Chapter 16
The Functional Role of the Conformational Changes in Arrestin Upon Activation

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Abstract G protein-coupled receptors (GPCR) signal primarily through G proteins and arrestins. Arrestin not only intervenes with G protein-mediated signaling, but also initiates a variety of G protein-independent functions. The functional diversity of arrestins has been attributed to their multiple active conformations after interaction with ligand-activated receptors, which are regulated by both the ligand-specific conformational states and the phosphorylation patterns of the receptors. Here we reviewed the current knowledge of the structural features of arrestins that underlie selective arrestin-mediated signaling. Recent breakthroughs in the functional correlation of the conformational changes in arrestin to downstream effector molecules were also highlighted.

Keywords GPCR · Arrestin · Structure · Crystallography · Conformational change · Activation

Introduction

G protein-coupled receptors (GPCRs), also known as seven-transmembrane domain receptors (7TMRs), are the largest family of membrane proteins that communicate extracellular stimuli to intracellular signals and play critical roles in mediating most known physiological functions (Pierce et al. 2002). The classical paradigm of

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GPCR signaling is based on a ligand-induced conformational change of the receptor (Farrens et al. 1996), which is recognized by heterotrimeric G proteins to regulate levels of second messengers. The activated receptor is also phosphorylated by GRKs, which results in subsequent recruitment of arrestins for endocytosis and/or G protein-independent signaling (Walther and Ferguson 2013; Thomsen et al. 2016).

The arrestin family consists of four members. In mammals, arrestin-1 and arrestin-4 are located almost exclusively in photoreceptor cells in the retina and interact with very few visual GPCRs (Wilden et al. 1986; Craft et al. 1994), whereas β-arrestin-1 and β-arrestin-2 (also called arrestin-2 and arrestin-3, respectively) are ubiquitously expressed in most tissues and interact with low specificity with the majority of non-visual GPCRs (Lohse et al. 1990; Attramadal et al. 1992). By binding to GPCRs, these four arrestins desensitize G protein-mediated signaling in many cases and also function as adapters to promote receptor internalization by recruiting endocytic proteins, such as clathrin (Goodman et al. 1996), adaptor protein 2 (AP2) (Laporte et al. 1999) and *N*-ethylmaleimide-sensitive fusion protein (NSF) (McDonald et al. 1999), linking the receptors to the clathrin-coated pit machinery (Shenoy and Lefkowitz 2003; Lefkowitz and Shenoy 2005). In addition to their roles in GPCR desensitization, internalization and trafficking, mounting evidence has revealed that β-arrestins also act as signaling transducers that interact with a growing list of intracellular effectors that include, but not limited to, signaling proteins such as c-Src, Raf-1, Akt, ERK1/2, JNK3 and IκB (Luttrell et al. 1999; Gao et al. 2004; Xiao et al. 2010; Reiter et al. 2012; Wang et al. 2014; Ning et al. 2015; Smith and Rajagopal 2016). Through interaction with these signaling molecules, β-arrestins are capable of initiating G-protein-independent signaling via MAPK, PI3K/AKT, and NF-κB pathways, connecting the activated receptor to diverse physiological responses. Although some studies indicate that G protein and arrestin signal synergistically (Thomsen et al. 2016), β-arrestin-dependent and G-protein-dependent signaling pathways could be pharmacologically separable. Biased ligands, which selectively activate one of these two signaling pathways have great therapeutic potential and have been extensively studied (Rajagopal et al. 2010; Reiter et al. 2012).

The remarkable functional diversity of arrestins raises the inevitable question of how distinct arrestin functions are precisely regulated. Aiming at the detailed mechanism, numerous biomedical and biophysical studies have been performed over the past decade using multiple novel techniques and approaches, leading to fruitful results. Recent structural studies of GPCR-arrestin complex provided considerable insight into the activation mechanism of arrestin (Kang et al. 2015). In the present chapter, we will review current knowledge of the structural features that underlie selective arrestin signaling.

Arrestin Undergoes Conformational Changes After Interaction with Activated Receptors

Based on the diverse functional capacities of arrestins, especially β-arrestins, it has been hypothesized that arrestins adopt multiple active conformations after activation that dictate their distinct functions (Gurevich and Gurevich 2006). In cells, the conformational change of β-arrestin-2 was detected by BRET-based biosensors after activation of the angiotensin II receptor type 1a (AT1aR), β_2 adrenergic receptor (β_2AR), and parathyroid hormone 1 receptor (PTH1R) (Shukla et al. 2008). In detail, different conformations of β-arrestin-2 induced by full agonists or β-arrestin-biased agonists in stimulated cells were observed with the BRET sensor. Later, the conformational biosensor of arrestin was improved by the insertion of the FlAsH (intramolecular fluorescein arsenical hairpin) in the specific loops of arrestin. Significant conformational features of arrestins that correlated with their downstream functions were revealed by this new version of probes (Lee et al. 2016). Further, using the FlAsH-decorated arrestins, a rapid activation/deactivation cycle of β-arrestins was disclosed by FRET analysis, which reshapes our thinking of the arrestin signaling (Nuber et al. 2016).

Phosphorylation of the GPCRs plays a key role in the recruitment and activation of arrestins. Since the 1970s, the finding of phosphorylation of rhodopsin and β_2AR on their C-termini during desensitization has led to the identification of a seven-member family of G protein-coupled receptor kinases (GRKs) (Weller et al. 1975; Benovic et al. 1986; Pitcher et al. 1998). Although subsequent studies revealed that receptors could be phosphorylated by multiple intracellular kinases other than GRKs, such as PKA (Tran et al. 2004) and PKC (Garcia et al. 1998), it has been suggested that the phosphorylation mediated by GRKs initiates arrestin recruitment and receptor internalization (Tobin et al. 2008; Walther and Ferguson 2013). Whereas GRK1 and 7 are confined to the retina and GRK4 is distributed primarily in the reproductive system, GRKs 2, 3, 5, and 6 are ubiquitously expressed and thus regulate the phosphorylation patterns of most GPCRs (Krupnick and Benovic 1998; Pitcher et al. 1998). Studies of different GPCRs revealed that siRNA-mediated knockdown of specific GRK or combinations of GRKs leads to clearly distinguishable effects on receptor functions, suggesting that different sets of GRKs might phosphorylate distinct sites on the receptors. Many of the regulatory roles of GRKs on receptors are through arrestin-mediated functions, including receptor desensitization, internalization and non-receptor-binding partners activation (Kim et al. 2005; Ren et al. 2005). Studies regarding specific GRK-regulated GPCR functions through arrestin brought up the barcode concept of the phosphorylation-regulated receptor function, of which the phosphorylation pattern of the receptor resembles the barcode of the beer can, transducing specific information to downstream effectors (Tobin et al. 2008; Nobles et al. 2011). Using the prototypical GPCR β_2AR as a model, mass spectrometry-based quantitative proteomics and phosphorylation site-specific antibodies were exploited to map the phosphorylation sites of GRK2 and GRK6 at C-terminus and the third intracellular

loop of the β_2AR expressed in HEK293 cells, whereas RNAi-induced GRK silencing and BRET assay were employed to delineate conformation-specific β-arrestin capabilities imparted by specific phosphorylation patterns induced by GRK2 and GRK6. Results indicated that whereas both GRK2 and GRK6 contribute to desensitization of the β_2AR through β-arrestin-2, GRK2 phosphorylation sites (T360, S364, S396, S401, S407, and S411) are primarily responsible for receptor internalization, whereas GRK6 sites (S355 and S356) are required for β-arrestin2-mediated ERK1/2 activation. Notably, phosphorylation of GRK2 sites appears to inhibit phosphorylation of GRK6 sites as well as β_2AR signaling to ERK1/2. It was also demonstrated that isoproterenol, the full agonist of β_2AR, initiates phosphorylation on both GRK2 and GRK6 sites, whereas carvedilol, a β-arrestin-biased ligand of β_2AR (Wisler et al. 2007), induces phosphorylation only at the GRK6 sites. Moreover, the BRET data suggested that distinct phosphorylation patterns on the β_2AR induced by either GRK2 or GRK6 result in different β-arrestin-2 conformations. Collectively, these results provide evidence that the receptor phosphorylation patterns induced by different GRKs establish a 'barcode' that is related to its functional capabilities. This barcode hypothesis has been strengthened by growing evidence from the studies of many other GPCRs, such as M3 muscarinic receptor (Butcher et al. 2011), cannabinoid 1 receptor (Delgado-Peraza et al. 2016), ghrelin receptor (Bouzo-Lorenzo et al. 2016), and fatty acid receptor GPR120 (Prihandoko et al. 2016).

In vitro, binding of the fully phosphorylated vasopressin 2 receptor (V2R) C-tail induces significant conformational changes of both β-arrestin-1 (Nobles et al. 2007) and β-arrestin-2 (Xiao et al. 2004). Recently, a flute model of how phosphorylation pattern of GPCRs was recognized by arrestin and thereafter transduced to downstream effectors through specific conformational changes were revealed by ^{19}F-NMR and unnatural amino acid incorporation approaches (Yang et al. 2015). Not only the phosphorylation states of the receptor, but the ligand-induced specific receptor conformations also contribute to specific arrestin conformations and thus activation. Studies comparing the binding of both visual and non-visual arrestins to four functional forms (inactive unphosphorylated receptor, inactive phosphoreceptor, active unphosphorylated receptor, and active phosphoreceptor) of rhodopsin, β_2AR and M2 muscarinic receptor suggested the existence of an 'activation sensor' in arrestin, which is proposed to mediate the interaction of arrestin with the ligand-induced activated conformation of the receptor (Gurevich et al. 1995; Gurevich and Gurevich 2004). Correspondingly, electron paramagnetic resonance (EPR) studies on rhodopsin-arrestin interactions revealed strong intermolecular contacts between the finger loop, a highly flexible loop connecting β-strands V and VI of arrestin, and the transmembrane core of light-activated phosphorylated rhodopsin, but not that of inactive phosphorylated rhodopsin (Hanson et al. 2006). Moreover, the light-activated unphosphorylated rhodopsin has been further demonstrated to independently induce a unique conformational change in arrestin, which is different from that induced by phosphorylated rhodopsin (Zhuang et al. 2013). Similarly, A NAPol-reconstituted V2R was demonstrated to interact with purified β-arrestin-2 after stimulation with full agonist AVP or β-arrestin-biased

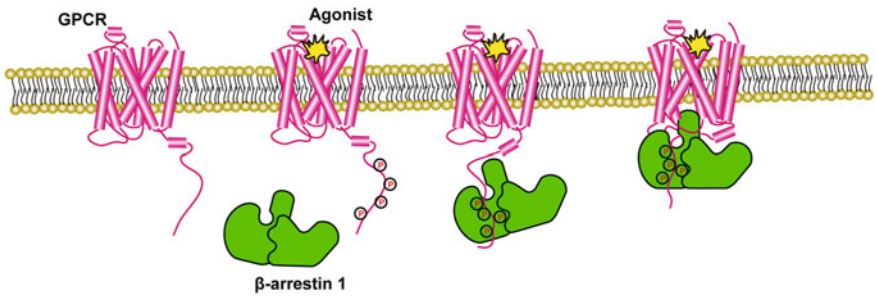


Fig. 16.1 A model of arrestin recruitment and activation by phosphorylated GPCRs. GPCRs are phosphorylated after activation, leading to the recruitment of arrestins. The phosphorylation states of the receptor and ligand-induced receptor conformations collectively determine the active conformations of the arrestins

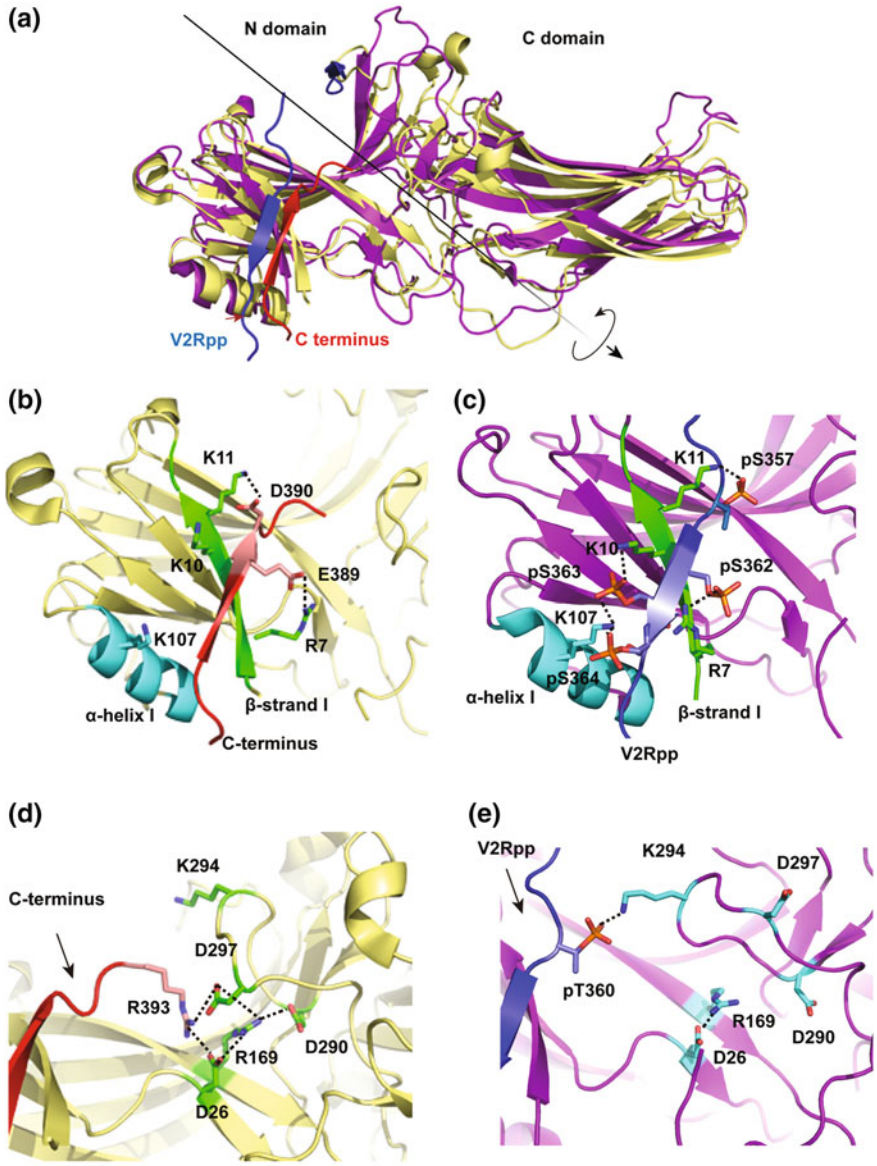
ligand SR121463, but not with the Gs-biased ligand MCF14 (Rahmeh et al. 2012). Therefore, ligand-specific conformational states of GPCR might not only determine the GRK-mediated phosphorylation pattern but also be directly correlated with the conformation changes of arrestin (Fig. 16.1).

Taken together, these findings revealed how arrestin is activated and then directs distinct functions downstream of the receptor. Ligand-dependent stabilization of distinct receptor conformations leads to specific phosphorylation patterns at the C-terminus and/or intracellular loops, which, together with ligand-specific conformational states of the receptor, define distinct arrestin conformations that might dictate selective signaling pathways (Fig. 16.1).

Structural Insight into Arrestin Conformational Change by Crystallography

Crystal structures of all four arrestins at inactive or basal state have been determined and they demonstrate high sequence and structural homology (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011). The arrestins essentially consist of N- and C-domains that are built almost entirely from antiparallel β sheets and linked by a relatively flexible 'hinge' region (Fig. 16.2a). The inactive conformation of the arrestin has been suggested to be constrained by two distinct subsets of intramolecular interactions: polar core that consists of five interacting charged residues in the center of arrestin (D26, R169, D290, D297, and R393 in β-arrestin-1) (Vishnivetskiy et al. 1999) and three-element interactions that comprises the β-strand I, α-helix I, and C-terminus of arrestin (Fig. 16.2b, d) (Vishnivetskiy et al. 2000).

Multiple mutagenesis and biophysical studies suggested that visual arrestin undergoes a conformational change upon binding to light-activated phosphorylated



rhodopsin (Gurevich and Gurevich 2003, 2004). Subsequently, direct evidence of a substantial conformational change of both β-arrestins upon activation was obtained using limited tryptic proteolysis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry analysis in the presence of a phosphopeptide derived from the C-terminus of V2R (Xiao et al. 2004; Nobles et al. 2007). Collectively, results from these studies support a model in which both polar core

◂**Fig. 16.2** Comparison of inactive β-arrestin-1 structure and active β-arrestin-1 structure. **a** Overall comparison of the inactive β-arrestin-1 structure (PDB accession code 1G4M; *gold*) and the active β-arrestin-1 structure (PDB accession code 4JQI; *purple*). The β-arrestin-1 C-terminus and the V2Rpp are highlighted in *red* and *blue*, respectively. The three-element interaction in the inactive β-arrestin-1 structure (**b**) and active β-arrestin-1 structure (**c**). The β-arrestin-1 C-terminal b-strand lying along the three-element interaction in the inactive conformation is displaced by the C-terminus of V2Rpp upon activation. The polar core in the inactive β-arrestin-1 structure (**d**) and active β-arrestin-1 structure (**e**). Upon V2Rpp binding, C-terminal residue R393 of β-arrestin-1is displaced, contributing to the disruption of the polar core

and three-element interactions might be disrupted during the activation of the arrestin (Gurevich and Gurevich 2006; Ostermaier et al. 2014).

Two X-ray crystal structures of activated arrestins, including pre-activated arrestin-1 (Kim et al. 2013) and V2R phosphopeptide (V2Rpp)-bound β-arrestin-1 (Shukla et al. 2013), were determined in 2013. In the latter study, the crystal structure of activated β-arrestin-1 revealed the displacement of β-arrestin-1 C-terminus by V2Rpp that binds to the N-domain as an antiparallel β-strand at a similar location to the β-arrestin-1 C-terminus in inactive structure but with a reversed direction (Fig. 16.2a, c). The replacement of the C-terminus of arrestin with V2Rpp disrupts both 3-element interaction and the polar core that restrain the arrestin in the inactive state (Fig. 16.2c, e). It was observed that two highly conserved residues on the β-stand I, the K10 and K11, which interact with F388 and F391 and are proposed to be a key component of three-element interaction in the inactive structure (Gurevich and Gurevich 2006), make instead charge-charge interactions with phosphates of V2Rpp, which therefore disrupt the 3-element interaction (Fig. 16.2c). Displacement of C-terminus of β-arrestin-1 also removes the R393 of the C-terminus, therefore disables the charge-charge interaction of the R393 with the D26 and D297, and distorts the lariat loop to form the new interaction between the pT360 and K294 (Fig. 16.2e). These changes disrupt the polar core. In the active structure of the V2Rpp/arrestin complex, the disruption of the 3-element interaction and the polar core in arrestin release the N-/C-domain associations and enable 20° twisting of the domains relative to each other. The twisting of these two domains of arrestin not only repositions three loops, including the finger loop, the middle loop and the lariat loop, which may facilitate receptor core interactions; but also exposes substantial regions that might enable downstream effectors recruitment (Sommer et al. 2012; Vishnivetskiy et al. 2013; Zhuo et al. 2014).

A similar twisting between the N-/C-domains of the visual arrestin (arrestin-1 or v-arrestin) was also observed in the recently solved crystal structure of rhodopsin/v-arrestin-phosphorylation-independent-active-mutant fusion complex obtained by serial femtosecond X-ray laser crystallography (Fig. 16.3) (Kang et al. 2015). Conformational changes of the finger loop, middle loop and the region between the I157-K168 enable the interaction of the rhodopsin with the v-arrestin. In detail, whereas the interaction between the rhodopsin C-terminus and v-arrestin N-domain was not observed, this structure revealed three patches of

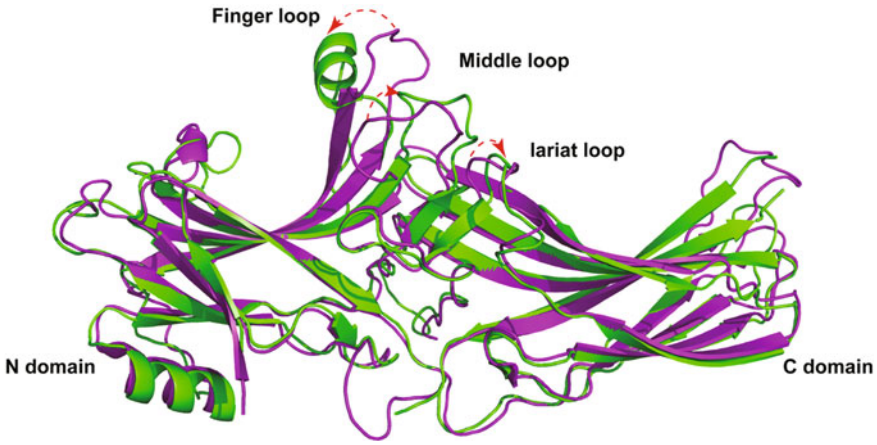


Fig. 16.3 Structural comparison of the active β-arrestin-1 and visual arrestin-1. The phospho-tail (V2Rpp)-activated β-arrestin-1 strucutre (PDB accession code 4JQI; *purple*) and the activated visual arrestin structure (PDB accession code 4ZWJ; *green*) in the complex with rhodopsin are superimposed

rhodopsin-v-arrestin interfaces, which include the interaction of the finger loop of v-arrestin with the intracellular loop-1 (ICL1), TM7 and H8 of the rhodopsin, the accommodation of rhodopsin ICL2 by the middle and C-loops of v-arrestin, and the engagement of β-strand following finger loop and the N-terminal β-strand 6 with the TM5, TM6, and ICL3 of rhodopsin. Interestingly, the structure of the v-arrestin bound to rhodopsin and β-arrestin-1 bound to V2Rpp are very similar other than the receptor interaction regions, suggesting activated arrestins assume similar conformations (Fig. 16.3). However, this result does not support the idea that distinct arrestin functions result from different receptor activation and phosphorylation by different GRKs. Therefore, the low-resolution crystal structures lack important detailed information of structural changes in arrestin to explain how it mediates differential signaling.

Conformational Changes in Arrestin and the Correlation with Downstream Functions

Although mounting evidence has indicated that different arrestin conformations are coupled to distinct functional outcomes, the precise correlation between them is not well established. Since the identification of clathrin as the first non-receptor binding partner of arrestins, a variety of trafficking and signaling proteins, such as c-Src, MAPK, and ASK1, have been reported to interact with arrestin (Goodman et al. 1996; Lefkowitz and Shenoy 2005). The binding sites of clathrin and AP2 on arrestin have been well characterized: both are localized in the C-terminus of

β-arrestins (Kim and Benovic 2002; Schmid et al. 2006; Kang et al. 2009). In contrast, few binding sites of other non-receptor binding partners have been precisely mapped, although particular residues in β-arrestins are suggested to participate in the binding of MEK1 or Raf1 (Meng et al. 2009; Coffa et al. 2011a, b). It has been demonstrated that most of these non-receptor binding partners except for clathrin and AP2 interact with both N-/C-domains of arrestin (Song et al. 2007, 2009) and therefore, the relative rotation between these two domains and rearrangement of the arrestin conformation, which have been confirmed in the structural studies, might explain the structural preference of certain partners that serve as effector molecules leading to distinct functional outcomes (Gurevich and Gurevich 2003, 2013). However, despite the continuous improvement and strengthening of barcode hypothesis, the detailed mechanism by which arrestin transmits signals encoded in the receptor to downstream effector molecules still remains largely unknown.

To better detect the conformational changes in arrestin and further explore how arrestin recognizes specific phosphorylation patterns in the receptor and translates them into distinct functional outcomes, we developed a series of structural sensors in β-arrestin-1 by incorporating the unnatural amino acid 3, 5-difluorotyrosin (F2Y) at specific locations and detected phospho-interaction patterns and residue-specific structural information in β-arrestin-1 using ^{19}F-NMR spectroscopy (Yang et al. 2015). In addition to V2Rpp, we also synthesized specific GRK2-, GRK6-, or PKA-phosphorylated β_2AR C-terminal fragments (GRK2pp, GRK6pp, and PKApp, respectively) and examined their effects on the biochemical properties of β-arrestin-1. We demonstrated that β-arrestin-1 reads phospho-messages in receptor C-tail with its concave surface, which harbors at least ten potential phosphate-binding sites (numbered 1–7 according to the binding mode of V2Rpp to β-arrestin-1 in the V2Rpp/β-arrestin-1 complex (Shukla et al. 2014), with A1–A3 indicating potential additional phosphate-binding sites at the N-terminus). Although all the GRK-phosphopeptides (GRKpps) but not PKApp interact with phosphate-binding site 1 of β-arrestin-1, our results revealed distinct phospho-interaction patterns between different GRKpps and β-arrestin-1 at the other binding sites, which are coupled to selective functional outcomes (Fig. 16.4a). Whereas GRK2pp interacts with β-arrestin-1 through binding sites 1–4–6–7 and promotes clathrin recruitment and receptor endocytosis, GRK6pp interacts with β-arrestin-1 in a different 1–5 pattern and elicits Src signaling (Fig. 16.4a). Mutations of key residues in specific phosphate-binding sites selectively eliminate corresponding β-arresin-1-mediated functions downstream of several GPCRs, including β2AR, cholecystokinin type-A receptor (CCKAR), and somatostatin receptor type 2 (SSTR2). These data suggested that specific phospho-patterns were recognized by arrestins and then translated to different cellular signaling pathways through distinct downstream effectors.

Using 7 ^{19}F-NMR probes incorporated into the specific arrestin sites other than phospho-interaction regions, we were able to detect different conformational states that were induced by binding of different GRKpps. Binding of the two distinct GRK2pp phospho-peptides induced conformational change at the Y249 in the loop

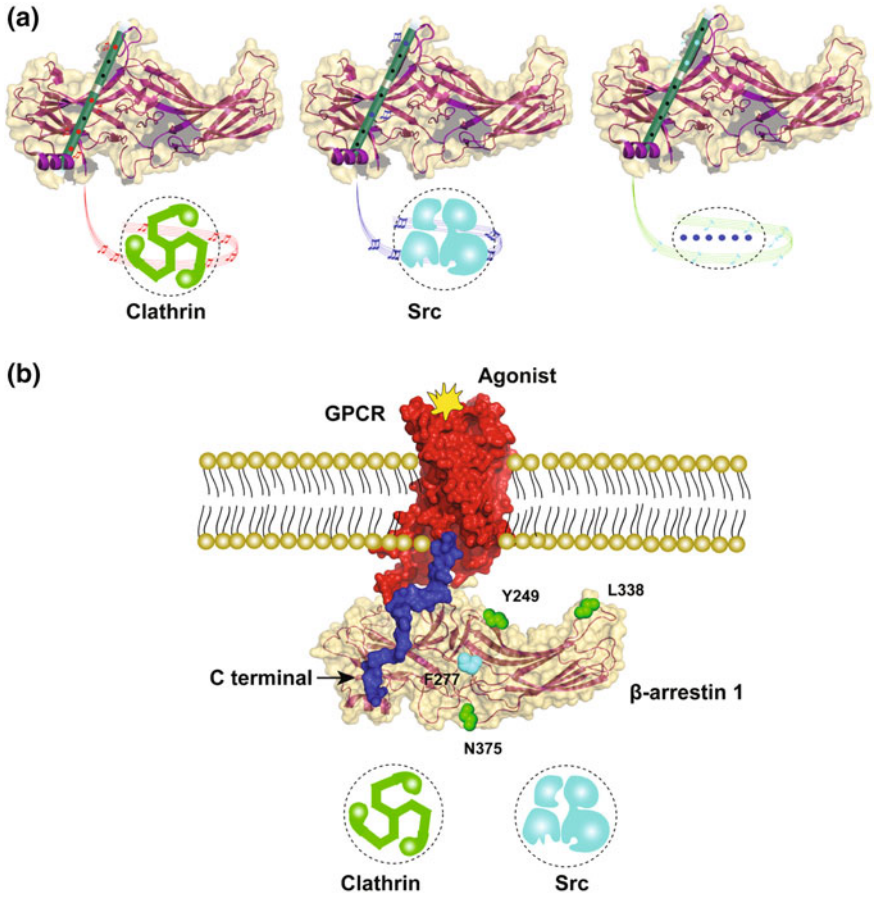


Fig. 16.4 Conformational changes of arrestin induced by different receptor phosphorylation patterns and their correlation with distinct cellular functions. **a** The flute model for the phospho-decision mechanism of the arrestin signaling. A phospho-barcode of 1–4–6–7 directs clathrin recruitment and endocytic function of arrestin, whereas a phospho-barcode of 1–5 directs Src recruitment and signaling. There are potentially more than 1000 phospho-patterns that a single arrestin could recognize. **b** Conformationl changes of arrestin correlate to its distinct functions. The conformational states of the Y249, L338 and N375 are recognized by clathrin and correlated to the endocytic function of β-arrestin-1, whereas the conformational state of F277 is correlated to arrestin-mediated Src signaling

between β-strands XV and XVI, the L338 in the splice loop, and the N375 in the C-terminal region of β-arrestin-1 (Fig. 16.4b). These structural states could be specifically recognized by the subsequent binding of clathrin. In contrast, binding of the GRK6pp to β-arrestin-1 caused a chemical shift at F277 in the end of the lariat loop, whereas binding of the GRK2pps caused no significant conformational change at this site (Fig. 16.4b). Deletion of the partial lariat loop encompassing

F277 abolished the GRK6pp-induced recruitment of Src to arrestin. Collectively, these data have clarified the receptor phospho-coding mechanism, by which changes in a GPCR phosphorylation pattern are translated to distinct conformations of arrestin that could be further recognized by different downstream effector molecules.

In addition to β-arrestin-1, the conformational change of β-arrestin-2 have also been investigated downstream of several receptors in cellular systems. A very recent study monitored the conformational changes of β-arrestin-2 using a panel of intramolecular fluorescein arsenical hairpin (FlAsH) BRET reporters in cells (Lee et al. 2016). Studies of six different types of GPCRs demonstrated the existence of β-arresin-2 "conformational signature" that was indicated by the changes in BRET efficacy from multiple vantage points. This β-arresin-2-FlAsH signature is conserved between GPCRs with similar arrestin binding/signaling characteristics and the changes in BRET efficacy at selected positions correlate with distinct arrestin functions, for example, trafficking pattern of GPCR-arrestin complex and arrestin-dependent ERK1/2 activation. Therefore, these data, together with the results of our study, provide considerable insight into the correlation between specific arrestin conformations and selective arrestin functions and pave the way to the future studies of the detailed functional roles of the conformational changes of arrestin.

Although receptor binding and arrestin conformational change are necessary for the engagement of arrestin with multiple downstream effectors, such as ERK1/2 (Luttrell et al. 2001; Coffa et al. 2011a, b), it has been reported that some binding partners could interact with arrestin in both inactive and active conformations (Song et al. 2009; Ahmed et al. 2011; Gurevich and Gurevich 2013), with some proteins, such as MEK1, even binding to arrestins in both conformations equally well (Coffa et al. 2011a, b; Gurevich and Gurevich 2014). This has added a new layer of complexity in the arrestin-dependent conformational signaling. Moreover, the two isoforms of β-arrestin, which share more than 70% sequence identity and high structural similarity, have been demonstrated to be functionally non-redundant in GPCR regulation by accumulating evidence (Srivastava et al. 2015). Whereas both β-arrestins facilitate the signaling in c-Raf1-MEK1-ERK1/2 cascade, only β-arrestin-2 promotes the signaling in ASK1-MKK4-JNK3 cascade (McDonald et al. 2000). Receptor-specific reciprocal regulation of selective signaling pathways by these two β-arrestins has also been reported. For example, siRNA knockdown of β-arrestin-2 attenuates PTH1R-mediated ERK1/2 activation, whereas knockdown of β-arrestin-1 yields opposite effect (Ahn et al. 2004). Therefore, the interaction between arrestin and effector molecules and the arrestin-dependent signal transduction might be much more complicated than expected. To further decipher the molecular mechanism underlying arrestin conformational signaling, more detailed information from the potential structures, using crystallography, electron microscopy or NMR approaches, of GPCR with arrestin and effectors is required.

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