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# Differential manipulation of arrestin-3 binding to basal and agonist-activated G protein-coupled receptors

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ABSTRACT

Non-visual arrestins interact with hundreds of different G protein-coupled receptors (GPCRs). Here we show that by introducing mutations into elements that directly bind receptors, the specificity of arrestin-3 can be altered. Several mutations in the two parts of the central "crest" of the arrestin molecule, middle-loop and C-loop, enhanced or reduced arrestin-3 interactions with several GPCRs in receptor subtype and functional state-specific manner. For example, the Lys139Ile substitution in the middle-loop dramatically enhanced the binding to inactive $M_2$ muscarinic receptor, so that agonist activation of the $M_2$ did not further increase arrestin-3 binding. Thus, the Lys139Ile mutation made arrestin-3 essentially an activation-independent binding partner of $M_2$, whereas its interactions with other receptors, including the $\beta_2$-adrenergic receptor and the $D_1$ and $D_2$ dopamine receptors, retained normal activation dependence. In contrast, the Ala248Val mutation enhanced agonist-induced arrestin-3 binding to the $\beta_2$-adrenergic and $D_2$ dopamine receptors, while reducing its interaction with the $D_1$ dopamine receptor. These mutations represent the first example of altering arrestin specificity via enhancement of the arrestin-receptor interactions rather than selective reduction of the binding to certain subtypes.

## 1. Introduction

Dysfunction of G protein-coupled receptor (GPCR) signaling plays an important role in the pathogenesis of numerous human diseases [1,2]. For example, gain-of-function mutations that result in excessive activity or elevated ligand production that over-stimulates normal receptors can each cause endocrinological disorders and malignant tumors [1,3,4]. Because receptor activity is crucial for normal cell function, it is tightly controlled by several regulatory mechanisms. Active receptors are phosphorylated by specific GPCR kinases, whereupon arrestin proteins bind active phosphoreceptors [5]. Conceivably, the introduction of strong negative regulators into pathological cells, such as enhanced arrestins that bind unphosphorylated GPCRs, can suppress excessive signaling and potentially correct these problems.

Wild type (WT) arrestins bind active phosphorylated GPCRs [6,7], thereby terminating G protein signaling [8], and facilitating receptor internalization [9]. Thus, enhanced arrestins that do not require receptor phosphorylation can potentially be used in compensational therapy aiming at suppression of excessive G protein-mediated signaling [10,11]. Vertebrates have > 800 GPCRs [12,13] and only four different arrestins [14]. Arrestin-1 and -4 are expressed in the visual system [15], whereas arrestin-2 and -3 (also known as β-arrestin1 and 2) are ubiquitously expressed, and each regulates hundreds of receptors. Previous work established that arrestins can be "pre-activated" by mutations destabilizing their basal conformation, thereby increasing their binding to GPCRs [16–18]. Enhanced arrestin-1 was shown to partially compensate for defects of rhodopsin phosphorylation in vivo [11]. Similarly, enhanced non-visual arrestins could reduce the signaling of hyperactive GPCRs. But as arrestin-2 and -3 are fairly promiscuous [19], enhanced non-visual arrestins would likely have unwanted side effects as they would simultaneously affect the function of all receptors expressed in the same cell. Thus, to make enhanced non-visual arrestins useful as therapeutic agents, their receptor specificity must be dramatically increased.

The feasibility of engineering arrestin-2 and -3 with enhanced receptor selectivity is suggested by strict selectivity of visual arrestin-

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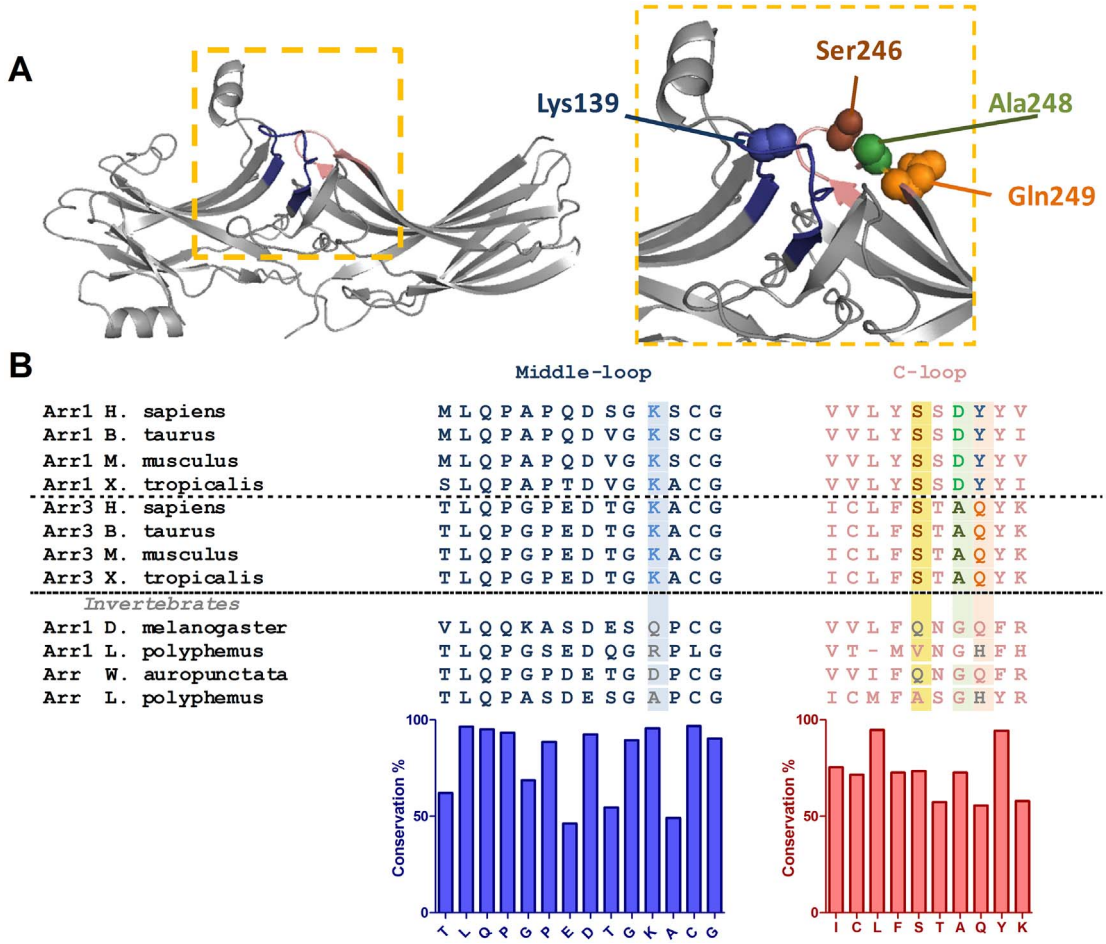


**Fig. 1.** Structure and sequence of the middle and C-loops of arrestins.A. Crystal structure of rhodopsin-bound arrestin-1 (Protein Data Bank entry 4ZWJ [27]). The middle and C-loops are shown in blue and pink, respectively. Residues mutated in this study are shown as CPK models. B. Multiple sequence alignment of arrestin-1 (Arr1), arrestin-3 (Arr3) and arrestin homologs (Arr) from invertebrate species. Residues mutated in this study are highlighted. Bar graph under the alignment shows the extent of residue conservation at each position. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1, exclusively expressed in photoreceptor cells, for photopigments. Arrestin-1 binds to phosphorylated light-activated (P-Rh*) rhodopsin much better than other receptors [20,21]. Earlier studies identified elements [21] and individual residues on the receptor-binding surface [22] responsible for receptor preference of arrestins. The exchange of ten non-identical residues on the receptor-binding surface can change arrestin-1 preference to that of arrestin-2, and vice versa [22]. Point mutations at these positions were shown to increase the preference of arrestin-3 for some cognate receptors over others up to 4-fold, whereas double mutations yielded 50-fold differential in the binding to $M_2$ muscarinic over $\beta_2$-adrenergic receptor [23]. Comparison of crystal structures [24,25] and primary sequences of arrestin-1 and -2 additionally identifies a key valine residue in arrestin-1 that stabilizes its N-domain, and likely contributes to its high selectivity for rhodopsin, as demonstrated by a dramatic increase in the binding to non-cognate $M_2$ muscarinic receptor of arrestin-1 with a single Val83Ser mutation [24]. However, mutations of the ten "receptor-discriminator" residues identified in the arrestin-1/arrestin-2 chimeras did not affect arrestin-3 binding to some of the GPCRs tested, including the $D_1$ dopamine [23] or $Y_1$ neuropeptide [26] receptors, while many of the same mutations dramatically change arrestin-3 binding to $D_2$ dopamine and $Y_2$ neuropeptide receptors. Here we took advantage of the recent crystal structure of arrestin-1 in complex with rhodopsin [27], which identified additional elements, including the middle-loop and C-loop, both of which engage the receptor and therefore might play a role in arrestin selectivity. We used four model GPCRs ($M_2$ muscarinic, $\beta_2$-adrenergic, $D_1$ dopaminergic, and $D_2$ dopaminergic) and show that mutations in these regions of arrestin-3 differentially affect its basal and agonist-induced binding.

## 2. Material and methods

### 2.1. Materials

Restriction endonucleases and other DNA modifying enzymes were from New England Biolabs (Ipswich, MA). Cell culture reagents and media were from Mediatech-Corning (Manassas, VA), Life-technologies (Carlsbad, CA), or PAA Laboratories GmbH (Pasching, Austria). Luciferase substrate coelenterazine *h* was from NanoLight Technology (Pinetop, AZ). DNA purification kits were from Zymo Research (Irvine, CA). All other reagents were from Amresco (Solon, OH) or Sigma-Aldrich (St Louis, MO).

### 2.2. Mutagenesis and plasmid construction

Plasmids encoding short splice variant of bovine arrestin-3 [28] with unique restriction sites introduced by silent mutations were used to introduce mutations on the background of Ala87Val base mutant (which is expected to be predisposed to higher receptor selectivity), as described [23,26]. To generate all mutants, oligonucleotides harboring the desired substitutions were used as forward primers and an oligonucleotide downstream from the far restriction site was used as a reverse primer for PCR. Resulting fragments of various lengths and an appropriate primer upstream of the near restriction site were then used