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Arrestin Mobilizes Signaling Proteins to the Cytoskeleton and Redirects their Activity

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Arrestins regulate the activity and subcellular localization of G protein-coupled receptors and other signaling molecules. Here, we demonstrate that arrestins bind microtubules (MTs) *in vitro* and *in vivo*. The MT-binding site on arrestins overlaps significantly with the receptor-binding site, but the conformations of MT-bound and receptor-bound arrestin are different. Arrestins recruit ERK1/2 and the E3 ubiquitin ligase Mdm2 to MTs in cells, similar to the arrestin-dependent mobilization of these proteins to the receptor. Arrestin-mediated sequestration of ERK to MTs reduces the level of ERK activation. In contrast, recruitment of Mdm2 to MTs by arrestin channels Mdm2 activity toward cytoskeleton-associated proteins, increasing their ubiquitination dramatically. The mobilization of signaling molecules to MTs is a novel biological function of arrestin proteins.

Keywords: arrestin; microtubules; ERK; Mdm2; G-protein coupled receptor

Introduction

As their name implies, arrestins were described originally as proteins that terminate G protein-mediated signaling by binding the activated phosphorylated forms of their cognate G protein-coupled receptors (GPCRs).[1–3] Recent discoveries of their interactions with numerous other binding partners revealed the role of arrestins as multi-functional regulators of cell signaling.[4,5] Arrestins redirect GPCR signaling to G protein-independent pathways and determine the intracellular localization of key regulatory proteins. In particular, arrestin retains ERK2 and JNK3 in complex with the receptor in the cytoplasm and removes Mdm2 and JNK3 from the nucleus.[4,6,7]

Structurally, arrestins are elongated two-domain molecules with an overall fold that is remarkably conserved between different subtypes. Receptor binding "unfastens" two critical "clasps" that hold the molecule in its basal state, inducing a global conformational change that involves the movement of the two arrestin domains.[2] Most non-receptor partners bind the arrestin–receptor complex, engaging arrestin elements that are not involved in receptor binding.[4,7] Recently, we identified microtubules (MTs) as an interaction partner of visual (rod) arrestin.[8,9] The difference in microtubule affinity between the two splice variants of visual arrestin expressed in bovine rods[10] determines their differential subcellular localization.[8]

Here, we demonstrate that all arrestin subtypes bind microtubules and we identify the arrestin elements involved. Receptors and microtubules engage the same side of the arrestin molecule, leaving the interaction sites for non-receptor binding partners accessible. We found that arrestins recruit ERK1/2 and ubiquitin ligase Mdm2 to microtubules, differentially affecting their activity. Arrestin-dependent mobilization of signaling molecules to the cytoskeleton is an earlier unappreciated link in the network of cellular regulatory pathways.

Abbreviations used: GPCR, G protein-coupled receptor; MT, microtubule; WT, wild-type; SDSL, site-directed spin labeling; EPR, electron paramagnetic resonance.

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Results

Arrestin binding to microtubules in living cells

Visual arrestin binds MTs in rod photoreceptors *in vivo*. This interaction determines its subcellular localization in membrane microdomains and in the compartments of the rod cell.[8,11] Remarkable structural homology between arrestin family members suggests that other arrestin subtypes may also interact with MTs.[12] To test this hypothesis, we fractionated HEK-293 cells expressing different arrestins. As expected, most arrestin was present in the cytosol, but a noticeable proportion of all arrestin subtypes co-fractionated with MTs. In fact, other arrestins bind microtubules better than rod (Figure 1(a) and (b)). The proportion of arrestin in the cytoskeletal fraction depends on the subtype, with arrestin3 demonstrating the highest level of binding. The quantification of soluble and cytoskeleton-associated arrestins by Western blot demonstrates that about 2–3 % of wild-type rod, cone, and arrestin2 are associated with MTs, and this proportion reaches ~8% for arrestin3 (Figure 1(b)). The deletion of the arrestin2 C-tail (cf. Figure 2(c)) (A2Tr), as well as a seven-residue deletion in its interdomain hinge region (A2D7) that locks arrestin in its basal conformation enhance its binding to microtubules (Figure 1(a)).[13]

To visualize this phenomenon in intact cells, we performed colocalization experiments on HEK cells overexpressing arrestin3 using a fixing method that preserves MTs.[14] The pattern of arrestin3 immunoreactivity overlaps partially with MT staining (Figure 1(c); arrows). The apparent extent of the colocalization is consistent with the proportion of this arrestin found in the cytoskeletal fraction (Figure 1(b)). In contrast, when microtubules are depolymerized by incubation on ice, the disappearance of visible microtubule bundles is accompanied by the loss of the "structured" appearance of arrestin3 immunofluorescence (Supplementary Data Figure S1). To better visualize the cytoskeleton, for arrestin2 we also used COS7 cells, which have a more extended cytoplasmic area. We found that truncated arrestin2, which binds MTs better than the wild-type (WT) (Figure 1(a)), localizes with MTs to a much greater extent (Figure 1(c)), demonstrating that sedimentation of this soluble protein with the cytoskeleton in our fractionation assay reflects its colocalization with MTs in cells. Thus, the association with MTs in cells is a common characteristic of all arrestin subtypes.

The conformation of MT-bound arrestin

Next, we used purified arrestins and MTs polymerized *in vitro* from pure tubulin to test whether this interaction is direct. This assay confirmed that both non-visual arrestins bind MTs better than rod arrestin (Figure 2(a)). The deletion of the arrestin C-tail (Figure 2(c)) (Tr) or its detachment from the body of the molecule by a triple alanine substitution (3A) increases arrestin flexibility,[15] and enhances its binding to receptors dramatically.[16–18] Interestingly, the deletion of the C-tail also enhances MT binding (Figure 2). Importantly, the relative binding of different purified arrestins and mutants reproduces our results in cells (compare Figures 1 and 2). For further structure–function studies, we used a relatively high-throughput direct binding assay with radiolabeled arrestins expressed in cell-free translation,[9] similar to the assay we use to measure arrestin binding to purified GPCRs.[19,20] As shown in Figure 2(b), this method reproduces the relative binding of full-length and truncated forms of different arrestins that we detected in cells (Figure 1) and with purified proteins (Figure 2(a)).

The similar effects of C-tail deletion and its detachment by 3A mutation (Figure 2(c)) on MT binding in all arrestins (Figure 2(b)) suggest that the mechanism of microtubule binding is conserved in the arrestin family. Arrestin N and C-domains are independent folding units that can be expressed separately and retain functional activity (Figure 2(c)).[19,21] We tested the relative role of the two arrestin domains in MT binding and found that the N-domains of rod, arrestin2, and arrestin3 bind substantially better than the full-length proteins, whereas the binding ability of the C-domains varies (Figure 2(b)). Apparently, the arrestin N-domain binds MTs better when it is not impeded by the C-domain, although both domains clearly participate in this interaction.

Numerous lines of evidence demonstrate that the conformations of free and receptor-bound arrestin are substantially different.[2,22,23] The N and C-domains of arrestin are connected by a 12-residue loop termed the hinge region (Figure 2(c)).[13] Progressive deletions in the inter-domain hinge severely impede receptor binding (Figure 2(e)), indicating that the movement of the two domains relative to each other is required in this process. Interestingly, hinge deletions actually enhance MT binding of all arrestins (Figure 2(f)), suggesting that the conformation of MT-bound arrestin differs from that of the receptor-bound form. The effect of hinge deletions on both receptor and MT binding is more dramatic in rod arrestin than in the non-visual subtypes, possibly due to the greater inherent flexibility of the non-visual arrestins.[24] Importantly, the MT association of arrestin hinge deletion mutants was similarly enhanced in cells (Figure 1(a)).

The MT-binding site in arrestin2

We took advantage of the significant difference between the MT binding of rod arrestin and arrestin2, and used a series of rod/arrestin2 chimeras to further define the specific elements responsible for MT binding (Figure 2(d); Supplementary Data Figure S2).[25] We found that the exchange of two elements on the concave sides of the N and C-domains (Figure 2(c)) simultaneously reverses receptor specificity and the relative ability