displayed robust binding to AP-2 under both basal and stimulated conditions (Fig. 8, A and C). Understandably, the weak interaction of β-arrestin2XK with clathrin upon isoproterenol stimulation could be a major factor in its inability to promote receptor endocytosis. Unlike the previously reported β-arrestin1-(319–418), which binds clathrin but lacks receptor interaction (22), β-arrestin2XK did not act as an inhibitor of receptor internalization.

**Impact of Ubiquitination on Raf and ERK Scaffolding Properties of β-Arrestin2—**Previous studies have shown that β-arrestin-Raf complexes are stable, since their isolation is possible by gel filtration as well as by coimmunoprecipitation (18, 23). We tested the interaction of the above three β-arrestin forms with the MAPK kinase kinase, c-Raf (Fig. 9A), and did not observe any differences between the WT and β-arrestin2XK in their ability to bind Myc-c-Raf1. In these assays, β-arrestin2Ub, however, bound more c-Raf than the WT (Fig. 9A). The amount of c-Raf in the immunoprecipitate normalized to total input levels was significantly higher with β-arrestin2Ub than with the wild type, as indicated by the quantification of bands from three independent experiments (data not shown).

Previous studies have also shown that by merely coexpressing β-arrestin2 with an MAPK kinase kinase (such as
c-Raf or ASK-1) and a MAPK (such as ERK2 or JNK3), robust activation of MAPK could be achieved (18, 24, 25). This property of \( \beta\)-arrestin2 is attributed to its capacity to simultaneously bind component enzymes of a kinase cascade, thus bringing them into proximity and allowing robust phosphorylation to occur. Accordingly, cotransfection of \( \beta\)-arrestin2, c-Raf, and GFP-ERK2 could enhance precipitation of phosphorylated ERK2 with FLAG-\( \beta\)-arrestin2 (18). To examine whether \( \beta\)-arrestin20K was capable of a similar function, we transfected COS-7 cells with WT, \( \beta\)-arrestin20K, or \( \beta\)-arrestin2-Ub along with RFP-ERK2 and increasing amounts of Myc-c-Raf-1. As shown in the Western blots (Fig. 9B) and the bar graphs depicting quantification of pERK in \( \beta\)-arrestin2 precipitates (Fig. 9C), \( \beta\)-arrestin20K could scaffold pERK to the same extent as the WT. Interestingly, \( \beta\)-arrestin2-Ub precipitates contained 60–80% more pERK than the WT, suggesting a greater level of kinase activation and/or a stronger interaction of \( \beta\)-arrestin2-Ub with pERK. All of the above coimmunoprecipitation data suggest that ubiquitination is not required for \( \beta\)-arrestin interaction with c-Raf and pERK and that despite the 31 lysine mutations, \( \beta\)-arrestin20K can interact with these \( \beta\)-arrestin partners.

Role of \( \beta\)-Arrestin Ubiquitination in the Formation and Subcellular Targeting of Receptor Signalosomes—We next examined the effects of \( \beta\)-arrestin2, \( \beta\)-arrestin2-Ub, and \( \beta\)-arrestin20K on the assembly of receptor-\( \beta\)-arrestin2-ERK complexes. As depicted in Fig. 10A, a significant amount of
PERK was associated with β2AR immunoprecipitates from COS-7 cells upon coexpression of β-arrestin2-Ub. Lesser amounts of pERK were detected upon wild type β2-arrestin2 expression, although this amount was still higher than the pERK detected with endogenous β-arrestin2 in the mock-transfected samples (Fig. 10, A and B). Expression of β-arrestin20K resulted in a significant decrease in pERK in receptor complexes compared with that obtained with endogenous β-arrestin2 as seen in the bar graph representing the quantification of signals from five independent experiments (Fig. 10B). This decrease was not due to a decline in overall ERK activation, since the level of activation in whole cell lysates was identical in all transfection conditions.

In general, β-arrestin-mediated ERK signals are retained in the cytosol and are prevented from entering the nucleus. To determine if β-arrestin ubiquitination plays a role in the subcellular localization of agonist-stimulated pERK, we performed confocal immunofluorescence microscopy and examined the relative distribution of agonist-activated receptors, β-arrestins, and pERK. An antibody that specifically recognizes Thr202/Tyr204-phosphorylated ERK1/2 was employed to detect activated endogenous ERK. If ubiquitination of β-arrestin2 indeed plays a role in determining the spatial distribution of active ERK, then differences should be observed in the cellular distribution of pERK stimulated in the presence of β-arrestin2-Ub versus β-arrestin20K.

As depicted in Fig. 11A, unstimulated cells show a uniform cytosolic distribution of β-arrestin2-GFP (green), a membrane distribution of HA-β2AR (blue), and a negligible amount of pERK (red). When the cells were stimulated for 5 min with isoproterenol, β-arrestin2 redistributed to the cell membrane to colocalize with the activated receptors. A robust increase in the level of pERK was observed in both cytosol and nucleus along with a clearly demarcated pERK signal on β-arrestin-studded cell membranes (Fig. 11A, second row). After 30 min of isoproterenol, the β2-ARs were visualized in intracellular vesi-
cells overexpressing both HA-5 min time point, unlike the case of WT cell membrane, some cells did have small vesicles in the vicinity detected (Fig. 11 proterenol treatment, a negligible amount of pERK was recycled and/or noninternalized receptors. After 30 min of iso-
receptors persist at the membrane, which most likely represent but are retained at the cell membrane. A small percentage of little active ERK was distributed in the nucleus with
nucleus. PMA stimulation does not lead to either activation of ERK, which is distributed in both cytoplasm and

treated with phorbol 12-myristate 13-acetate (PMA) are shown

Effects of \( \beta \)-arrestin overexpression on the time course of isoproterenol-stimulated pERK.

HEK-293 cells stably expressing the \( \beta \)-AR were transiently transfected with vector, \( \beta \)-arrestin2, \( \beta \)-arrestin2-Ub, or \( \beta \)-arrestin20K. Monolayers of cells in 12-well dishes were stimulated with isoproterenol (10 \( \mu \)M) for the indicated times, and whole cell lysates were analyzed for ERK phosphorylation by Western blotting. A, bar graphs representing the quantification of pERK bands normalized to ERK levels obtained from 4–6 experiments. Maximal signal from an individual experiment was used as 100%. ***, \( p < 0.001; **, p < 0.01;\) two-way analysis of variance, Bonferroni post-tests, all compared with vector only condition. B, representative Western blots for phospho-ERK in the top panels. The same blots were stripped (Restore stripping buffer; Pierce) probed for ERK1/2 levels (middle), and restriped and reprobed for \( \beta \)-arrestin levels (bottom).

The results of similar experiments performed with GFP-\( \beta \)-arrestin2-Ub and HA-\( \beta \)-ARs are shown in Fig. 11B. Under unstimulated conditions, the subcellular distributions are identical to what is observed with the WT \( \beta \)-arrestin2. Quite strikingly, at 5 min of stimulation, a distinct and robust ERK activation is observed at the cell membrane coinciding with the distinct membrane recruitment of \( \beta \)-arrestin2-Ub. Although a majority of the cells (~80%) displayed such distribution at the cell membrane, some cells did have small vesicles in the vicinity of the cell membrane, which contained \( \beta \)-arrestin2-Ub, \( \beta \)-AR, and pERK as shown in Fig. 11B (second row). Surprisingly, at the 5 min time point, unlike the case of WT \( \beta \)-arrestin2 expression, little active ERK was distributed in the nucleus with \( \beta \)-arres-
tin2-Ub overexpression. We do not know the exact mechanism by which this occurs, but possibly, \( \beta \)-arrestin2-Ub can simul-
taneously promote \( \beta \)-arrestin-dependent cytosolic ERK and curb the G protein ERK pathway, leading to less nuclear ERK.

After 30 min of isoproterenol treatment, a dramatic redistribution of \( \beta \)-arrestin2-Ub, \( \beta \)-AR, and pERK was seen in intracellular vesicles. These data clearly indicate that a stably ubiquitinat-
ed \( \beta \)-arrestin can remain associated with a Class A receptor (i.e. \( \beta \)-AR) and target activated ERK to early endosomes, resulting in a pool of pERK complexed with internalized receptors.

In the absence of agonist, \( \beta \)-arrestin20K-GFP is mainly cytoplasmic, with HA-\( \beta \)-AR at the plasma membrane and very little active ERK (Fig. 11C, top row). After 5 min of isoproterenol stimulation, a robust activation of ERK occurs, which is seen distributed in both cytoplasmic and nuclear compartments. However, none of this active ERK is localized with \( \beta \)-arrestin20K. Possibly, much of this activity is G protein-mediated and is excluded from receptor complexes, since less pERK is complexed with the \( \beta \)-AR in the presence of \( \beta \)-arrestin20K (see Fig. 10). At 30 min, levels of pERK decreased but were not abolished (Fig. 11C, bottom row). This situation contrasts with what is observed with the stably ubiquitinated \( \beta \)-arrestin2-Ub (Fig. 11B), where pERK signals are stabilized and localized on endosomal vesicles at 30 min of isoproterenol stimulation. As seen in the 30 min panels of Fig. 11C, \( \beta \)-AR internalized into endosomes, which is consistent with our internalization data (Fig. 7, A–D), which indicate the inability of \( \beta \)-arrestin20K to inhibit receptor internalization.

We also determined the kinetics of ERK phosphorylation in HEK-293 cells expressing the \( \beta \)-AR (1 pmol/mg of cellular protein) upon transfection of vector, \( \beta \)-arrestin2 WT, \( \beta \)-arrestin20K, or \( \beta \)-arrestin2-Ub. As shown in Fig. 12, expression of \( \beta \)-arrestin2-Ub significantly increased ERK activity at 20 min of isoproterenol treatment, \( \beta \)-arrestin2 led to a modest augmentation, and \( \beta \)-arrestin20K had no effect over mock conditions (Fig. 12B). Previous studies have demonstrated that later ERK activity induced by 7TMRs is actually \( \beta \)-arrestin-mediated (reviewed in Ref. 26). These results further support the idea that \( \beta \)-arrestin ubiquitination status underlies some aspects of \( \beta \)-arrestin-dependent signaling.

Ubiquitination Favors \( \beta \)-Arrestin Distribution in Membrane Compartments—The \( \beta \)-arrestin isoforms are mainly cytosolic proteins and are translocated to the plasma membrane upon 7TMR activation. Thus far, no lipid modifications in \( \beta \)-arrestins favoring macromolecular membrane interactions have been identified. One well accepted mechanism that keeps them in a membrane environment is their binding to phosphorylated domains of receptors (5). Our current and previous results indicate that ubiquitination could be an important factor
that determines the longevity of β-arrestin interactions with receptors leading to colocalization on endosomal vesicles. Interestingly, when we analyzed the distribution of the ubiquitinated form of β-arrestin by subcellular fractionation, we found that the ubiquitination status of β-arrestin favors its partitioning to membrane fractions. When COS-7 cells expressing either β-arrestin2 or β-arrestin2-Ub were lysed in a detergent free low salt buffer (40 mM NaCl) and the soluble and insoluble fractions were further separated by differential centrifugation, nonubiquitinated β-arrestins were mainly cytosolic. Most of the exogenously expressed β-arrestin2 as well as YFP-β-arrestin2 was detectable in the soluble fraction (Fig. 13). The YFP-β-arrestin2 band in the membrane fraction with a slightly slower mobility is unreactive to ubiquitin antibodies, such as FK2, P4D1, and FK1, and its identity remains to be elucidated. On the other hand, ubiquitinated β-arrestin2 was distributed mostly in the insoluble membrane fractions (Fig. 13, A and B). As seen in Fig. 4B, β-arrestin2-Ub appears to be uniformly distributed in the cytosol in an undisturbed cell. Accordingly, the membrane fractionation of β-arrestin2-Ub is not due to its presence in inclusion bodies but rather due to its affinity for membrane components. These results suggest that ubiquitination increases the propensity of β-arrestin for membrane association, thus favoring prolonged localization of β-arrestin in membrane microdomains. Although ubiquitination is dispensable for β-arrestin interactions with cytosolic partners, it may be necessary to facilitate the formation of functional 7TMR-β-arrestin endocytic and signaling complexes in a membrane environment.

**DISCUSSION**

β-Arrestin1 and -2 are ubiquitously expressed proteins that function to desensitize G protein mediated signals, which arise upon the stimulation of 7TMRs (2, 3). β-Arrestins also bind to clathrin and AP2 and serve as endocytic adaptors for several 7TMRs to promote internalization via clathrin-coated vesicles (7–9). In addition, β-arrestin2 functions as a receptor-regulated scaffold for MAPK pathways (e.g. for JNK3 and ERK2) (18, 25). Recently, it has been demonstrated that β-arrestin2 can specifically initiate ERK pathways even when receptors do not couple to G proteins (17, 26, 27). Moreover, β-arrestin-dependent signaling plays important roles in diverse cellular processes, including cell proliferation, membrane ruffling, chemotaxis, and metastasis (28–31).

A consequence of agonist stimulation of several 7TMRs is the ubiquitination of β-arrestins, which is required for rapid receptor internalization (12). Ubiquitination is a post-translational modification that was originally described in the context of regulated destruction of many proteins by the proteasomal machinery (32). However, recent years have witnessed the discovery of a plethora of nonproteasomal roles of ubiquitin (33, 34), and ubiquitination has been shown to play an important role in the lysosomal degradation of 7TMRs (35). Moreover, monoubiquitination of adaptor proteins, such as Eps15, and mono-/multiubiquitination of cell surface receptors are implicated in endocytosis, whereas polyubiquitination of the adaptor protein TRAF6 is suggested to be crucial for triggering NF-κB signaling pathways (36–38). β-Arrestin functions as both an endocytic and a signaling adaptor for 7TMRs and bears a functional analogy to proteins such as Eps15 and TRAF6. Currently, the nature of β-arrestin2 ubiquitination as to being poly- or mono- ubiquitination is unknown. Nevertheless, β-arrestin ubiquitination facilitates both receptor internalization and MAPK activation.

The kinetics of β-arrestin ubiquitination and deubiquitination appear to determine the stability and duration of β-arrestin-receptor interactions, which in turn determine its trafficking pattern (13). Our studies indicate that stable β-arrestin-receptor interaction leading to cotrafficking of receptors and β-arrestins into endosomes not only results in sustained ubiquitination but also in the enhanced activation of ERK. β-Arrestin ubiquitination also plays an important role in promoting receptor internalization (12, 13). Are these cellular processes (namely receptor internalization, β-arrestin trafficking, MAPK activation, and β-arrestin ubiquitination) independent or related events? Could β-arrestin ubiquitination serve as a locus of control for these various pathways? To understand the integration of ubiquitination into the traffick-
ing and signaling functions of β-arrestins, we sought to compare wild type β-arrestin2 with a nonubiquitinated form as well as a stably ubiquitinated form. To generate β-arrestin2 totally defective in ubiquitination, we had to replace all of the 31 lysines within β-arrestin2 with arginine residues. Sometimes it takes only one mutation to generate a completely misfolded protein, so it is a concern to study a protein with 31 lysine-arginine changes. However, we believe that the conservative nature of the introduced changes allowed β-arrestin20K to be functional in both in vitro assays and our protein–protein interaction studies. For the most part, β-arrestin20K behaved like the wild type β-arrestin2, since its binding to recombinant β2AR in vitro and to c-Raf and ERK was unchanged from the wild type. Interestingly, β-arrestin20K bound AP-2 much more robustly than the wild type, whereas its interactions with clathrin and the β2AR were impaired in vivo. Previous studies have shown that certain arginine residues in β-arrestin2 (Arg394 and Arg396) are involved in AP-2 binding (39). It is possible that by introducing 31 arginines in the place of lysines in β-arrestin20K, we introduced additional AP-2 binding sites leading to a more robust interaction. Although the AP-2 binding domain in the wild type β-arrestin2 is exposed only after a receptor-induced conformational change, it is possible that for the β-arrestin20K, some of the arginines could present an interaction domain constitutively.

Although β-arrestin20K is capable of equivalent protein–protein interactions with the β2AR as the nonubiquitinated wild type in vitro, in a cellular context, it shows impairment in binding, since unlike the wild type, it cannot be ubiquitinated at the proper site(s). β-Arrestin20K did not support internalization of either β2AR or V2R, confirming that ubiquitination of β-arrestin is crucial for its role in promoting receptor endocytosis (Fig. 7). Additionally, this mutant was only transiently recruited to the plasma membrane upon stimulation of the β2AR or the V2R (Fig. 6). In contrast, a β-arrestin-Ub chimera that remains stably ubiquitinated can enhance β2AR internalization (13) and is stably recruited to endosomal compartments with the β2AR (Fig. 4B). Although both ubiquitinated and nonubiquitinated forms of β-arrestin can form complexes with pERK (Fig. 9), only the ubiquitinated form is capable of this function in a receptor complex (Figs. 10 and 11). In other words, β-arrestin ubiquitination plays a central role in stabilizing kinase activity in receptor signalosomes. However, whether β-arrestin ubiquitination acts as a “trigger mechanism” for activating the c-Raf-MEK1-ERK2 cascade remains to be determined. Thus far, TRAF6 autoubiquitination is the only example in which the adaptor protein ubiquitination initiates kinase signaling (38).

One important consequence of β-arrestin-dependent MAPK activation is the compartmentalization of the signals. Thus, AT1AR-stimulated pJNK3 scaffolded by β-arrestin2 is retained on endocytic vesicles (25). Similarly, AT1AR-stimulated ERK is concentrated on endosomes, which are also the destination for internalized receptor–β-arrestin complexes (18). As evidenced by our microscopy experiments, this subcellular location of MAPKs is indeed directed by the ubiquitination status of β-arrestin2 (Fig. 11, A–C). Thus, a stably ubiquitinated β-arrestin not only confers a Class B trafficking pattern on a Class A recepto-

### β-Arrestin Ubiquitination and 7TMR Signalosomes

Our cell fractionation experiments indicate that ubiquitin moieties on β-arrestin somehow favor its membrane distribution. We currently do not know the exact mechanism by which ubiquitin chains favor membrane interactions that facilitate subsequent signalosome formation. It is possible that although the concave domains of β-arrestin interact with the phosphorylated domains of the receptor, ubiquitin chains elsewhere on β-arrestin help its retention in a membrane environment. Furthermore, the ubiquitinated domains of β-arrestin could favor a tighter interaction with c-Raf, allowing robust kinase activation at the membrane. This activity is then passed down through the cascade, leading to ERK phosphorylation within the β-arrestin scaffold.

The subcellular distribution of visual arrestins as well as β-arrestins is also influenced by their binding to various phosphoinositides (40, 41). Inositol 1,4,5-bisphosphate and inositol 1,4,5-trisphosphate binding facilitates plasma membrane recruitment of β-arrestins (42), whereas the soluble ligand IP6 regulates oligomerization of β-arrestin1 and β-arrestin2 as well as nuclear localization of β-arrestin1 (43). Interestingly, mutation analyses of bovine β-arrestins as well as structural studies of β-arrestin1–IP6 co-crystals indicate the presence of lysine residues within the phosphoinositide-binding domains in β-arrestin (42, 43). Since our results indicate that β-arrestin ubiquitination favors its membrane interactions (Fig. 13), it will be of great interest to determine if ubiquitination at distinct lysine(s) plays any role in β-arrestin oligomerization and/or modulates β-arrestin–phosphoinositide binding.

Our data suggest that β-arrestin and the ERK protein bound to it have to remain at the membrane for an optimal duration before receptor signalosomes are formed. Moreover, a subsequent stable interaction of receptor and β-arrestin is required for sustaining and targeting this activity to subcellular compartments. Interestingly, previous studies have shown that the membrane recruitment of β-arrestin2 itself can lead to ERK signaling and that a 7TMR-β-arrestin1 chimeric protein can act as a constitutive signalosome (44, 45). Our findings indicate that β-arrestin ubiquitination controls not only receptor trafficking but the nature, stability, and subcellular localization of active ERK signals. It seems overwhelmingly likely that this regulation also extends to the nature of the ERK substrates and hence of the cellular consequences of receptor mediated activation of ERK. Seen in this light, β-arrestin ubiquitination may be viewed as the “glue” that holds the receptor “signalosome” together and directs the ultimate destination of its cellular journey. It will be of interest to determine which other signaling pathways may be regulated in this way.

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