Extensive shape shifting underlies functional versatility of arrestins

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Abstract

Among four vertebrate arrestins, only two are ubiquitously expressed. Arrestins specifically bind active phosphorylated G protein-coupled receptors (GPCRs), thereby precluding further G protein activation. Recent discoveries suggest that the formation of the arrestin-receptor complex initiates the second round of signaling with comparable biological importance. Despite having virtually no recognizable sequence motifs known to mediate protein-protein interactions, arrestins bind a surprising variety of signaling proteins with mind-boggling range of functional consequences. High conformational flexibility allows arrestins to show many distinct "faces" to the world, which allows these relatively small ~45 kDa proteins to bind various partners under different physiological conditions, organizing multi-protein signaling complexes and localizing them to distinct sub-cellular compartments.

Introduction

Exquisitely timed precisely regulated protein-protein interactions determine every aspect of cell behavior. If we clearly understood what makes proteins bind each other and what prevents their interactions, we would be able to tell the cell what to do in a language it cannot ignore. Arrestins are a family of only four proteins in mammals with remarkably similar structures [1–4]. Their only function in life is to bind an amazing variety of partners at appropriate times, organizing multi-protein complexes and localizing them to proper sub-cellular compartments, thereby orchestrating cell signaling [5,6]. Because of unimpressive size (~45 kDa), an arrestin molecule can accommodate no more than 5–6 other proteins simultaneously, suggesting that at any given moment it interacts only with select few out of hundreds of possible partners [7]. Thus, arrestins provide a perfect model for elucidating how the structural changes in proteins make them "decide" which partners to bind and in what combinations. Extensive crystallographic, biophysical, and mutagenesis studies of arrestin proteins are beginning to shed light on the molecular mechanisms underlying these critical choices.

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[1]Different systems of arrestin names are used in the field. We use the 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).

Basal does not mean inactive

The protein that specifically binds active phosphorylated rhodopsin was discovered less than 30 years ago [8]. The fact that receptor binding induces a global conformational change in this protein was established soon thereafter [9]. This idea was one of the cornerstones of the first mechanistic model (which still holds water) coherently explaining how arrestin can bind with high affinity to the receptor that is active and phosphorylated at the same time, but neither to inactive phosphorylated nor active unphosphorylated form of the same protein [10]. Therefore, subsequently solved remarkably similar crystal structures of all four arrestins [1–4,11,12] were believed to represent their basal conformation, which was deemed "inactive". In essence, arrestins were assumed to exist in this "waiting" state, ready to be "activated" by receptor binding [13], whereupon they would acquire the ability to interact with various non-receptor partners [14]. This view was largely shaped by the pioneering reports in 1999–2001 that arrestin-assisted activation of Src [15,16], MAP kinases JNK3 [17] and ERK1/2 [15,16,18,19], and arrestin ubiquitination [20] happened only in response to receptor activation. Although the first evidence contradicting this view emerged as early as 2001, when Miller at al reported receptor-independent facilitation of JNK3 phosphorylation by arrestin-3 [21], the idea that only GPCR-bound arrestin can interact with non-receptor partners is still popular. In fact, it is an example of unwarranted generalization: it is correct in some cases, but misleading in others. Careful comparison of ERK1/2 and JNK3 activation in the same cell showed that while ERK1/2 phosphorylation is strictly dependent on receptor stimulation and is only mediated by arrestins that can bind receptors, the activation of JNK3 is not affected by receptor activity or the ability of arrestin to interact with GPCRs [22]. This finding was consistent with previous demonstration that ERK1/2 preferentially binds receptor-associated arrestins [23], whereas JNK3 binds free arrestins in their basal state perfectly well [24], and its activation is facilitated even by arrestin-3 mutant with the deletion in the inter-domain hinge that precludes receptor binding [22,25]. Admittedly, cell-based assays cannot yield unambiguous answers, because every cell expresses thousands of different proteins that can mediate indirect interactions and significantly affect outcomes. Recently the ability of arrestin-3 to facilitate JNK3 activation by its upstream kinases was demonstrated in the system reconstituted from purified proteins in the absence of receptors [26,27], proving beyond reasonable doubt that this is a function of free arrestin-3.

In fact, remarkably few signaling proteins were shown to preferentially interact with receptor-bound arrestins: in addition to ERK1/2 [23], this short list includes clathrin and AP2 [28,29], E3 ubiquitin ligase AIP4 [30], and cRaf1 [23]. Several partners strongly prefer the basal conformation of arrestins: microtubules [31], calmodulin [32], E3 ubiquitin ligases Mdm2 [24] and parkin [33]. Some proteins apparently bind arrestins in both basal and "active" conformations comparably: JNK3 [24], ASK1, MKK4, MEK1 [25], and PDE4D [34] (Table 1). Unfortunately, we do not know the conformational preferences (if any) of the great majority of arrestin-binding proteins. This issue must be addressed experimentally for each partner, and it is safe to predict that for various proteins the answers will be very different. It seems logical that if the interaction site of a particular partner overlaps with the receptor footprint, this partner would only bind free arrestins. This prediction is supported by the evidence in case of calmodulin [32] and microtubules [31]: both interact with the concave sides of the two domains engaged by GPCRs, and therefore receptors successfully outcompete them [32,35]. Unfortunately, arrestin residues mediating the interaction were identified in very few cases (Fig. 1), which makes the usability of this logic very limited. To complicate things further, different functional forms of the same receptor apparently interact with distinct arrestin elements, as has been recently shown by comparing the residues engaged by active unphosphorylated and inactive phosphorylated rhodopsin on arrestin-1 [36] (Fig. 2). Thus, it is entirely possible that certain partners would compete with some

forms of the receptor, but not others. Precise identification of the binding sites of different functional forms of GPCRs and other signaling proteins on arrestins is yet another unmet need in arrestin biology.

Receptor-bound conformation: what does it look like?

It became clear that the shape of receptor-bound arrestins must be quite different from the basal state long before any crystal structures were solved [9]. The release of the C-tail was the first receptor-induced conformational rearrangement documented [37]. However, the finding that the deletion of the C-tail does not make arrestin bind all forms of the receptor indiscriminately [10] suggested that additional changes are likely. Recently several attempts to determine receptor-bound "active" conformation of arrestins were made using various methods, such as NMR [36], EPR [38], and crystallography [39,40] (Fig. 3). Each of these approaches has obvious caveats: biophysical studies of arrestin complexes with real receptor cannot yield atomic resolution [36,38], whereas real receptors are absent in beautiful crystal structures of "active" arrestins [39,40]. Nonetheless, collectively these attempts proved very informative. As could be expected, every study detected significant conformational rearrangements in arrestins. EPR documented the movement of four flexible loops on the receptor-binding side [38], some of which were predicted, while others were quite unexpected (Fig. 3B). NMR confirmed the engagement of distinct arrestin elements by inactive phosphorylated and active unphosphorylated forms of the receptor [36] (Fig. 2), which was predicted earlier based on mutagenesis and EPR data [10,41]. Unexpectedly, NMR study also revealed global increase in arrestin flexibility upon the binding to active phosphorylated receptor [36]. General "melting" of arrestin structure upon receptor binding was never predicted, but biologically it makes perfect sense: receptor-bound arrestin interacts with numerous signaling proteins, and protein-protein interactions are often mediated by unstructured elements, where the binding to an appropriate partner promotes folding [42,43]. The global conformational change induced by receptor binding was hypothesized to include the movement of the two arrestin domains relative to each other [13], partially based on the finding that the deletions in the 12-residue inter-domain hinge impair receptor binding of all arrestins [31,44]. However, proposed clam-like movement of the two domains was not detected by EPR [38] (Fig. 3B). Recently two crystal structures of presumably "active" arrestins were reported: one of truncated arrestin-2 (previously shown to be constitutively active [45]) associated with multi-phosphorylated C-terminus of vasopressin V2 receptor [40](Fig. 3C) and the other of short arrestin-1 splice variant p44 also lacking the C-tail [39] (Fig. 3D). These very similar structures suggest that the conformational changes include the rotation of the two domains relative to each other by 20–21° [39,40] (Fig. 3C,D). Both also revealed a large movement of the "139 loop" in the central crest of the receptor-binding surface, away from the "finger loop" implicated in receptor binding [41,46] (Fig. 3). This movement was earlier discovered using EPR [38] and confirmed by mutagenesis [47]. Because the receptor is not present in the "active" structures, we do not know how much arrestin domains actually move upon GPCR binding, but domain rotation allows to rationalize hinge deletion results [31,44], which distance measurements by EPR [38] left unexplained.

It is worth noting that back in 2006 this type of domain movement was proposed as a possibility due to hydrophobic nature of the inter-domain interface [48] and even suggested as the most likely based on molecular modeling [49]. The extent of domain rotation was estimated at ~20° based the length of the hinge in WT arrestins [49]. The coincidence is remarkable, even though this model was originally proposed to explain how two GPCRs can fit a single arrestin [49]. A series of recent studies proved that monomeric class A (rhodopsin-like) receptor is necessary and sufficient to perform all known signaling functions: activate cognate G protein [50–52], become phosphorylated by G protein-coupled

receptor kinases [53,54], and bind arrestins [53–56]. Interestingly, while the high-affinity binding invariably involves one-to-one arrestin-receptor stoichiometry, an alternative mode, where a single arrestin-1 molecule apparently engages two rhodopsins, one with high and one with low affinity, was recently reported in the visual system [57,58]. It remains to be elucidated whether this is a special adaptation in rods, where half of the surface of disc membranes is occupied by rhodopsin, with the concentration in rod outer segments of ~ 3 mM [6], or reflects the capability of all arrestin proteins.

Although the true shape of "active" arrestins will be revealed only by the structure of the arrestin-receptor complex, recent studies provided interesting clues (Fig. 3). Unless the real structure proves otherwise, domain rotation appears the most likely global conformational change, accompanied by the movement of several loops on the receptor-binding side. This model, along with the identification of the docking sites of nonreceptor partners, which is sadly missing in most cases, should help us understand their conformational preferences.

Microtubule-bound state: the third face of Janus?

All arrestins bind microtubules [31,35], and this interaction apparently triggers a conformational change different from that induced by GPCRs [59], because hinge deletion mutants impaired in receptor binding showed higher affinity for microtubules [31]. Microtubule binding was proposed to determine the localization of visual arrestin-1 in dark-adapted photoreceptors [35] and to play a role in sub-cellular distribution and activity of signaling proteins associated with non-visual arrestins [31]. Several proteins, including ERK1/2 and Mdm2, interact with arrestins in microtubule-bound conformation (and/or hinge deletion mutants mimicking this form) better than with free arrestins [23,24,31]. This was particularly surprising in case of ERK1/2 with strong preference for the receptor-bound form [23]. We know even less about the actual shape of microtubule-bound arrestins than about their receptor-bound conformation. Since in this case crystallization is not an option, only careful biophysical studies can give additional clues. This information is biologically important, because it might give us tools to direct signaling proteins to the cytoskeleton and/ or specifically regulate their activity in this compartment.

Elusive transitions and signaling bias

Among many arrestin conformations we know well only the free state, with one structure affording better than 2Å resolution [2]. Several recent studies [36,38–40] provided clues about receptor-induced conformational changes, although the structure of the arrestin-receptor complex, preferably several revealing different "flavors" of these complexes that likely exist [60], would be most welcome. The shape of the microtubule-bound arrestin remains to be elucidated. Recent finding that arrestin-3 binding to JNK3α2 differentially affects its affinity for the upstream kinases, enhancing the interaction with MKK4, while decreasing MKK7 binding [27], strongly suggests that many partners induce conformational changes in arrestins, so that GPCRs and microtubules are not unique in this regard. Thus, the shape of arrestins in complex with different partners must be investigated. Most importantly, we know virtually nothing about the process of conformational transitions in arrestins, which can be only elucidated by biophysical methods revealing protein dynamics, because it is virtually impossible to deduce them from inherently static crystal structures.

Identification of arrestin elements engaged by various partners is another challenge. Mapping of the receptor-binding site on arrestins [41,46] (Fig. 1A) and even partial elucidation of the mechanics of the arrestin-receptor interactions [1–4,61] led to the construction of enhanced arrestins that bind unphosphorylated active GPCRs, change receptor trafficking pattern [62], and even compensate for the lack of receptor

phosphorylation *in vivo* [63]. Reengineering of the receptor-binding surface of inherently promiscuous non-visual arrestin-3 yielded mutants with >50-fold preference for some GPCRs over others [64] (Fig. 1A). Similarly, even imperfect identification of MAP kinase interaction sites led to the construction of arrestin-2 that does not facilitate ERK1/2 activation [65] (Fig. 1B), as well as arrestin-3 mutant that acts as a dominant-negative silent scaffold, recruiting the kinases of JNK activation cascade away from productive scaffolds and suppressing JNK activity in the cell [22] (Fig. 1B). Considering how many different proteins arrestins bind [7], these pioneering attempts show that there are many possible ways of constructing arrestins with signaling bias [60], which can be used to change cell signaling in the desired manner.

Conclusions

Arrestins are typical scaffolds that bind numerous partners, organizing multi-protein signaling complexes and ensuring their proper sub-cellular localization via interactions with "anchoring" proteins, such as GPCRs and microtubules. Arrestins exist in at least three (very likely more) distinct conformations: free, GPCR-associated, and microtubule-bound. This flexibility underlies their functional versatility: each conformational state preferentially recruits particular partners. Current challenge is to elucidate the shape of arrestins in each state, the mechanism of their transition from one state to another, and the sites where other proteins bind consistent with their conformational preferences. This will pave the way to engineering arrestins that bind the partners we want and recruit them to sub-cellular locations of our choosing in order to channel cell signaling in desired directions.

Acknowledgments

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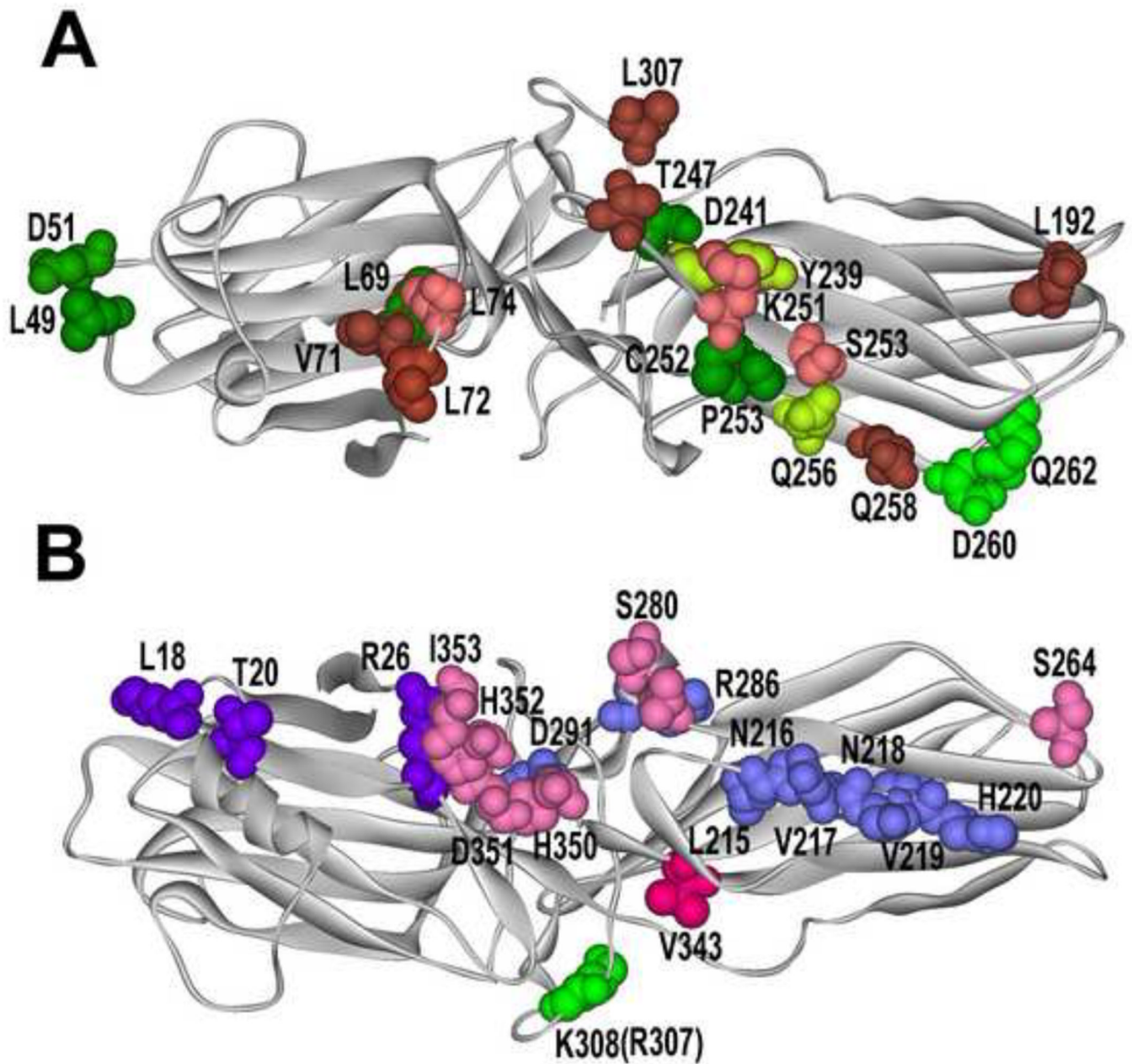
Fig. 1. What arrestin elements do GPCRs and other partners engage?
A. Arrestin from "receptor viewpoint". Arrestin residues that determine receptor specificity [46,64] are shown in different shades of green: yellow-green, those where mutations (Y239T and Q256Y) result in reduced dopamine D2 receptor binding; bright green, those where mutations (D260K and Q262P) result in high preference for other GPCRs over β2-adrenergic receptor. Elements engaged by Ca^{2+}-liganded calmodulin are shown in pink and brown (darker color indicates greater immobilization by calmodulin [32]). Residues in green with red border (L69 and T239) are shared by receptors and calmodulin. **B.** Non-receptor-binding side of arrestins. Residues engaged by all forms of PDE4D are shown in blue, those specific for PDE4D5 in bright violet [34], the residue critical cRaf1-binding is in bright green (mutation R307A in arrestin-3 prevents cRaf1 interaction; homologous mutation K308A in arrestin-3 does not) [65], residues responsible for the ability of arrestin-3 to

promote JNK3 activation are shown in bright red (critical V343) and pink (supporting residues) [67]. Arrestin-3 structure 3P2D [4] was used to generate this figure (since calmodulin-binding elements were identified in arrestin-2 [32], homologous arrestin-3 residues are highlighted).
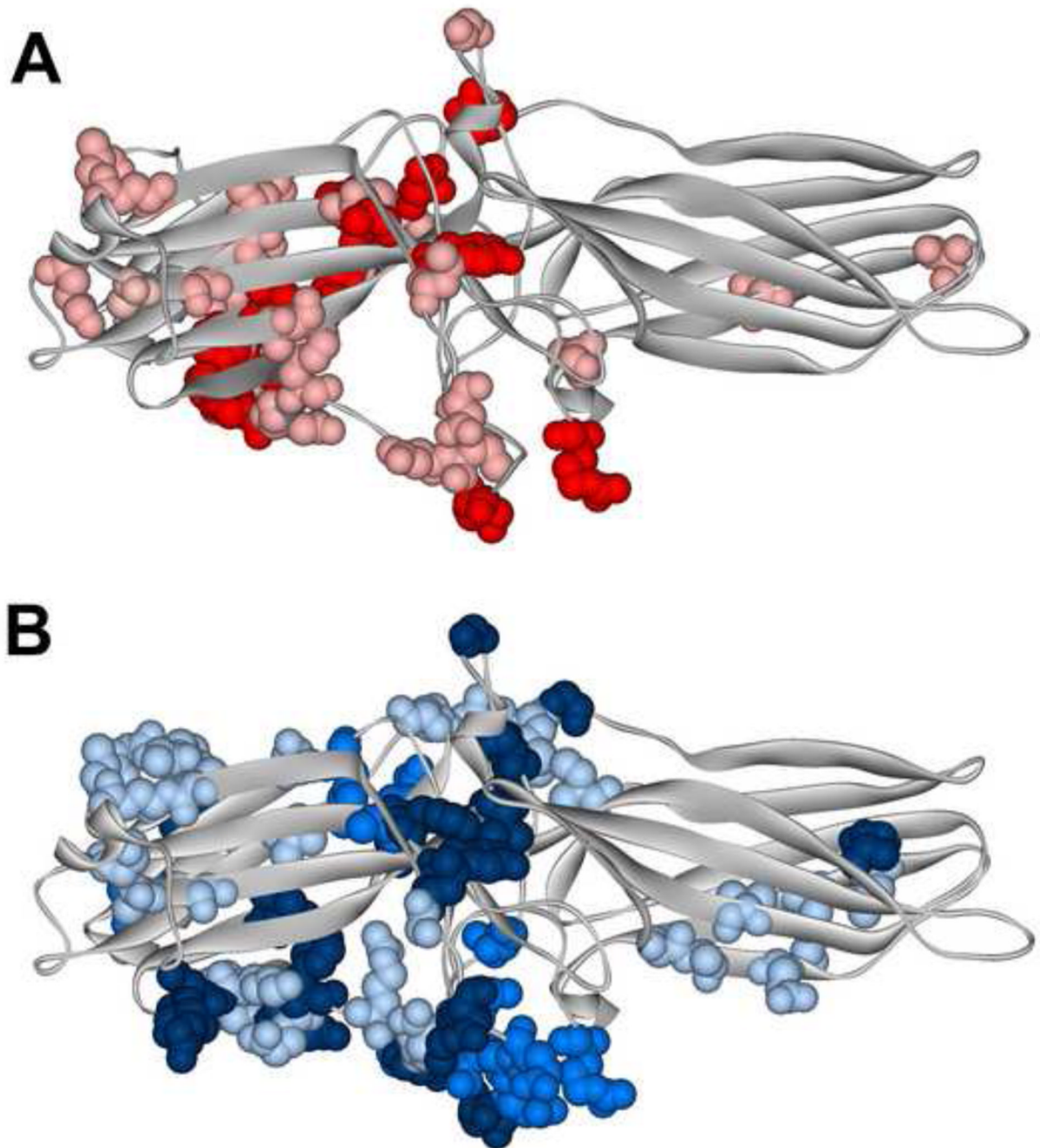
Fig. 2. Different functional forms of the same receptor engage distinct arrestin elements
Arrestin-1 residues demonstrating significant chemical shift changes in NMR spectra upon the binding of light-activated unphosphorylated (**A**) or inactive phosphorylated (**B**). **A.** The magnitude of binding-induced changes is shown by color coding: bright red, shifts greater than 0.01; pink, shifts greater than 0.005, but smaller than 0.01. **B.** The magnitude of binding-induced changes is shown by color coding: dark blue, peaks disappeared; blue, shifts greater than 0.01; light blue, shifts greater than 0.005, but smaller than 0.01. The two forms of rhodopsin affect distinct arrestin-1 elements that only partially overlap [36].
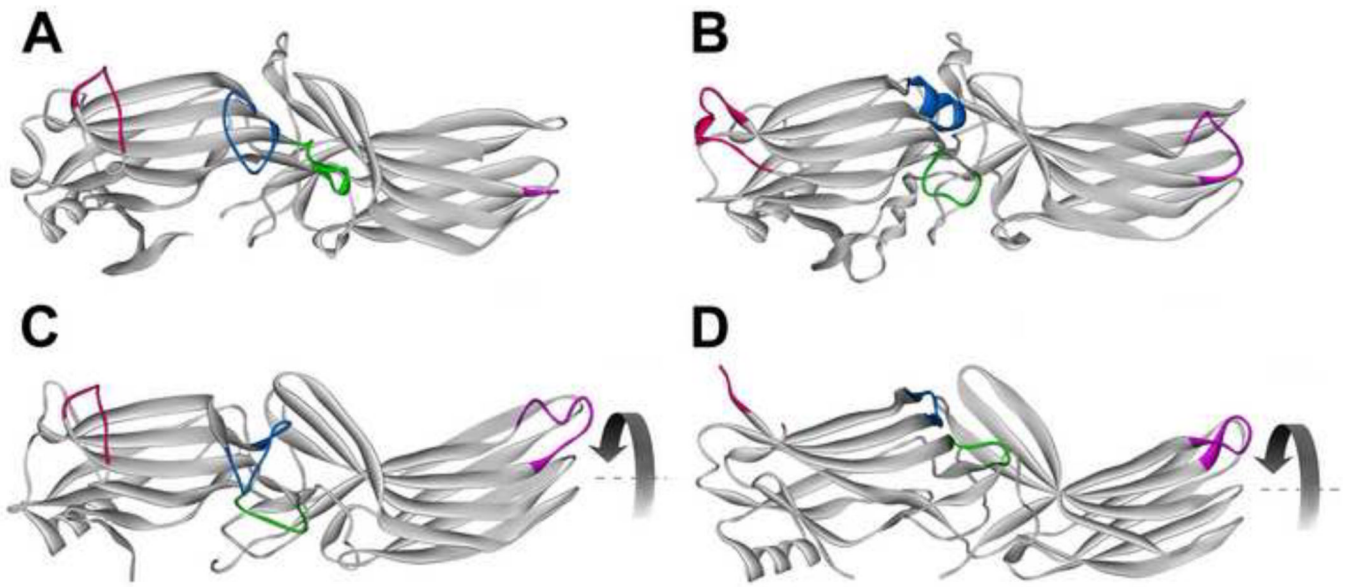
Fig. 3. Receptor binding-induced structural changes in arrestins
A. Basal arrestin conformation (based on arrestin-2 structure 1G4M, which has the highest resolution among published arrestin structures [2]). **B.** A model of arrestin-1 bound to lightactivated phosphorylated rhodopsin based on intra-molecular distance measurements in free and bound arrestin-1 [38]. **C.** Crystal structure 4JQI of truncated arrestin-2-(1–382) in complex with multi-phosphorylated C-terminus of vasopressin V2 receptor receptor [40]. **D.** Crystal structure of p44, truncated splice variant of arrestin-1. Arrestin elements that change conformation upon receptor binding are color-coded, as follows: blue, the "finger loop" (G68-S78 in arrestin-1; G65-T74 in arrestin-2); green, "139 loop" (P134-S142 in arrestin-1; P131-A139 in arrestin-2); red, "157 loop" (H155-P165 in arrestin-1; E152-H159 in arrestin-2); magenta, "344 loop" (S336-S344 in arrestin-1; S330-S340 in arrestin-2). Residue numbers for corresponding bovine proteins are given. The rotation of the two domains relative to each other by 20–21° is indicated by curved arrow in **C** and **D**.

Table 1

Known conformational preferences of arrestin binding partners.

Arrestin Binding Partner	Arrestin isoform	Preferred Arrestin Conformation	References
ERK1/2	Arrestin-2, arrestin-3	receptor-bound	[23]
clathrin	Arrestin-2, arrestin-3	receptor-bound	[28,29]
AP-2	Arrestin-2, arrestin-3	receptor-bound	[28,29]
E3 ubiquitin ligase AIP4	Arrestin-2	receptor-bound	[30]
cRaf1	Arrestin-2	receptor-bound	[23]
microtubules	Arrestin-1, arrestin-2, arrestin-3	basal	[31,59]
calmodulin	arrestin2	basal	[32]
E3 ubiquitin ligase Mdm2	Arrestin-1, Arrestin-2, arrestin-3	basal	[24]
E3 ubiquitin ligase parkin	Arrestin-2, arrestin-3	basal	[33]
JNK3	Arrestin-1, arrestin-2, arrestin-3	either	[24]
ASK1	Arrestin-2, arrestin-3	either	[25]
MKK4	Arrestin-3	either	[25]
MEK1	Arrestin-2, arrestin-3	either	[25,66]
c-Raf1	Arrestin-3	either	[23]
PDE4	Arrestin-3	either	[34]