MINIREVIEW—MOLECULAR PHARMACOLOGY IN CHINA

Phosphorylation of G Protein-Coupled Receptors: From the Barcode Hypothesis to the Flute Model

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ABSTRACT

Seven transmembrane G protein-coupled receptors (GPCRs) are often phosphorylated at the C terminus and on intracellular loops in response to various extracellular stimuli. Phosphorylation of GPCRs by GPCR kinases and certain other kinases can promote the recruitment of arrestin molecules. The arrestins critically regulate GPCR functions not only by mediating receptor desensitization and internalization, but also by redirecting signaling to G protein-independent pathways via interactions with numerous downstream effector molecules. Accumulating evidence over the past decade has given rise to the phoso-barcode hypothesis, which states that ligand-specific phosphorylation patterns of a receptor direct its distinct functional outcomes. Our recent work using unnatural amino acid incorporation and fluorine-19 nuclear magnetic resonance (19F-NMR) spectroscopy led to the flute model, which provides preliminary insight into the receptor phosphocoding mechanism, by which receptor phosphorylation patterns are recognized by an array of phosphate-binding pockets on arrestin and are translated into distinct conformations. These selective conformations are recognized by various effector molecules downstream of arrestin. The phosphobarcoding mechanism enables arrestin to recognize a wide range of phosphorylation patterns of GPCRs, contributing to their diverse functions.

Introduction

Seven transmembrane-spanning G protein-coupled receptors (GPCRs) comprise the largest known membrane protein family encoded by the human genome, and GPCRs regulate almost all the known physiologic processes in humans by converting a broad range of extracellular stimuli (ranging from light to hormones and neurotransmitters) to intracellular signals (Ritter and Hall, 2009; Manglik and Kobilka, 2014; Wisler et al., 2014; Dohlman, 2015). Upon ligand binding and activation, most GPCRs “floating” on the plasma membrane are phosphorylated at sites located on intracellular loops or C-terminal tails (Table 1).

Many different phosphorylation sites in different GPCRs have been identified, mostly by mass spectrometry or phospho-specific antibodies. By contrast, the functions of receptor phosphorylation are often established by mutagenesis both in vitro and in vivo (Budd et al., 2000; Jones et al., 2007; Busillo et al., 2010; Bradley et al., 2016). In cells, the phosphorylation process is mediated by at least two classes of serine/threonine kinases, including the second messenger-dependent (e.g., PKA and protein kinase C [PKC]) and -independent kinases (i.e., GPCR kinases [GRKs]) (Lefkowitz, 1998). As a classic paradigm, phosphorylation of receptors by the former type of kinases is independent of ligand binding and directly uncouples the receptors from their cognate G proteins, leading to heterologous desensitization (Hausdorff et al., 1990). In contrast, receptor phosphorylation

ABBREVIATIONS: Akt, protein kinase B; β2AR, β2-adrenergic receptor; AT1aR, angiotensin II receptor type 1; BRET, bioluminescence resonance energy transfer; ERK, extracellular signal-regulated kinase; 19F-NMR, fluorine-19 nuclear magnetic resonance; GPCR, G protein-coupled receptor; GPR120, G-protein coupled receptor 120; GRK, G protein-coupled receptor kinase; GRK2, G protein-coupled receptor kinase 2, or β-adrenergic receptor kinase 1; GRK2pp, GRK2-phosphopeptides; ICL3, third intracellular loop; M3-mACHR, M3-muscarinic acetylcholine receptor; PKA, protein kinase A; PKC, protein kinase C; Thr, threonine; V2R, V2 vasopressin receptor.
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<td></td>
<td>S345, S346</td>
<td>PAK</td>
<td>Mut</td>
<td>Mouse L cells</td>
<td>Desensitization</td>
<td>(Clark et al., 1989)</td>
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<td></td>
<td>S355, S356, S364</td>
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<td></td>
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<td>T347</td>
<td>ND</td>
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<td></td>
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<td></td>
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<td>S431, T439, T446</td>
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<td>β-arrestin-2 recruitment and calcium mobilization</td>
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<td>G protein-coupled receptor 120 (GPR120)</td>
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<td>PKC/GRK6</td>
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<td>β-arrestin-2 recruitment and calcium mobilization</td>
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<td>S357, S361</td>
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<td></td>
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<td></td>
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<td>Mut</td>
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<td></td>
<td>Cluster S406-T409, T427-T431</td>
<td>GRK</td>
<td>Mut</td>
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<td>Receptor-β-arrestin1/2-ubiquitin complex formation</td>
<td>(Jaeger et al., 2014)</td>
</tr>
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AMPK, 5′-AMP-activated protein kinase; CaMKII, Ca2+/calmodulin-dependent protein kinase II; CHO, Chinese hamster ovary cells; CR2, casein kinase II; COS-1, CV-1 in origin with SV40 genes monkey kidney cells; EDA, Edman degradation analysis; HEK293, human embryonic kidney 293 cells; Mut, mutagenesis; MS, mass spectrometry; ND, not determined; P-SA, phospho-specific antibody; PAA, phospho-amino acid analysis.
Phosphorylation Barcoding of the GPCR

Phosphorylation of the GPCR at Multiple Sites

Stimulus-induced GPCR phosphorylation was first reported for rhodopsin in 1972, which led to the subsequent identification and isolation of rhodopsin kinase (GRK1) (Kühn and Dreyer, 1972; Weller et al., 1975). A similar observation was made in the β2-AR system, where the deactivation of agonist-occupied β2-AR was found to be tightly associated with receptor phosphorylation by a β-adrenergic receptor kinase (ARK1 or GRK2) (Benovic et al., 1986; Studer et al., 1983). These findings identified the pivotal role of phosphorylation in the desensitization mechanism of GPCRs. Since then, seven isoforms of GRKs, referred to as GRK 1-7, have been identified (Pitcher et al., 1998). GRK 2, 3, 5, and 6 are ubiquitously expressed and serve as important determinants of phosphorylation patterns in most nonvisual system GPCRs (Krupnick and Benovic, 1998; Butcher et al., 2012).

In addition to GRKs, GPCR can also be phosphorylated by second messenger-regulated kinases and some other kinases (Benovic et al., 1985; Pitcher et al., 1992; Kelly et al., 2008; Tobin, 2008). For example, casein kinase-induced phosphorylation plays an important role for M3-mAChR and thyrotropin-releasing hormone receptor, whereas Ca2+/calmodulin-dependent protein kinase II (CaMKII)–induced phosphorylation has been identified in the dopamine receptor D3, GABAAR receptor, and μ-opioid receptor (Table 1 and Table 2) (Budd et al., 2001; Hanyaloglu et al., 2001; Torrecilla et al., 2001; Liu et al., 2009; Guetg et al., 2010; Chen et al., 2013). These kinases are important “composers” of receptor phosphorylation patterns and, therefore, specifically regulate receptor functions.

The very first mutagenesis analyses of rhodopsin and β2-AR determined that these receptors are phosphorylated at multiple sites (Wilden and Kühn, 1982; Bouvier et al., 1988). As discussed previously, the involvement of a variety of protein kinases suggests that undergoing multiple phosphorylation events might be a common phenomenon among the GPCR superfamily, and this has been confirmed by numerous studies mapping phosphorylation sites on GPCRs using different techniques and approaches.

Whereas site-directed mutagenesis was considered the primary method for identifying the potential phosphorylation residues in early studies, multiple novel techniques have been

by GRKs, a kinase family consisting of seven members, is ligand-stimulation dependent and is followed by the recruitment of arrestin molecules to the receptor that sterically inhibits G protein coupling, initiating homologous desensitization (Pitcher et al., 1992; Tobin et al., 2008; Gurevich et al., 2012).

The functional importance of receptor phosphorylation has been demonstrated in many aspects of GPCR regulation other than desensitization. Studies of the PKA-regulated β2-adrenergic receptor (β2AR) phosphorylation have shown that it not only decreases the coupling of β2AR to the Gi protein but also switches this coupling to the G protein (Daaka et al., 1997; Lefkowitz et al., 2002). Receptor phosphorylation by GRKs promotes the coupling of arrestins to the activated receptors, which then mediate G protein-independent signaling.

Four isoforms of arrestin have been identified: arrestin-1 and arrestin-4 are restricted to the visual system and accordingly are named “visual arrestins” (Wilden et al., 1986; Craft et al., 1994). The other two isoforms, β-arrestin-1 and β-arrestin-2, are ubiquitously distributed (Lohse et al., 1990; Attramadal et al., 1992). Arrestin binding facilitates the internalization of nonvisual receptors via clathrin-dependent endocytic machinery (McDonald and Lefkowitz, 2001; Shenoy and Lefkowitz, 2003).

Moreover, arrestin-mediated receptor trafficking initiates a second wave of receptor signaling via interactions with a growing list of signaling molecules such as SRC proto- oncogene, non-receptor tyrosine kinase (SRC), Raf-1 proto- oncogene, serine/threonine kinase (Raf-1), protein kinase B proto- oncogene, non-receptor tyrosine kinase (SRC), Raf-1 proto- kinases. Since then, seven iso- forms of GRKs, referred to as GRK 1-7, have been identified (Pitcher et al., 1998). GRK 2, 3, 5, and 6 are ubiquitously expressed and serve as important determinants of phosphor- ylation patterns in most nonvisual system GPCRs (Krupnick and Benovic, 1998; Butcher et al., 2012).
**TABLE 2.** Examples of receptor phosphorylation on intracellular loops and corresponding functions

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<td>PKA</td>
<td>Mut</td>
<td>Mouse L cells</td>
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<tr>
<td>Dopamine receptor D1 (DRD1)</td>
<td></td>
<td>ICL3</td>
<td>PKA</td>
<td>Mut</td>
<td>Rat C6 Glioma cells</td>
<td>Desensitization and $\beta$-arrestin-2 recruitment and desensitization</td>
<td>(Jiang and Sibley, 1999)</td>
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<tr>
<td>Dopamine receptor D3 (DRD3)</td>
<td>S229, S229, S257</td>
<td>ICL3</td>
<td>CaMKII</td>
<td>Mut</td>
<td>HEK293</td>
<td>Inhibit ERK1/2 activation</td>
<td>(Liu et al., 2009)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>PKC</td>
<td>Mut</td>
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<td>$\beta$-arrestin-independent desensitization and internalization</td>
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<tr>
<td>Follicle-stimulating hormone receptor (FSHR)</td>
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<td>ND</td>
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<tr>
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<td>V2 vasopressin receptor (V2R)</td>
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<td>JNK kinase activation</td>
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<td></td>
<td></td>
<td></td>
<td>CK1$\alpha$</td>
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<td>CHO</td>
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CHO, Chinese hamster ovary cells; CK2, casein kinase II; COS7, derived from CV-1 in origin with SV40 genes monkey kidney cells; CPM, chymotryptic phospho-peptide mapping; HEK293, human embryonic kidney 293 cells; JNK, c-Jun N-terminal kinase; Mut, mutagenesis; ND, not determined.
developed and applied for more precise mapping of phosphorylation sites in an increasing number of GPCRs (Table 1). For example, using mass spectrometry analysis and phospho-specific antibodies, three serines and two threonines at the C-tail of the ghrelin receptor and 14 serines and two threonines at the C-tail and on the third intracellular loop (ICL3) of the M3-mAChR were identified as phosphorylation sites (Butcher et al., 2011; Bouzo-Lorenzo et al., 2016). Furthermore, whereas the phosphorylation sites of GPCRs are primarily located at the C-tail and on the ICL3, there is increasing evidence that phosphorylation events could also occur on other cytoplasmic regions, including the first and second intracellular loops (Table 2) (Nakamura et al., 1998; Celver et al., 2001; Nobles et al., 2011).

One interesting feature of GPCR phosphorylation is its dynamics and sequential fashion. For example, the phosphorylation of the primary sites in rhodopsin permits the phosphorylation of the other residues, and this has also been observed in several other GPCRs (Ohtubo et al., 1993). Studies of the phosphorylation profile of the D1 dopamine receptor indicated that although the mutation of a cluster of serines within the ICL3 resulted in severely impaired ligand-induced receptor phosphorylation and desensitization, C-terminal truncation at a selective threonine (Thr347) led to reduced receptor phosphorylation but normal arrestin-mediated desensitization (Kim et al., 2004). This result suggested that primary phosphorylation of the specific sites on the C-tail of the D1 dopamine receptor is required for secondary phosphorylation of the residues within ICL3, which leads to receptor desensitization. This type of hierarchical phosphorylation has also been described for the A3 adenosine receptor (Palmer and Stiles, 2000) and the δ-opioid receptor (Kouhen et al., 2000), suggesting that it is a general phenomenon.

Taken together, these findings indicate that agonists promote GPCR phosphorylation at multiple sites, including the C-tail, the ICL3, and the first and second intracellular loops. The phosphorylation of the receptor is highly heterologous and dynamic, and this can generate different phosphorylation patterns at distinct cellular locations and specific time points after agonist stimulation, thereby providing a physical basis for a phosphorylation barcode hypothesis.

Diverse Signaling Regulated by Phosphorylation of GPCRs: A Phospho-Barcode Hypothesis

By binding to phosphorylated receptors, arrestins regulate the desensitization and internalization of most GPCRs and redirect signaling to numerous G protein-independent pathways. Many receptors share similar signaling pathways, such as arrestin-mediated ERK1/2 or SRC signaling (Luttrel et al., 1999; Ahn et al., 2004; Wang et al., 2014; Ning et al., 2015; Yang et al., 2015). Given this similarity, it is possible that the coupling of arrestins to activated and phosphorylated receptors is nonspecific and leads to the same signaling downstream of different receptors. However, studies have shown that different GRKs regulate distinct functions of GPCRs. For instance, research on the AT1aR and V2 vasopressin receptor (V2R) showed that GRK2 and GRK3 are indispensable for agonist-dependent β-arrestin recruitment and receptor desensitization, whereas GRK5 and GRK6 are primarily responsible for β-arrestin-2-mediated ERK1/2 signaling (Kim et al., 2005; Ren et al., 2005). These findings have raised the question of whether these distinct functional outcomes are generated by signaling arising from different receptor phosphorylation events or by the activity of these GRKs toward other substrates. Therefore, an elegant study was subsequently performed to investigate the phospho-coding of the β2AR using mass spectrometry and cellular approaches (Nobles et al., 2011).

In β2AR-expressing human embryonic kidney 293 (HEK293) cells, GRK6-dependent phosphorylation promotes β-arrestin-2-mediated ERK1/2 signaling, whereas GRK2-dependent phosphorylation functions in the opposite manner yet plays a more important role in receptor internalization. Consistent with these findings, a full agonist of β2AR stimulates robust phosphorylation at both GRK2 and GRK6 sites, whereas a β-arrestin-biased ligand is able to initiate β2AR phosphorylation only at GRK6 sites. Moreover, bioluminescence resonance energy transfer (BRET) analysis has shown that distinct phosphorylation patterns of β2AR induced by GRK2 or GRK6 are correlated with different β-arrestin-2 conformations (Nobles et al., 2011).

Similarly, different GRK subtypes also encode specific functional capabilities and conformational changes of β-arrestin-1, as shown by our recent studies. Our data indicate that by interacting with β-arrestin-1, the GRK2-induced β2AR phosphorylation pattern selectively recruits clathrin, whereas the GRK-6-regulated phosphorylation pattern selectively activates SRC (Yang et al., 2015). Collectively, these studies provide evidence that different GRKs phosphorylate distinct sites on the receptors and thereby establish a phosphorylation barcode, which in turn affects the conformation of the recruited β-arrestins by changing the topology of the intracellular face of the receptor, which further dictates β-arrestin-related cellular functional outcomes (Fig. 1).

The barcode hypothesis has been supported by numerous data from studies of different types of GPCRs, including C-C motif chemokine receptor 7 (Zidar et al., 2009), the free fatty acid receptor G-protein coupled receptor 120 (GPR120) (Prihandoko et al., 2016), and the ghrelin receptor (Bouzo-Lorenzo et al., 2016). For example, the phosphorylation of five residues that are clustered in two separable regions of the C-tail of GPR120 is pivotal for β-arrestin-2 recruitment (Butcher et al., 2014). The phosphorylation of residues within cluster 1 (Thr347, Thr349, and Ser350) is indispensable for Akt activation, but the phosphorylation of residues within cluster 2 (Ser357 and Ser361) is specifically responsible for arrestin-mediated receptor internalization (Prihandoko et al., 2016). Moreover, studies of subtypes of another multifunctional therapeutic target, the orexin receptor, have demonstrated that phosphorylation of an additional serine/threonine cluster in the C-tail of the orexin-2 receptor establishes a phospo-barcode that is different from that of the orexin-1 receptor, enabling the orexin-2 receptor to form more stable complexes with β-arrestin and ubiquitin (Dalrymple et al., 2011; Jaeger et al., 2014).

With all the increasing evidence, most of it derived from in vitro studies, supporting the barcode hypothesis, one outstanding question has been how the phosphorylation barcode contributes to the physiologic responses of GPCRs. Recently, progress has been made toward answering this question via a series of studies on M3-mAChR. By combining phosphopeptide mapping, mass spectrometry, and phospho-specific
antibodies, Tobin and colleagues showed that M3-mAChR is differentially phosphorylated in three cell lines, including Chinese hamster ovary (CHO) cells transfected with M3-mAChR, mouse insulinoma (MIN6) cells, and cerebellar granule neurons that endogenously express M3-mAChR (Torrecilla et al., 2007; Butcher et al., 2011). Accordingly, different phosphorylation patterns of three serine residues, two of which (Ser384 and Ser412) are within ICL3 and another (Ser577) is at the C-terminus of M3-mAChR, were revealed in the central nervous system, pancreas, and salivary glands of the mouse. Moreover, the phosphorylation profiles at these residues of M3-mAChR, especially at Ser577, were different in response to full or partial agonists administration (Butcher et al., 2011). The kinases that contribute to different phosphorylation patterns and the potential role of arrestins in these processes remain elusive; however, these findings provide compelling evidence that the phosphorylation status of M3-mAChR is ligand dependent and both cell type and tissue specific, thus suggesting a substantial correlation between the phospho-barcode and the functional outcomes of receptor in different physiologic contexts.

To understand the physiologic relevance of receptor phosphorylation, a series of studies was performed using transgenic knock-in mice. Removal of the phosphorylatable sites on M3-mAChR by mutation resulted in the abolition of arrestin recruitment and arrestin-mediated receptor internalization but had little effect on Gq-dependent signaling pathways in terms of PKC activation and calcium mobilization. Intriguingly, compared with the normal mice, the transgenic mice carrying the phospho-deficient M3-mAChR mutant displayed significant deficiencies in pancreatic insulin secretion (Kong et al., 2010), hippocampal learning and memory (Poulin et al., 2010), and bronchoconstriction regulation (Bradley et al., 2016). In contrast, the transgenic mice behaved normally in terms of M3-mAChR-mediated salivary secretion and weight gain (Bradley et al., 2016), indicating that these physiologic responses are independent of M3-mAChR phosphorylation.

Collectively, these data provide primary insight into the physiologic roles of receptor phosphorylation. However, more animal models that harbor mutants for specific phosphorylation sites of target receptors are required to better understand the significance of barcode hypothesis in vivo and to further link distinct phosphorylation patterns of GPCRs to different physiologic functions. Taken together, these novel findings indicate that the different phosphorylation patterns of GPCRs that might be generated by different kinases, potentially resembling a barcode in the intracellular regions of the receptor, could transduce specific information and dictate distinct functional outcomes (Fig. 1).

However, despite these developments and breakthroughs, it should be noted that the phosphorylation barcode hypothesis is not supported by all studies. For example, a cytoplasmic tail truncation mutant of AT1aR that cannot be phosphorylated by either GRK or PKC was shown to recruit β-arrestin, albeit in a weaker manner than the wild-type receptor. Strikingly, phosphorylation-deficient AT1aR elicits normal ERK signaling upon agonist stimulation (DeWire et al., 2007). Similarly, for some other GPCRs, such as luteinizing hormone receptor and the D6 chemokine receptor, phosphorylation is not necessarily required for arrestin recruitment (Min and Ascoli, 2000; Galliera et al., 2004). Intriguingly, studies have also suggested that negatively charged amino acids located

![Fig. 1. A model of receptor phospho-barcoding. Most GPCRs are phosphorylated by different kinases at multiple sites upon ligand stimulation, resulting in the recruitment of arrestins, which mediate receptor desensitization and internalization. Different GPCR phosphorylation patterns encoded by different GRKs transduce specific information to arrestins to dictate distinct functional outcomes. Whereas the GRK2-mediated phosphorylation pattern specifically recruits clathrin, the GRK6-mediated phosphorylation pattern selectively activates SRC or ERK1/2. The interactions between arrestin and other effectors, such as phosphodiesterase (PDE) and diacylglycerol kinase (DGK), are potentially regulated by different phospho-barcodes.](image-url)
in the ICL3 or C-tail might function as phosphate mimics in these nonphosphorylated receptors and thereby interact with arrestins, contributing to GPCR regulation (Mukherjee et al., 2002; Galliera et al., 2004; Gurevich and Gurevich, 2006; Tobin, 2008).

Nevertheless, considering the relatively low sequence homology, particularly in the intracellular loops and C-terminal domains, among different receptors, it is still difficult to understand how the phospho-barcode selectively directs different arrestin functions. To provide further mechanistic insight, a detailed structural analysis of the interaction between phosphorylated receptors and arrestins is urgently needed.

**Structural Basis of GPCR Phospho-Barcoding: A Flute Model**

Mammalian genomes encode 16 Ga, 5 Gb, and 12 Gγ subunits (Downes and Gautam, 1999; Khan et al., 2013). In contrast, there are only two β-arrestin isoforms that are ubiquitously distributed. It is therefore unclear how arrestins decipher the phosphorylation barcode and regulate numerous GPCR functions by selectively interacting with a large repertoire of downstream signaling molecules. Early in vitro studies using limited tryptic proteinolysis and mass spectrometry analysis revealed that upon binding to a phospho-peptide derived from the C-tail of V2R, both subtypes of the β-arrestins undergo significant conformational changes (Xiao et al., 2004; Nobles et al., 2007). The C terminus of β-arrestin, which harbors the clathrin-binding site and is primarily responsible for receptor endocytosis, was observed to be exposed upon activation. Moreover, a subtle difference in conformational changes was observed between the two subtypes of β-arrestins, predominantly located in the connecting region between the N and C domains.

It was then hypothesized that arrestins are able to adopt multiple conformations, which connect to different signaling pathways (Gurevich and Gurevich, 2006). Subsequently, structural changes in β-arrestin-2 were detected using an intramolecular BRET-based biosensor upon stimulation of AT1AR, β2AR, or parathyroid hormone 1 receptor (PTHR1) by agonists (Shukla et al., 2008). Further evidence supporting this notion came from a recent study using an improved BRET sensor with a panel of intramolecular fluorescein arsenical hairpins (FLaSH) inserted in specific loops of β-arrestin-2, revealing that different β-arrestin-2 conformational changes generated distinct “conformational signatures” correlated with different downstream functions (Lee et al., 2016).

The crystal structures of V2R phospho-peptide-bound β-arrestin-1 and a fusion complex of constitutively active rhodospin bound to a preactivated visual arrestin were recently determined (Shukla et al., 2013; Kang et al., 2015). In comparison with the inactive conformation, the activated arrestin molecule undergoes displacement of its C terminus from the N domain and approximately 20° twisting between its N and C domains that repositions several important loops, including the finger, middle, and lariat loops, and thereby allows interactions with the seven-transmembrane core of the GPCR. Importantly, the interaction between β-arrestin-1 and β2V2R (modified β2AR with its C terminus replaced by that of V2R) was recently visualized by negative stain electron microscopy, which has substantially improved our understanding of GPCR-arrestin interaction (Shukla et al., 2014).

Collectively, these studies suggest that β-arrestin first interacts with the phosphorylated C-tail of the activated receptor via its N domain and subsequently forms tighter engagement with the transmembrane core of the receptor. These marked conformational changes of arrestin and the biphasic mechanism of GPCR-β-arrestin interaction shed the first light on structural information during arrestin activation. However, the crystal structure represents a static profile of only a single activated arrestin molecule, limiting any in-depth analysis of the structural changes of arrestin that correlate with differential signaling. Therefore, the detailed mechanism by which arrestins precisely transmit the phospho-coding information to downstream effectors remains to be elucidated.

To better characterize the conformational changes in arrestin and to uncover the phospho-barcoding mechanism for selective signaling, we incorporated 19F-NMR probes at seven potential phosphate-binding pockets to sense negatively charged interactions and at seven other sites to monitor the conformational changes in β-arrestin-1 by using unnatural amino acid 3,5-difluorotyrosine (F2Y) incorporation (Yang et al., 2015). We showed that β-arrestin-1 interacts with different types of phospho-peptides, which were synthesized to mimic different phospho-barcodes corresponding to the C terminus of β2AR phosphorylated by GRK2, GRK6, or PKA, through different phospho-interaction patterns correlated with selective functional outcomes. Intriguingly, whereas GRK2-phosphopeptides (GRK2pp) bind to β-arrestin-1 in a 1-4-6-7 pattern and mediate the clathrin interaction,
GRK6-phosphopeptides (GRK6pp) interact with β-arrestin-1 in a 1:5 pattern and specifically elicit SRC signaling.

Moreover, analyses of \(^{19}F\)-NMR spectra at specific β-arrestin-1 sites other than phospho-interaction regions enabled us to show that phosphate binding to specific sites generates different conformations of arrestin at remote locations. For example, the binding of GRK2pp induces a specific conformational change of β-arrestin-1 at Y249 and L338 located in different loops. These structural states were specifically recognized by the subsequent binding of clathrin. Conversely, the binding of GRK6pp to β-arrestin-1 elicits a unique structural alteration at F277 located in the lariat loop. Deletion of the partial lariat loop encompassing F277 abolishes the GRK6pp-induced recruitment of SRC.

These findings collectively support a potential receptor phospho-coding mechanism by which changes in the GPCR phosphorylation pattern induced by specific ligand stimulation events are recognized by an array of phosphate-binding pockets located in the N-terminal region of arrestin. These changes are subsequently translated to distinct conformations of arrestin that could be recognized by different downstream effector molecules.

To further examine whether the identified arrestin-involved phospho-coding mechanism could be generally applied to many GPCR members, we performed alanine substitution of specific phosphate-binding sites residues in β-arrestin-1 and tested their functionalities upon interactions with different GPCRs, including β2AR, cholecystokinin type-A receptor (CCKAR), and somatostatin receptor type 2 (SSTR2). In accordance with the in vitro data, phosphate-binding site 4 was important for the β-arrestin-1/clathrin association, whereas site 5 was essential for the β-arrestin-1/SRC interaction, indicating the generality of the phospho-decision mechanism.

Taken together, as shown in Fig. 2, our results can be summarized as the flute model, in which phosphate-binding sites along the N terminus of β-arrestin-1 resemble the holes in a flute. Different phosphorylation patterns of the receptor function as “fingers” and instruct the conformational changes within β-arrestin-1, resulting in different “melodies” that are correlated with distinct downstream signaling. At least 10 potential phosphate-binding sites in β-arrestin-1 have been identified, which could potentially produce more than 1000 different sequence combinations \((2^{10} - 1 = 1023)\) in a single arrestin molecule. In theory, these binding patterns are able to generate enough conformations of arrestin to facilitate its numerous downstream signaling events, thereby contributing to the divergent functionalities of the more than 800 known human GPCRs.

The barcode hypothesis arose from the observation that GPCRs are heterogeneously phosphorylated at multiple sites, which correlate with different cellular contexts and functions. It provides a theoretical basis for understanding how a single receptor could engage multiple distinct functions through arrestin. The dissection of the underlying mechanism of the barcode hypothesis led us to generate the flute model, a detailed illustration of and powerful complement to the barcode hypothesis. For the first time, this model reveals the mechanism by which the phospho-barcode on the ligand-occupied GPCR is precisely recognized by arrestin N-terminal phosphate-binding concave and turned into functional selective arrestin conformations that are correlated with distinct signaling pathways. According to the barcode hypothesis, a “biased” ligand for a GPCR modulates specific phosphorylation patterns and might activate only a selective set of signaling pathways in contrast to a “balanced” ligand, which nonselectively activates both G protein-mediated and phosphorylation/arrestin-regulated physiologic responses (Luttrell, 2014; Wisler et al., 2014). Therefore, the barcode hypothesis has great potential in the development of therapeutic compounds that activate pathways that mediate beneficial effects over those initiating adverse responses. The newly proposed flute model provides mechanistic insight into this biased agonism in terms of arrestin conformational signaling and should further contribute to barcode-based novel drug design.

**Conclusion**

GPCR phosphorylation plays an important role in regulating GPCR function. The phospho-barcode concept developed over the past decade explains the multidimensional nature of the signaling network downstream of GPCRs and provides a potential mechanism by which GPCR functions are regulated through their interaction with arrestins. The flute model for phospho-barcodng further expands our knowledge, and preliminary data reveal that arrests precisely recognize and transduce the phospho-message from the receptor by reading the message through its N-terminal phosphate-binding concave and generating specific conformations to recruit multiple effector molecules. The phospho-barcoding mechanism might function together with ligand-induced receptor core conformations, dictating arrestin-mediated signaling networks.

Because the phosphorylation patterns of a given receptor are ligand-specific, the phospho-barcoding machinery has great potential to be used in pharmaceutical development. These novel drugs are expected to selectively elicit signaling and therefore have enhanced therapeutic potentials.

However, despite accumulating in vitro evidence supporting the phospho-barcode, additional in vivo studies are required to further link the phosphorylation patterns of receptors to specific physiologic responses. Moreover, with more than 220 downstream effectors, the molecular mechanism underlying arrestin conformational signaling remains to be deciphered. More explicit structural information on receptor-arrestin-effector complexes, which might be disclosed by crystallography, electron microscopy, or NMR, is highly desirable.

**Authorship Contributions**

**Participated in research design:** Z. Yang, F. Yang, Yu, Sun.

**Wrote or contributed to the writing of manuscript:** Z. Yang, F. Yang, Zhang, Z. Liu, Lin, C. Liu, Xiao, Yu, Sun.

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