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Arrestins: Critical Players in Trafficking of Many GPCRs⋆

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Abstract

Arrestins specifically bind active phosphorylated G protein-coupled receptors (GPCRs). Receptor binding induces the release of the arrestin C-tail, which in non-visual arrestins contains high-affinity binding sites for clathrin and its adaptor AP2. Thus, serving as a physical link between the receptor and key components of the internalization machinery of the coated pit is the best-characterized function of non-visual arrestins in GPCR trafficking. However, arrestins also regulate GPCR trafficking less directly by orchestrating their ubiquitination and deubiquitination. Several reports suggest that arrestins play additional roles in receptor trafficking. Non-visual arrestins appear to be required for the recycling of internalized GPCRs, and the mechanisms of their function in this case remain to be elucidated. Moreover, visual and non-visual arrestins were shown to directly bind *N*-ethylmaleimide-sensitive factor, an important ATPase involved in vesicle trafficking, but neither molecular details nor the biological role of these interactions is clear. Considering how many different proteins arrestins appear to bind, we can confidently expect the elucidation of additional trafficking-related functions of these versatile signaling adaptors.

1. ARRESTINS AND GPCR TRAFFICKING

Preferential binding of arrestins to active phosphorylated receptors was discovered about 30 years ago.[1] The finding that arrestin binding suppresses receptor coupling to cognate G proteins was made soon after in the visual system.[2] The mechanism turned out to be remarkably simple: direct competition between arrestin and G protein for overlapping sites.[3,4] For some time, it appeared that the only function arrestins have is to bind active phosphorylated G protein-coupled receptors (GPCRs), precluding receptor interactions with G proteins by direct competition.[3,4] The first described non-GPCR binding partners of arrestins were trafficking proteins: clathrin in 1996[5] and clathrin adaptor AP2 a few years later.[6] These data demonstrated that arrestins play an essential role not only in GPCR desensitization[7] but also in receptor endocytosis,[8] via trafficking signals added by receptor-bound arrestins. The discovery that arrestins are ubiquitinated upon receptor binding and regulate ubiquitination of GPCRs[9] revealed yet another mechanism, whereby arrestins regulate receptor trafficking indirectly. Here, we discuss several known mechanisms of arrestin effects on GPCR trafficking and highlight observations that suggest that there are many other mechanisms that still remain to be elucidated.

⋆We use systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons, its gene is called “arrestin 3” in the HUGO database).

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2. NON-VISUAL ARRESTINS MEDIATE GPCR INTERNALIZATION VIA COATED PITS

Arrestins promote GPCR internalization by virtue of recruitment of clathrin and AP2 via fairly well-mapped binding sites in the C-tail of non-visual arrestins[5,6,10,11] (Fig. 1). Interestingly, the C-tail in the basal conformation of all arrestins is anchored to the N-domain,[12–16] whereas receptor binding triggers its release.[17–19] The expression of separated arrestin C-tail carrying these sites inhibits GPCR internalization, apparently by winning the competition with the arrestin–receptor complexes for clathrin and AP2.[20] This finding provided the first clear evidence of functional significance of shielding of the arrestin C-tail in the basal conformation and its release upon receptor binding. In free arrestins, the C-tail is anchored to the body of the molecule, which makes it inaccessible, preventing its competition with the receptor-bound arrestins for the components of internalization machinery (reviewed in Ref. 21).

Another known mechanism of arrestin recruitment to the coated pit is its direct binding to phosphoinositides, which was reported to be necessary for GPCR internalization.[22] Since resident coated pit protein AP2 is also recruited to this part of the membrane via phosphoinositide binding,[23] one might think that as soon as the arrestin–receptor complex is formed, it has no choice but to move to the coated pit. However, this does not appear to be the case. In muscarinic M2 receptor, which was among the first shown to bind arrestins,[24] two Ser/Thr clusters in the third cytoplasmic loop were identified as critical for arrestin binding and receptor desensitization.[25] Yet the elimination of these clusters, and even dominant-negative dynamin K44A mutant that blocks the internalization of β2AR in the same cells, did not prevent M2 endocytosis, suggesting that M2 receptor does not use coated pits and internalizes in an arrestin-independent manner.[25] Interestingly, overexpression of non-visual arrestins can redirect some M2 to coated pits,[25] suggesting that this receptor can use more than one route. Many other GPCRs were shown to have that choice. For example, chemo-kine receptor CCR5 uses both phosphorylation- and arrestin-dependent and -independent pathways.[26] Cysteinyl leukotriene type 1 receptor internalizes normally in mouse embryonic fibroblasts lacking both non-visual arrestins, yet arrestin expression facilitates its internalization,[27] apparently directing it to the arrestin-dependent pathway, which is usually not preferred, similar to M2 receptor.[25] Metabotropic glutamate receptor mGluR1a constitutively internalizes via arrestin-independent mechanism, whereas its agonist-dependent internalization appears to be mediated by arrestin-2.[28] Endogenous and overexpressed serotonin 5HT4 receptor internalizes via arrestin-dependent pathway, but the deletion of Ser/Thr cluster targeted by G protein-coupled receptor kinases (GRKs) redirects it to an alternative pathway and even facilitates its internalization.[29]

Thus, it appears that the ability of GPCRs to use more than one internalization pathway is a general rule, rather than an exception, likely representing one of the many backup mechanisms cells usually have. Many receptors have recognizable internalization motifs in their sequence, so arrestin binding simply adds new ones. The relative strength of these motifs, as well as the arrestin expression levels, likely determines the pathway(s) each receptor chooses in a particular cell. The dominant internalization pathway of a particular

receptor is not necessarily the same in different cell types, or even at different functional states of the same cell (reviewed in Ref. 8). Variety, rather than uniformity, characterizes the world of GPCR signaling and trafficking.[30]

3. VISUAL ARRESTINS AND TRAFFICKING PROTEINS

In vertebrate rod photoreceptors, rhodopsin is localized on the discs, which are detached from the plasma membrane[31] and therefore are topologically equivalent to vesicles with internalized non-visual GPCRs. Thus, vertebrate rhodopsin is not supposed to be internalized. Indeed, arrestin-1, which is the prevalent arrestin isoform in both rods and cones,[32] does not have conventional clathrin- or AP2-binding elements in its C-tail.[33] However, sequence comparison of arrestin-1 and non-visual subtypes shows that in the region homologous to AP2-binding motif in arrestin-2 and -3, only one positive charge is missing.[34] Therefore, it is hardly surprising that arrestin-1 also binds AP2, albeit with ~30 times lower affinity.[34] Constitutively active rhodopsin–K296E is a naturally occurring mutant that causes autosomal dominant retinitis pigmentosa in humans, apparently due to constitutive phosphorylation and formation of a stable complex with arrestin-1.[35] The concentration of rhodopsin in the outer segment of rods reaches ~3 mM.[31] Rods also express roughly 8 arrestin molecules per 10 rhodopsins,[36–38] so the concentrations of both proteins and their complex formed in bright light are very high. It turns out that at these concentrations even low affinity matters: the presence of WT arrestin-1 facilitates rod death in animals expressing rhodopsin–K296E, with visible accumulation of AP2 in the outer segment, where it is not observed in normal mice.[34] In contrast, truncated arrestin-1 lacking the C-tail containing the low-affinity AP2-binding site protects photoreceptors in these animals and preserves their function.[34] Thus, in rod and cone photoreceptors, both of which express very high levels of arrestin-1,[32] even relatively low-affinity interactions, which would not matter in other cells, with submicromolar concentrations of both non-visual arrestins,[39,40] can become biologically relevant.

Interestingly, the localization of rhodopsin on invaginations of the plasma membrane in flies, in contrast to detached discs in vertebrate rods, is one of the many differences between vertebrate and invertebrate photo-receptors. Another difference directly follows from this localization: *Drosophila* rhodopsin is internalized, like “normal” vertebrate GPCRs, via clathrin- and AP2-mediated mechanism.[41] In fly photoreceptors, arrestin is evenly distributed, whereas in dark-adapted vertebrate rods, it is concentrated in the inner segment, with fairly small fraction in the outer segment, where rhodopsin resides.[36–38] However, in both types of photoreceptors upon illumination, arrestin translocates to rhodopsin-containing membranes.[36–38,42–45] Like non-visual arrestins, and in contrast to vertebrate visual arrestin, [22] visual arrestin in *Drosophila* has high-affinity phosphoinositide-binding site.[43] It was proposed that due to phosphoinositide binding, *Drosophila* arrestin translocates to rhodopsin on phosphoinositide-rich vesicles moved with the help of *Drosophila* myosin III (NINAC).[42] The participation of NINAC in metarhodopsin inactivation in *Drosophila* was independently confirmed,[46] but arrestin translocation was found to be largely driven by its binding to rhodopsin in flies,[44] just like in mice.[45] Thus, the internalization of invertebrate rhodopsin apparently follows the same rules as many non-visual GPCRs: active receptor recruits

arrestin via direct binding,[47] which then links it to the key components of the coated pit.[5,6,41]

4. UBIQUITINATION AND DEUBIQUITINATION IN GPCR CYCLING AND SIGNALING

Monoubiquitination of many proteins regulates their trafficking and signaling, rather than proteasomal degradation.[48] Two GPCRs, β2AR[9] and chemokine receptor CXCR4,[49] were shown to be ubiquitinated in response to agonist activation. Arrestin ubiquitination upon receptor binding, as well as the role of arrestin in GPCR ubiquitination, was discovered a few years later than the interactions of non-visual arrestins with clathrin and AP2.[9] It appears that arrestin ubiquitination by Mdm2 prolongs the life of the arrestin–receptor complex.[50] As only receptor-bound arrestins facilitate ERK1/2 activation,[51,52] it is natural that arrestin ubiquitination increases ERK1/2 activation induced by GPCR stimulation.[53] Slow deubiquitination of the receptor-bound arrestin prolongs the dwell time of the complex inside the cell and slows down receptor recycling.[50] However, receptor or arrestin ubiquitination per se does not appear to be necessary for arrestin-dependent internalization: virtually complete suppression of agonist-induced ubiquitination of arrestin-2 does not appreciably affect endocytosis of β2AR.[54] Arrestin-2 recruits ubiquitin ligase AIP4 to ubiquitinate CXCR4, which affects endosomal sorting of this receptor.[55] Receptor-bound arrestin-3 recruits yet another ubiquitin ligase, Nedd4, which ubiquitinates β2AR, and this receptor modification is required for lysosomal targeting of internalized β2AR,[56] although arrestin domain-containing protein 3 was also suggested as the mediator of the interaction of Nedd4 with β2AR.[57,58] Finally, both non-visual arrestins bind a fourth ubiquitin ligase, parkin.[54] Interestingly, parkin binding enhances arrestin interactions with Mdm2, but paradoxically strongly reduces arrestin ubiquitination in response to receptor activation.[54] The possible role of parkin in receptor modification remains to be elucidated. To further complicate matters, arrestins were found to recruit deubiquitinating enzymes USP20 and USP33 to β2AR, which facilitate receptor recycling and resensitization.[59,60]

To summarize, it is clear that arrestins bind several ubiquitin ligases and recruit them at least to some GPCRs. Both arrestins and GPCRs are ubiquitinated upon receptor stimulation. Receptor ubiquitination appears to play a role in sorting and lysosomal targeting, whereas the ubiquitination of arrestins likely affects their affinity for receptors. However, arrestin-mediated recruitment of some deubiquitinating enzymes suggests that their role in GPCR trafficking is more complex and includes postendocytotic steps. Interestingly, the role of arrestins in recruiting deubiquitinases was shown on β2AR,[59,60] which appears to contradict the idea that arrestins bound to this particular receptor dissociate from it very quickly.[61] Thus, the biological functions of arrestin-assisted ubiquitination and deubiquitination of GPCRs and similar modifications of non-visual arrestins need to be further clarified. One should also keep in mind that the role of the same processes in trafficking of different GPCRs is not necessarily the same: the very fact that animals have so many members of this superfamily suggests that variety, rather than uniformity, is the key.[30]

5. FASTER CYCLING PREVENTS RECEPTOR DOWNREGULATION

With very few exceptions, the fate of internalized receptors is not predetermined: they can be recycled back to the plasma membrane and reused, or sent to lysosomes and destroyed.[7] The latter process leads to the reduction of overall receptor number, usually termed downregulation. We do not know how the choice between recycling and elimination is made, but it appears that the intensity and/or duration of signaling can tip the scales one way or another. In the process of internalization and recycling, most receptors transition through several functional states. First, in case of GPCRs that internalize via arrestin-dependent pathway, after phosphorylation by GRKs and arrestin binding receptors, move into coated vesicles and then to endosomes. The internal pH in endosomes is much lower than on the extracellular side of the membrane.[62] It is likely (but remains unproven) that acidification facilitates the dissociation of the ligand. The loss of the bound agonist and consequent transition into inactive state is the only conceivable mechanism of subsequent release of bound arrestins: both non-visual subtypes demonstrate lower binding to inactive phosphoreceptors,[63–65] even though the difference is not as dramatic as in the case of visual arrestin-1.[66,67] Arrestin dissociation is necessary to make receptor-attached phosphates accessible to phosphatases,[68] so it must precede receptor dephosphorylation. Since both non-visual arrestins require at least two phosphates for high-affinity binding,[63] dephosphorylation has to be a multistep process. It must be completed, as it appears that only fully dephosphorylated receptors are recycling competent.[69,70] One conceivable model is that only certain functional states of the receptor can be diverted to lysosomes and destroyed; and the other is that every state can be transported to lysosomes, so that the longer the time that a GPCR spends in the endosomal compartment, the higher the probability that it will be transported to lysosomes and destroyed.

Similar to visual arrestin-1, both non-visual arrestins can be made to bind active unphosphorylated GPCRs by mutations destabilizing the main phosphate sensor, the polar core, by mutations detaching the C-tail from the body of the molecule, or by C-tail deletions.[64,65,71] The effect of two different arrestin-2 mutants, one activated by polar core mutation and the other by the C-tail detachment, on cycling of β2AR was tested in cells.[72] Since these forms of arrestin-2 bind the same active receptor as GRKs, they actually compete with GRKs and suppress receptor phosphorylation both *in vitro*, in the system reconstituted from purified proteins, and in cells.[72] It turned out that in cells, these preactivated arrestin-2 mutants bind unphosphorylated β2AR and induce its internalization. Interestingly, unphosphorylated β2AR internalized in complex with these mutants recycles very rapidly, much faster than in the presence of WT arrestin-2 that only binds phosphorylated receptor.[72] Importantly, the expression of phosphorylation-independent arrestin-2 mutants protected the receptor from downregulation, so that, in sharp contrast to cells expressing WT arrestin-2, even after 24 h of agonist exposure virtually no β2AR was lost.[72] This was the first study of the effect of the nature of the arrestin–receptor complex on the fate of internalized receptor. It did not answer all questions. The results can be interpreted in the context of both models: (1) as an indication that rapid cycling reduces the chances of the receptor to be diverted to lysosomes, or (2) as a suggestion that only phosphorylated forms of the receptor are diverted

to that compartment and destroyed. The use of nonphosphorylatable β2AR mutants in similar experiments is necessary to resolve this issue.

6. ARRESTINS IN RECEPTOR RECYCLING AND VESICLE TRAFFICKING: QUESTIONS WITHOUT ANSWERS

The mechanism whereby arrestin-2 and -3 participate in GPCR internalization is fairly well established: the C-tail of both non-visual arrestins is released upon receptor binding,[19] which increases the accessibility of clathrin and AP2-binding sites in this element.[10,73,74] In addition, arrestins appear to recruit ubiquitin ligases to GPCRs, and receptor ubiquitination plays a role in receptor sorting.[9,55,56] Yet it is still unclear how arrestins participate in other steps of GPCR trafficking. *N*-Formyl-peptide receptor binds arrestin-2 and -3 in an activation- and phosphorylation-dependent manner,[75,76] yet it was reported to internalize in the absence of both non-visual arrestins.[77] However, in mouse embryonic fibroblasts lacking both non-visual arrestins, internalized *N*-formyl-peptide receptor does not recycle.[77] The receptor travels to the perinuclear recycling compartment and gets stuck there, but its recycling can be rescued by the expression of either arrestin-2 or -3.[77] These data suggest that, as far as *N*-formyl-peptide receptor recycling is concerned, the two non-visual arrestins are functionally redundant. Yet we do not have many clues how exactly are arrestins involved in GPCR recycling. One conceivable scenario is that arrestins bind to this receptor after internalization and recruit deubiquitinating enzymes necessary for recycling, as was shown in the case of β2AR,[59,60] but this leaves open the question why arrestins do not bind it before endocytosis, similar to β2AR.[5,6,9] Existing evidence does not suggest any good answers to this question.

Another issue that needs experimental clarification is arrestin binding to the *N*-ethylmaleimide-sensitive factor (NSF), an ATPase involved in vesicle trafficking. Arrestin-2 binding to NSF was discovered 15 years ago,[78] but its functional significance in case of non-visual arrestins remains unclear. Interestingly, a few years ago, visual arrestin-1 was shown to interact with NSF in photoreceptors.[79] It appears that in rods, arrestin-1 is necessary to maintain proper NSF function and normal level of neurotransmitter release.[79] However, the molecular mechanism of this arrestin-1 effect remains to be elucidated.

7. CONCLUSIONS AND FUTURE DIRECTIONS

The role of non-visual arrestins in recruiting GPCRs to coated pits and facilitation of receptor internalization via this pathway is fairly well established. The case of ubiquitin modification of receptors and arrestins is less straightforward: arrestins seem to recruit enzymes responsible for ubiquitination and deubiquitination of GPCRs. These modifications play distinct roles in receptor trafficking, but the exact role of non-visual arrestins, which are also ubiquitinated in response to receptor stimulation, remains to be elucidated. The functions of non-visual arrestins in complex trafficking itineraries of individual GPCR subtypes might be different. How arrestins affect the recycling of internalized GPCRs, and how exactly arrestin binding regulates NSF function and vesicle trafficking, remains even less clear (Fig. 1). Cytoskeleton is intimately involved in trafficking of many proteins. Arrestins were shown to bind microtubules[80–82] and a very specialized structure containing

polymerized tubulin, the centro-some.[83] However, the role of these interactions in the transport of receptors and/or other molecules within the cell still needs to be defined. Most likely, recent finding that non-visual arrestins recruit clathrin to microtubules targeting focal adhesions, thereby facilitating integrin internalization and focal adhesion disassembly,[84] is only the tip of the iceberg.

ABBREVIATIONS

AIP4	atrophin-1-interacting protein 4
AP2	adaptor protein 2
β2AR	β_2-adrenergic receptor
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
Nedd4	neural precursor cell expressed developmentally down-regulated protein 4

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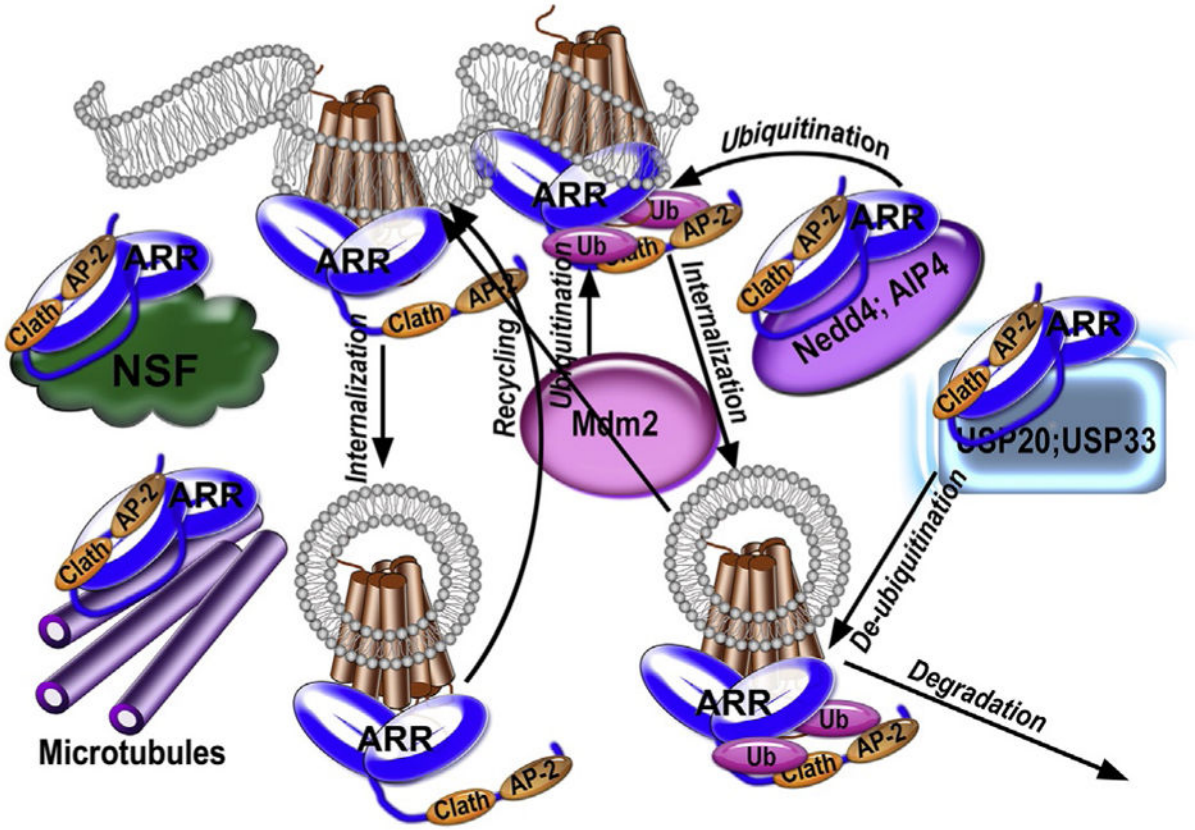

Figure 1.

Arrestins play many roles in GPCR trafficking. Arrestins (ARR) bind active phos-phorylated GPCRs (shown as a seven-helix bundle). Receptor binding induces the release of the arrestin C-tail, which carries binding sites for clathrin (Clath) and adaptor protein-2 (AP2). The interactions of these sites with clathrin and AP2 promote receptor internalization via coated pits. Arrestins also recruit ubiquitin ligases Mdfm2, Nedd4, and AIP4 to the complex, which favors ubiquitination of both non-visual arrestins and at least some GPCRs. Arrestins also recruit certain deubiquitination enzymes (USP20 and USP33 are shown), facilitating receptor deubiquitination. The role of arrestin interactions with microtubules, centrosome, and *N*-ethylmaleimide-sensitive factor (NSF) in trafficking of GPCRs and/or other proteins remains to be elucidated.