Chapter 6
Phosphate Sensor and Construction of Phosphorylation-Independent Arrestins

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Abstract Arrestins preferentially bind active phosphorylated GPCRs. Receptor binding is associated with a global conformational change in arrestins. These findings lead to a model where arrestins have distinct sensors for the receptor-attached phosphates and active receptor conformation, and that simultaneous engagement of both sensors by corresponding parts of the receptor induces binding-associated conformational change. Receptor-attached phosphates perturb two intra-molecular interactions in arrestins that stabilize their basal conformation: the polar core between the two domains and the three-element interaction that anchors the arrestin C-tail. Indeed, mutations that disrupt those interactions yield "enhanced" mutants capable of binding active receptors regardless of their phosphorylation. Structural and functional characterization of these mutants lead us to propose an allosteric regulation model for arrestin. Further, it was proposed that these mutants can compensate for defects in GPCR phosphorylation, including those caused by mutations, thereby serving as tools for gene therapy of these gain-of-function GPCR mutations. This idea so far was tested only in the visual system, where partial compensation for lack of rhodopsin phosphorylation was reported. These proof-of-concept experiments suggested that this approach works, but more powerful phosphorylation-independent mutants are needed in photoreceptors using the fastest, most sensitive, and most demanding GPCR-driven signaling system.

Keywords Arrestin · GPCR · Phosphorylation · Congenital disorders · Gene therapy

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V.V. Gurevich (ed.), *The Structural Basis of Arrestin Functions*,
DOI 10.1007/978-3-319-57553-7_6

Early Biochemistry and Structure-Function Characterization

Visual arrestin-1 (under the name of 48 kDa protein)[1] was first discovered as one of the retinal proteins that preferentially bind light-activated rhodopsin (Kuhn 1978). Later, Kuhn's group found that arrestin-1 binding is greatly enhanced by rhodopsin phosphorylation (Kuhn et al. 1984). These findings established arrestin specificity for active phosphorylated rhodopsin (P-Rh*), versus all other functional forms of rhodopsin: inactive unphosphorylated (Rh), active unphosphorylated (Rh*), and inactive phosphorylated (P-Rh). The finding that arrestin binding to P-Rh* has a steep Arrhenius activation energy ($\sim$39 kcal mol^{-1}) suggested that arrestin undergoes a global conformational change in the process (Schleicher et al. 1989). This implied that there must be a sophisticated molecular mechanism responsible for arrestin specificity, which allows binding-associated conformational rearrangements only when arrestin encounters P-Rh*.

With development of a femtomolar sensitive quantitative assay for the arrestin-rhodopsin interaction, it became clear that arrestin does specifically bind Rh* and inactive P-Rh, but its binding to P-Rh* is 10–20-fold higher than to either of these forms (Gurevich and Benovic 1993). This impressive binding differential suggested that the mechanism is more sophisticated than a simple cooperative two-site interaction. These findings led to a double-trigger model, which posited that arrestin has distinct sensors that detect (i) phosphorylation and (ii) the active state of the receptor, which mediate the binding to P-Rh and Rh*, respectively. The model explained the specificity for P-Rh* by the idea that arrestin acts as a coincidence detector; only when both sensors in arrestin are engaged by the receptor simultaneously (which only P-Rh* can do), all constraints are released, allowing arrestin to transition into a conformationally distinct high-affinity receptor-binding state (Gurevich and Gurevich 2004).

This double-trigger model stimulated a search for the two sensors. The expected difference between regular receptor-binding element and phosphate sensor was that the mutations in the former would decrease the binding to the receptor, whereas in the latter case, activating mutations that relieve the conformational constraints of arrestin would enable greater binding to non-preferred forms of rhodopsin. It was clearer what to look for in case of phosphate sensor: phosphates are negatively charged at physiological pH, so the sensor as a phosphate-binding element must have positive charges. At that stage, there were no structures, but all arrestins cloned by that time had one linear stretch rich in positive charges (Fig. 6.1). Naturally, lysines and arginines in that stretch were targeted first (Gurevich and Benovic 1995). Quite a few charge neutralization mutations in that region reduced

[1]Here 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).

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B. taurus arr1 (161)          EDKIPKKSSVRLLIRK
B. taurus arr2 (155)          EEKIHKRNSVRLVIRK
B. taurus arr3 (156)          EEKSHKRNSVRLVIRK
H. sapiens arr4 (152)         EETVSKRDYVRLVVRK
D. melanogaster arr2 (152)    DDRQHKRSMVSLVIKK
C. erythrocephala arr2 (152)  DDRQHKRSMVSLVIKK
L. migratoria arr2 (161)      EEKGHKRSAVTLAIKK
L. polyphemus arr (160)       DEKPHKRNSVSMAIRK
D. melanogaster arr1 (152)    CDRSHRRSTINLGIRK
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Fig. 6.1 Conserved motif rich in positive charges in arrestins. Aligned sequences (the number of the first residues is shown in parentheses) of cow (*Bos taurus*) arrestins 1, 2, and 3, human (*Homo sapiens*) arrestin-4, Drosophila melanogaster sensory arrestins 1 and 2, blowfly (*Calliphora erythrocephala*) arrestin-2, locust (*Locusta mogratoria*) arrestin-2, and horseshoe crab (*Limulus polyphemus*) arrestin. Positively charged residues are shown in *bold*. Note that this 16-residue element contains 5–6 positive charges, most of which are highly conserved in evolution. Interestingly, a homologous element capable of phosphate binding was found in an unrelated protein ataxin-7 (Mushegian et al. 2000)

arrestin-1 binding to both P-Rh and P-Rh*, identifying those charges as phosphate-binding residues. One mutant Arg175Gln, behaved in a strikingly different manner: it demonstrated much higher binding to Rh*, which is consistent with Arg175 being the phosphate sensor (Gurevich and Benovic 1995). However, the binding of Arg175Gln mutant to inactive P-Rh and even P-Rh* was also enhanced. Thus, it was necessary to demonstrate that Arg175 actually binds phosphates, as phosphate sensor was expected to, rather than doing something different. Luckily, by that time a somewhat naively designed mini-arrestin, [residues 1–191 now known to be the arrestin N-domain (Granzin et al. 1998; Hirsch et al. 1999)] was described, which still binds best to P-Rh*, but where that binding appears to be the sum of its binding to inactive P-Rh and Rh* (Gurevich and Benovic 1992, 1993). In the context of that short form, the Arg175Gln mutation simply reduced the binding to both P-Rh and P-Rh*, but did not affect Rh* binding, demonstrating that Arg175 does interact with phosphates. Subsequent replacement of Arg175 with all other 19 residues showed that charge elimination or reversal "pre-activated" arrestin-1, enhancing its binding to Rh*, whereas conservative Arg175Lys substitution that preserved the charge did not (Gurevich and Benovic 1997). These data suggested that in arrestin-1 Arg175 has an intra-molecular negatively charged partner, and that the salt bridge between Arg175 and this putative partner acts as a phosphate sensor: receptor-attached phosphates neutralize the charge of Arg175, thereby breaking that bridge, which "tells" the rest of the molecule that the phosphates are in place (Gurevich and Gurevich 2004; Gurevich and Benovic 1997).

Naturally, this proposal called for a search for that putative negatively charged partner. Arrestin-1 is a 404 residue protein with 57 negatively charged Asp and Glu side chains. Thus, the partner of Arg175 could have been identified either by "carpet bombing"—mutating each one of these negative charges individually, or by

solving the structure that would reveal the neighbors of Arg175. Two crystal structures of arrestin-1 were solved within the next two years (Granzin et al. 1998; Hirsch et al. 1999). Both identified the same three "suspects": Asp30, Asp296, and Asp303 in the vicinity of Arg175. Interestingly, these residues along with Arg382, form the "polar core" right on the interface of the two arrestin domains (Hirsch et al. 1999) (Fig. 6.2). The functional significance of this arrangement was underscored not only by the presence of Arg175 there, but also by the fact that these charges were buried, whereas charged residues in soluble proteins are usually located at the protein-solvent surface, due to their hydrophilicity. Exhaustive mutagenesis of these residues established that the salt bridge between Arg175 and Asp296 acts as the key phosphate sensor in arrestin-1. Charge reversal of either residue greatly increased arrestin-1 binding to Rh*, whereas simultaneous reversal of both charges, restoring the salt bridge in the opposite configuration, yielded arrestin-1 with essentially wild type (WT) selectivity for P-Rh* (Hirsch et al. 1999; Vishnivetskiy et al. 1999).

These results unambiguously identified the phosphate sensor and established the mechanism of its function. In addition, the data solved a mystery in the field: how can just two non-visual arrestins present in vertebrates "serve" hundreds of different GPCRs with minimal sequence conservation of their cytoplasmic faces? The Arg-Asp salt bridge mechanism of phosphate recognition is sequence context-independent, so that arrestins can respond to the presence of receptor-attached phosphates regardless of the nature of surrounding residues (Vishnivetskiy et al. 1999). Thus, it was not surprising that virtually identical polar core was found in other subtypes: arrestin-2 (Han et al. 2001; Milano et al. 2002), arrestin-3 (Zhan et al. 2011), and arrestin-4 (Sutton et al. 2005).

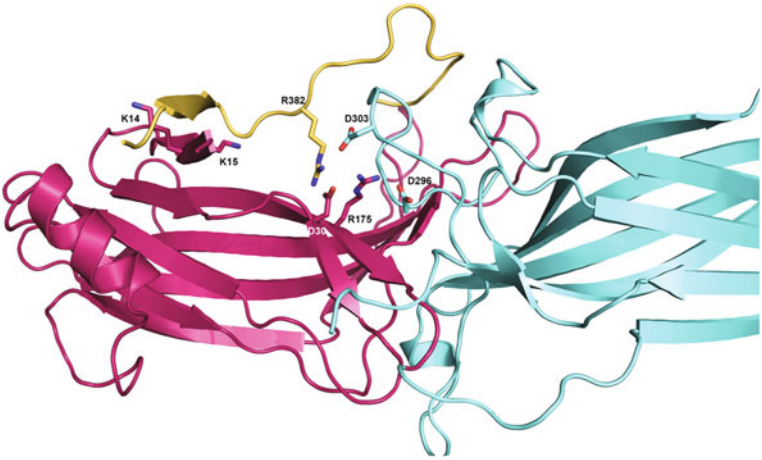

Fig. 6.2 Structure of basal WT arrestin. The N-domain is depicted in magenta while the C-domain is *cyan*. The C-tail is colored *yellow*. Residues mentioned in the text are shown, in particular those residues that are part of the polar core. Based on PDB 1CF1 (Hirsch et al. 1999)

Numerous lines of evidence suggest that for high-affinity arrestin binding, the receptor requires more than one phosphate (Gurevich et al. 1995; Mendez et al. 2000; Vishnivetskiy et al. 2007; Azevedo et al. 2015), whereas the postulated mechanism of the phosphate sensor function suggests that it should respond to a single phosphate. Subsequent mutagenesis revealed that there is more to the phosphate sensor than the polar core. In addition to several phosphate-binding residues identified earlier (Gurevich and Benovic 1995), which the crystal structures showed to be located in the β-strand X (Hirsch et al. 1999), two lysines in the β-strand I (Lys 14 and Lys15 in bovine arrestin-1) (Fig. 6.2) were identified as phosphate-binding residues (Vishnivetskiy et al. 2000). The elimination of these charges by mutagenesis turned out to be the most potent binding-reducing mutations described (Vishnivetskiy et al. 2000). Interestingly, in the context of phosphorylation-independent mutants this substitution did not affect arrestin binding to P-Rh* much (Vishnivetskiy et al. 2000).

Based on these data and localization of these two lysines on the surface of arrestin-1 (Fig. 6.2), a more sophisticated model of phosphate sensing was proposed. Exposed Lys14–15 on the β-strand I were proposed to "greet" receptor-attached phosphates first and guide them to the polar core (Vishnivetskiy et al. 2000). Thus, their function is indispensable in WT arrestin-1, but unnecessary in mutants where the polar core is already destabilized by mutations. The distance between these lysines, other phosphate-binding residues (Gurevich and Benovic 1995), and the polar core (Hirsch et al. 1999) shows that a single phosphate cannot reach all of these elements at the same time. This physical distance (16 Å) suggests why multiple [three in the case of rhodopsin (Mendez et al. 2000; Vishnivetskiy et al. 2007)] receptor-attached phosphates are needed for high-affinity arrestin binding and termination of G protein-mediated signaling.

The identification of the two lysines in the β-strand I as phosphate-binding residues also explained how receptor binding releases the arrestin C-tail from its basal position. The release of the arrestin C-tail by P-Rh* and poly-anions, such as heparin, was discovered long before crystal structures (Palczewski et al. 1991). Truncation (Gurevich and Benovic 1992, 1993), alternative splicing (Smith et al. 1994), or perturbation by a triple alanine (called 3A) substitution of the hydrophobic residues that anchor it to the N-domain (Gurevich 1998) facilitates the binding to any active form of rhodopsin, phosphorylated and unphosphorylated. For the two lysines in neighboring positions in the β-strand I to bind phosphates, this β-strand has to be deformed. This would likely move hydrophobic residues that mediate its interaction with the C-tail out of their basal position, facilitating the release of the arrestin C-tail (Vishnivetskiy et al. 2000). Thus, in addition to destabilizing the polar core, receptor-attached phosphates also break the three-element interaction that holds the C-tail in place (Hirsch et al. 1999). This explains why the deletion or detachment of the C-tail, similarly to the polar core mutations, makes arrestin phosphorylation-independent.

Towards an Allosteric Model for Arrestin

These collective findings suggest a way to frame arrestin regulation and the functional consequences in a classical allosteric scheme. Two extreme states can be postulated, namely a basal, largely inactive (*T* for tense) form and the active form (*R* for relaxed), that are in equilibrium:

$$T \leftrightarrow R.$$

Allosteric modulators then are the phosphorylated C-terminal tail and the activated receptor. The action of both modulators shifts an increased ensemble percentage of arrestin into the *R* state. The activation energy barrier between states is large, in keeping with the well-documented Arrhenius activation energy, i.e. a temperature-dependence in arrestin binding to receptor, noted earlier. The *R* state will be competent for specific, high affinity binding to activated receptor (P-Rh*). Thus, the *R* state would not be expected to have exactly the same conformation as seen in a receptor:arrestin complex (Kang et al. 2015). The various mutants discussed above then have increased population shifting between states.

Evidence for this model was obtained when we structurally characterized purified constitutively active arrestin mutants. Circular dichroism (CD) spectra of WT arrestin and three constitutively active mutants, i.e. Arg175Glu, 3A and ΔV8, arrestin proteolyzed by *Staphylococcus aureus* V8 protease, equivalent to p44, were taken at 4 °C (Fig. 6.3a). All four spectra show a negative band at 216 nm corresponding to β-sheet structures (Johnson 1990), consistent with the β-sheet structures that were presented in the crystal structures (Granzin et al. 1998; Hirsch et al. 1999). Notably, the WT arrestin CD spectrum was distinguished by a shoulder at 190 nm, detected also by Palczewski et al. (1992), which does not appear in the constitutively active mutants. This spectral signature, indicating a structural difference may confer the selectivity of WT to the phosphorylated light active rhodopsin (P-Rh*) that is compromised in the constitutively active mutants. We also measured the CD spectra of WT and mutants at different temperatures. Increasing temperature increments leads to an amplification of the negative band at 216 nm, as well as the positive band around 190 nm for both WT and mutants. Notably, the shoulder at 190 nm that characterized WT at 4 °C disappears as temperature increases, and the WT spectrum comes to resemble the constitutively activated mutants. Thus, WT arrestin at 35 °C has an almost identical spectrum as Arg175Glu at 4 °C (Fig. 6.3b). This shoulder reappears when the sample is cooled back to 4 °C (data not shown), indicating that it is a reversible structural feature, in agreement with our postulated equilibrium model.

In order to correlate these structural findings with functional data, we investigated the effect of temperature on arrestin binding. WT and two constitutively active mutants, namely Arg175Glu and 3A were tested for binding to light-activated and phosphorylated rhodopsin (P-Rh*) and to non-phosphorylated

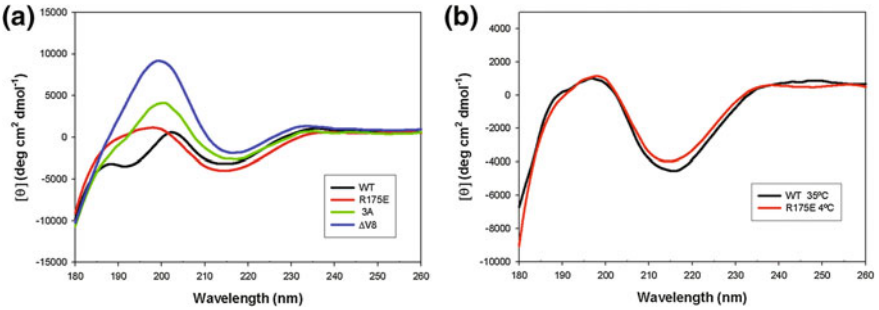

Fig. 6.3 CD spectra of WT arrestin and constitutively active mutants. All CD spectra were collected over the range of 260–180 nm at a scan rate of 1 nm s^{-1}. For all measurements, a cell with 0.1 mm path length was used. Each spectrum is an average of 5 scans. Accurate concentration of protein was obtained by monitoring the absorption of the 190 nm band using the corresponding extinction coefficient (Δ_{190} = 10560 L cm^{-1} mol^{-1} $amide^{-1}$) (Palczewski et al. 1992). **a** All spectra show bands at 216 nm consistent with β-sheet structure. As opposed to the constitutively active mutants, the WT has a shoulder at 190 nm. All the spectra shown in this panel were recorded at 4 °C. **b** A comparison of CD spectra between WT at 35 °C and the constitutively active mutant, R175E, at 4 °C. At these temperatures the WT and the R175E mutant have almost identical CD spectra

light activated rhodopsin (Rh*) at five different temperatures: 0, 20, 37, 40 and 45 °C (Fig. 6.4a, b). WT shows a steep increase in binding to P-Rh* as temperature increased until 37 °C. Binding at 37 °C to P-Rh* was 25-fold higher than at 0 °C. However, temperature effects for Arg175Glu mutant binding are moderate while for 3A there is almost no effect in comparison to WT. Moreover, the quantity of WT that bound to either Rh*-P or to Rh* at 37 °C was very similar to the amount of 3A mutant that bound to Rh*-P or to Rh* at 0 °C, respectively. Finally, the selectivity of WT for P-Rh* at physiological temperatures is similar to the constitutively active mutants at 4 °C (Fig. 6.4c).

These measurements correlate well with the CD data that display similar temperature effects (Fig. 6.3). Notably, WT arrestin has a shoulder around 190 nm in contrast to the constitutively active mutants, which may suggest a specific structural conformation, important for the WT arrestin inactive state. Indeed, functional data show minimal binding of arrestin to Rh*-P at 0 °C in comparison to the constitutively active mutants (Fig. 6.4a), indicating that the CD spectrum of WT at 4 °C corresponds to predominantly the *T* form of arrestin.

Thermal energy input to WT arrestin diminishes the differences between the CD spectrum of WT arrestin and the CD spectra of its constitutively active mutants, such that at 35 °C WT strongly resembles a mutant spectrum taken at 4 °C. The shoulder that characterizes the WT arrestin CD spectrum at 4 °C vanishes with temperature increases, suggesting some shift of the T form population towards the *R* form. Functional data of arrestin strongly support this conclusion. Accordingly, WT arrestin at 37 °C behaves like constitutively active mutant at 0 °C, i.e. WT arrestin binds P-Rh* like constitutively active mutant at 0 °C. Functional

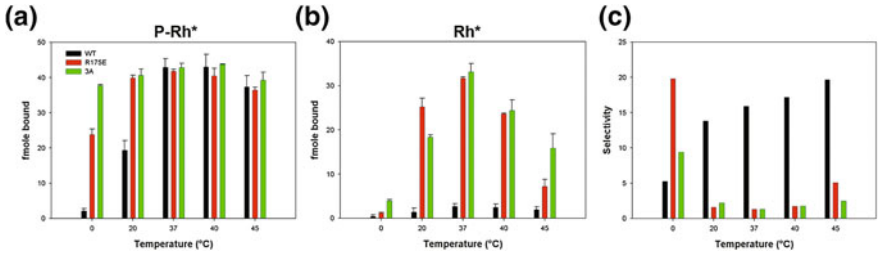

Fig. 6.4 Functional effects of temperature on arrestin binding to different states of rhodopsin. **a** Arrestin binding to P-Rh*. **b** Arrestin binding to Rh*. Binding experiments were performed as described (Vishnivetskiy et al. 2000). **c** Temperature effects on selectivity of WT arrestin and constitutively active mutants. Selectivity was defined as the ratio between binding to P-Rh* to Rh*

experiments with WT and constitutively active mutants at different temperatures demonstrate that the selectivity of WT arrestin for Rh*-P versus Rh* rises with temperature in contrast to the constitutively active mutants. Thus, at 37 °C WT arrestin is three fold more specific to Rh*-P than at 0 °C. In terms of selectivity, the mutants have maximal selectivity at 0 °C versus 37 °C for WT.

Warming the constitutively active mutants does not significantly increase the binding to Rh*-P but rather to Rh*. Therefore, we conclude that for the constitutively active mutants, the phosphorylated C-terminal segment of rhodopsin is sufficient to enable binding to rhodopsin regardless of the temperature. Nevertheless, binding to Rh* remains temperature-dependent, since the constitutively active mutants still need to undergo conformational changes, driven by thermal energy in the absence of the phosphorylated C-terminal segment of rhodopsin in order to bind Rh*. In contrast to the constitutively active mutants, WT arrestin requires both temperature and the presence of the phosphorylated C-terminal segment of rhodopsin in order to bind rhodopsin (low binding to Rh*-P at 0 °C and to Rh* at 37 °C).

As postulated above, an equilibrium pertains between the *T* and *R* states, which is appreciably shifted in favor of the *T* state. This form, we suggest, has a relatively rigid conformation, stabilized both by the intact polar core and the C tail interactions (Vishnivetskiy et al. 1999). Upon interaction of arrestin with an allosteric effector, the phosphorylated C-terminal segment of rhodopsin, the latter pushes the equilibrium in favor of the active *R* state. The phosphorylated C-terminal segment of rhodopsin shifts the equilibrium by destabilizing arrestin's inactive conformation (Vishnivetskiy et al. 2000). In addition, thermal energy also pushes the equilibrium towards the active state and indeed, physiological temperature is required in order to obtain maximal binding of arrestin to P-Rh* in vitro. At 0 °C, the natural allosteric effector cannot provide sufficient push to move the equilibrium in favor of the active state (very low binding to P-Rh* at 0 °C). Subsequently, the active conformation is stabilized by binding to the receptor, and as a result further pushes the equilibrium towards the active state. In contrast with WT arrestin, the mutations in constitutively active forms destabilize arrestin's inactive state, most probably by

disrupting the polar core or the C tail interactions, and as a result push the equilibrium in favor of the active state. Therefore, they do not require the phosphorylated C-terminal segment of rhodopsin, and can bind Rh*.

This framework appears to be in line with the more recent structural studies that have provided important insights into the conformation of constitutive arrestin mutants like p44 and R175E. Two crystal structures of p44 have been reported. One report, by Granzin and coworkers, described a structure that was remarkably similar to the basal, inactive WT structures (Granzin et al. 2012). The second report, by Sommers and coworkers, revealed p44 with a significant change in conformation, most notably, a rigid body C-domain shift and specific changes in the polar core (Kim et al. 2013). In addition, a crystal structure of R175E was reported last year, revealing conformations similar to the conformationally novel p44, but with a smaller inter-domain twist, suggesting that this is the structure of the activation intermediate (Granzin et al. 2015) (see Chap. 12 for details). All of these findings can be reconciled and organized by our thermodynamic allosteric model. The constitutive mutants sample both *T* and *R* states, residing more often than WT in the *R* state. Hence, upon crystallization, it is possible to capture in a given crystal form either the *T* or *R* state. Importantly, the p44 structure described in the *R* state had been incubated and crystallized with opsin, indicating that receptor assists in activating or shifting the p44 into that precise conformation. It appears that R175E is more shifted or destabilized than p44, again in line with our observations above.

Applications

All the experiments described above were performed on visual arrestin-1. However, the structural features identified by arrestin-1 mutations are conserved in the arrestin family at the level of sequence (Gurevich and Gurevich 2006) and 3D structures (Granzin et al. 1998; Hirsch et al. 1999; Han et al. 2001; Milano et al. 2002; Zhan et al. 2011; Sutton et al. 2005). Thus, it is hardly surprising that homologous mutations in other arrestin subtypes yield a similar biochemical phenotype: tight binding to unphosphorylated receptors and suppression of the G protein signaling without receptor phosphorylation (Gurevich et al. 1997; Kovoor et al. 1999; Celver et al. 2002). It was generally concluded that the mechanism of activation is conserved in the arrestin family (Gurevich and Gurevich 2004, 2006), raising two questions: are there generally applicable methods of generating "enhanced" arrestins and might they have translational use?

Humans have ~800 different GPCR subtypes. Naturally, there are quite a few genetic errors in these receptors, many of which lead to congenital disorders (Schoneberg et al. 2004; Stoy and Gurevich 2015). Mutations in genes fall into two broad categories: loss-of-function and gain-of-function. In case of loss-of-function mutations, a strategy for gene therapy is clear: restoration of the WT protein sequence should solve the problem. Moreover, loss-of-function mutations are

usually recessive, as enough functional protein is often generated from the normal second allele. Cases of haplo-insufficiency are rare, especially among signaling proteins, such as GPCRs.

In contrast, gain-of-function mutations are dominant, as mutant protein generates excessive signaling even in the presence of a perfectly normal product from the second allele. Until recently, it has not been obvious how to approach these mutations therapeutically. Today there may be options, at least conceptually. At the simplest level, faulty genes can now be edited to correct the errors [reviewed in Stoy and Gurevich (2015)]. However, such a strategy would make sense in stem cells generating rapidly dividing cells (e.g., skin, lung epithelium, etc.), but would hardly work in non-dividing cells, such as neurons, where the gene in every cell has to be edited. Alternatively, ribozymes specifically attacking mutant mRNA can be engineered. However, in many cases such as frame-shift and point mutations, normal and faulty mRNA differ by just one base (Fuchs et al. 1995). It is quite challenging to design ribozymes that combine this level of precision with high enough efficiency to significantly reduce excessive signaling. Therefore, in the case of mutated GPCRs, a compensational approach has been proposed: counteract excessive receptor activity with expression of an enhanced arrestin that has a greater than normal ability to suppress signaling (Song et al. 2009). Similar to the ribozyme strategy, this approach requires expression of the desired construct in the majority of affected cells.

Functionally, gain-of-function mutations in GPCRs fall into two categories: those that cause increased constitutive activity (signaling in the absence of activating agonists) and those that reduce the ability of GRK/arrestin system to quench G protein activation, such as defects in receptor phosphorylation (Mendez et al. 2000; Chen et al. 1995, 1999). Conceivably, in the latter case, expression of a phosphorylation-independent arrestin mutant could compensate and return signaling closer to normal.

The idea of compensational gene therapy has been experimentally tested to date only in the visual system. Excessive rhodopsin signaling in rod photoreceptors due to the absence of cognate GRK1 (Chen et al. 1999), arrestin-1 (Xu et al. 1997), the absence (Chen et al. 1995) or insufficient number of phosphorylation sites (Mendez et al. 2000) invariably leads to prolonged photo-responses. This excessive signaling results in night blindness (Fuchs et al. 1995; Yamamoto et al. 1997) and eventually leads to retinal degeneration. Transgenic mouse lines expressing an arrestin-1 3A mutant with higher than normal ability to bind Rh* were created (Nair et al. 2005). Transgenic mice were then bred in a GRK1 (rhodopsin kinase) knockout background and directly compared with WT arrestin-1 mice. The success of these proof-of-concept experiments was partial. On the one hand, enhanced arrestin-1 facilitated photoresponse recovery and improved photoreceptor survival (Song et al. 2009). On the other hand, the time of half recovery of "compensated" photoreceptors was still many times longer than that in WT mice (Song et al. 2009). Moreover, lower ($\sim$50% of WT) expression of the enhanced mutant worked better than higher ($\sim$240% of WT) expression (Song et al. 2009), and high levels of enhanced mutant turned out to be detrimental for photoreceptor health and survival

(Song et al. 2013). Independent experiments also showed that even relatively low affinity of arrestin-1 for clathrin adaptor AP2 can be detrimental for the rod health, whereas elimination of the AP2 binding site makes the mutant harmless and allows it to compensate for constitutive activity of rhodopsin mutant (Moaven et al. 2013). Hence, these pioneering experiments suggested that to be therapeutically usable the enhanced versions of arrestin-1 should be further improved in two ways. The mutant should have a (i) higher affinity for Rh* and (ii) lack an AP2 binding site. New and improved phosphorylation-independent versions of arrestin-1 need to be tested experimentally to determine how far the compensation can be pushed in the visual system (Vishnivetskiy et al. 2013).

However, rod photoresponse is the fastest and most sensitive GPCR-driven signaling system. Rods respond to single photons (Baylor et al. 1979) and turn off within 250 ms (Mendez et al. 2000), i.e., many times faster than any other GPCR-elicited response. Thus, an approach that yields partial compensation in rods is likely to ensure full compensation in other cells. Non-visual arrestins can be pre-activated by mutations homologous to those that pre-activate arrestin-1 (Kovoor et al. 1999; Celver et al. 2002). The key challenge is that while arrestin-1 is highly selective for rhodopsin, non-visual arrestins are quite promiscuous, binding many GPCRs with comparable affinity (Gimenez et al. 2012; Barak et al. 1997). Most cells express numerous GPCR subtypes, only one of which is likely to be an overactive GPCR mutant. Thus, a promiscuous enhanced non-visual arrestin meant to suppress excessive signaling by the mutant will likely affect the signaling by the other GPCRs in the same cell, causing unwanted side effects. Having generated the rhodopsin-specific arrestin-1, evolution demonstrated that high receptor specificity of arrestin can be achieved. Approaches to the problem of receptor selectivity of non-visual arrestins is discussed in more detail in Chap. 9.

To summarize, we now know where receptor-attached phosphates bind in arrestins and how they promote arrestin “activation”, i.e., a global conformational rearrangement necessary for the transition into high-affinity receptor-binding state. Several mutations targeting intra-molecular interactions that hold arrestins in their basal conformation were shown to facilitate the activation, yielding mutants that can bind any active receptor, phosphorylated or not. These mutants appear to have the ability to compensate for excessive signaling of GPCR with gain-of-function mutations, but their full therapeutic potential needs to be further explored.

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