Phosphorylation of G protein-coupled receptors: from the barcode hypothesis to the flute model

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Running title: Phosphorylation barcoding of the GPCR

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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 (GRKs) 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 signalling to G protein-independent pathways via interactions with numerous downstream effector molecules. Accumulating evidence over the past decade has given rise to the phospho-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 (\textsuperscript{19}F-NMR) spectroscopy, led to the flute model, which provides preliminary insight into the receptor phospho-coding 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 phospho-barcoding 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) compromise the largest known membrane protein family encoded by the human genome, and GPCRs regulate almost all known physiological processes in humans by converting a broad range of extracellular stimuli (ranging from light to hormones and neurotransmitters) to intracellular signals (Dohlman, 2015; Manglik and Kobilka, 2014; Ritter and Hall, 2009; Wisler et al., 2014). 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 (Bradley et al., 2016; Budd et al., 2000; Busillo et al., 2010; Jones et al., 2007). 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 PKC) and -independent kinases (i.e., GPCR kinases or GRKs) (Lefkowitz, 1998). As a classical 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 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 inhibit G protein coupling, initiating homologous desensitization (Gurevich et al., 2012; Pitcher et al., 1992; Tobin et al., 2008).

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 Gs protein but also switches this coupling to the Gi 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 signalling. Four isoforms of arrestin have been identified: arrestin-1 and -4 are restricted to the visual system and accordingly named ‘visual arrestins’ (Craft et al., 1994; Wilden et al., 1986), whereas the other two isoforms, β-arrestin-1 and -2, are ubiquitously distributed (Attramadal et al., 1992; Lohse et al., 1990). Arrestin binding facilitates the internalization of non-visual 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 signalling via interactions with a growing list of signalling molecules, such as SRC, Raf-1, Akt, ERK1/2, PDE, and DGKs (Barki-Harrington and Rockman, 2008; Dong et al., 2016; Kumari et al., 2016; Luttrell et al., 1999; Ning et al., 2015; Reiter et al., 2012; Wang et al., 2014; Xiao et al., 2010). In particular, activation of a single receptor, such as angiotensin II receptor (AT1aR), recruits more than 220 signalling proteins to the receptor/arrestin complexes (Xiao et al., 2010). Given the potential multiple phosphorylation sites located on GPCRs, the possibility of a mechanism that mediates the phosphorylation pattern of the receptor such that specific signalling molecules are recruited through arrestin is intriguing.

In 2011, two seminal studies brought the barcode hypothesis of GPCR phosphorylation to light by studying two prototypic GPCRs, β2AR and M3-muscarinic acetylcholine receptor (M3-mACHR) (Butcher et al., 2011; Nobles et al., 2011). Whereas distinct phosphorylation patterns of the β2AR induced by different GRKs are correlated with different cellular functions (Nobles et al., 2011), M3-mACHR is differentially phosphorylated in various cells and tissues, supporting a role for receptor phosphorylation in directing physiologically relevant receptor signalling (Butcher et al., 2011). Therefore, the phosphorylation of distinct sites on the GPCRs may constitute a barcode
that dictates the downstream signalling outcomes of the receptor. Because different ligands that
bind to a single receptor might induce distinct patterns of receptor phosphorylation, the pleiotropic
functionalities and therapeutic importance of ligand-specific, phosphorylation/arrestin-dependent
signalling have been emerging (Jean-Charles et al., 2016; Latorraca et al., 2016; Peterhans et al.,
2016; Smith and Rajagopal, 2016; Xiao and Liu, 2016). However, since there is little primary
phosphorylation pattern identity among different receptors, the mechanism by which the phosho-
barcode is recognized and then converted to specific signalling remains largely unknown, although
many of the GPCRs share similar signalling pathways, such as arrestin-mediated ERK or SRC
signalling.

Recently, using a newly developed unnatural amino acid incorporation technique
combined with fluorine-19 nuclear magnetic resonance (^{19}\text{F}-\text{NMR}) spectroscopy (Neumann-
Staubitz and Neumann, 2016), we showed that the phosphorylation barcode of the receptor is
specifically recognized by the N-terminal half of arrestin (Yang et al., 2015). The 10 phosphate-
binding sites located at the N-domain of arrestin act as sensors on the phosphorylated receptor C-
terminal tail, which in theory, enables more than 1000 specific arrestin conformations for
downstream signalling outcomes. Given this recent progress, we will review the current
knowledge of GPCR phosphorylation, summarize the recent studies that support the barcode
hypothesis, and highlight the emerging structural mechanism of GPCR phospho-coding.

**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) (Kuhn and Dreyer,
A similar observation was identified in the β2AR system, where the deactivation of agonist-occupied β2AR was found to be tightly associated with receptor phosphorylation by a β-adrenergic receptor kinase (βARK or GRK2) (Benovic et al., 1986; Stadel et al., 1983). These findings identified the pivotal role of phosphorylation in the desensitization mechanism of GPCRs. Since then, 7 isoforms of GRKs, referred to as GRKs 1-7, have been identified (Pitcher et al., 1998). GRKs 2, 3, 5, and 6 are ubiquitously expressed and serve as important determinants of phosphorylation patterns in most non-visual system GPCRs (Butcher et al., 2012; Krupnick and Benovic, 1998). In addition to GRKs, GPCR can also be phosphorylated by second messenger-regulated kinases and some other kinases (Benovic et al., 1985; Kelly et al., 2008; Pitcher et al., 1992; Tobin, 2008). For example, casein kinase-induced phosphorylation plays important roles for M3-mAChR and thyrotropin-releasing hormone receptor, whereas CaMKII-induced phosphorylation has been identified in the dopamine receptor D3, GABA_B receptor, and μ-opioid receptor (Table 1 and Table 2) (Budd et al., 2001; Chen et al., 2013; Guetg et al., 2010; Hanyaloglu et al., 2001; Liu et al., 2009; Torrecilla et al., 2007). These kinases are important 'composers' of receptor phosphorylation patterns and, therefore, specifically regulate receptor functions.

The very first mutagenesis analyses of rhodopsin and β2AR determined that these receptors are phosphorylated at multiple sites (Bouvier et al., 1988; Wilden and Kuhn, 1982). 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 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, 3 serines and 2 threonines at the C-tail of the ghrelin receptor and 14 serines and 2 threonines at the C-tail and on the third intracellular loop (ICL3) of the M3-mAChR were identified as phosphorylation sites (Bouzo-Lorenzo et al., 2016; Butcher et al., 2011). 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) (Celver et al., 2001; Nakamura et al., 1998; 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 rhosopsin permits the phosphorylation of the other residues, and this has also been observed in several other GPCRs (Ohguro et al., 1993). Studies of the phosphorylation profile of the D1 dopamine receptor (DRD1) 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 DRD1 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 promoted 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 signalling regulated by phosphorylation of GPCRs: a phoshpo-barcode hypothesis

By binding to phosphorylated receptors, arrestins regulate the desensitization and internalization of most GPCRs and redirect signalling to numerous G protein-independent pathways. Many receptors share similar signalling pathways, such as arrestin-mediated ERK1/2 or SRC signalling (Ahn et al., 2004; Luttrell et al., 1999; Ning et al., 2015; Wang et al., 2014; 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 signalling 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 signalling (Kim et al., 2005; Ren et al., 2005). These findings raised the question of whether these distinct functional outcomes are generated by signalling arising from different receptor phosphorylation events or by the activity of these GRKs towards 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 HEK293 cells, GRK6-dependent phosphorylation promotes β-arrestin-2-mediated ERK1/2 signalling, whereas GRK2-dependent phosphorylation functions in the opposite manner yet plays...
more important roles 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 showed 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 indicated that by interacting with β-arresin-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 and 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 CCR7 (Zidar et al., 2009), the free fatty acid receptor 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). Whereas the phosphorylation of residues within cluster 1 (Thr347, Thr349 and Ser350) is indispensable for Akt activation, 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 multi-functional therapeutic target, the orexin receptor, demonstrated that phosphorylation of an additional serine/threonine cluster in the C-tail of the orexin-2 receptor establishes a phospho-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 increasing evidence, most of which is derived from *in vitro* studies, supporting the barcode hypothesis, one outstanding question is how the phosphorylation barcode contributes to the physiological responses of GPCRs. Recently, progresses have been made towards answering this question in 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 CHO cells transfected with M3-mAChR, mouse insulinoma (MIN6) cells, and cerebellar granule neurons that endogenously express M3-mAChR (Butcher et al., 2011; Torrecilla et al., 2007). 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 roles 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 physiological contexts.
To understand the physiological 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 G_q-dependent signalling 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 physiological responses are independent of M3-mAChR phosphorylation. Collectively, these data provide primary insight into the physiological roles of receptor phosphorylation. However, more animal models that harbour 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 physiological functions.

Taken together, these novel findings indicate that 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 signalling 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 (Galliera et al., 2004; Min and Ascoli, 2000). Intriguingly, studies have also suggested that negatively charged amino acids located in the ICL3 or C-tail might function as phosphate mimics in these non-phosphorylated receptors and thereby interact with arrestins, contributing to GPCR regulation (Galliera et al., 2004; Gurevich and Gurevich, 2006; Mukherjee et al., 2002; 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 Gα, 5 Gβ, 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 signalling molecules. Early *in vitro* studies using limited tryptic proteolysis and mass spectrometry analysis revealed that upon binding to a phosphopeptide derived from the C-tail of VzR, both subtypes of the β-arrestins undergo significant conformational changes (Nobles et al., 2007; Xiao et al., 2004). The C-terminus of β-arrestin, which harbours 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 locating 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 signalling 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 PTH1R 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 phosphopeptide-bound β-arrestin-1 and a fusion complex of constitutively active rhodopsin bound to a pre-activated visual arrestin were recently determined (Kang et al., 2015; Shukla et al., 2013). In comparison to the inactive conformation, the activated arrestin molecule undergoes displacement of its C-terminus from the N-domain and an 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 firstly interact 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 signalling. 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 signalling, we incorporated 19F-NMR probes at 7 potential phosphate-binding pockets to sense negatively charged interactions and at 7 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 phosphopeptides, which were synthesized to mimic different phospho-barcodes corresponding to the C-terminus of β2AR phosphorylated by GRK2, GRK6, or PKA, through different phospho-interactions 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 signalling. Moreover, analyses of 19F-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 phosho-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 phosho-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 phosho-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 signalling. At least ten potential phosphate-binding sites in β-arrestin-1 have been identified, which could potentially produce more than 1,000 different sequence combinations (2^{10} – 1=1,023) in a single arrestin molecule. In theory, these binding patterns are able to generate enough conformations of arrestin to facilitate its numerous downstream signalling 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 signalling pathways. According to the barcode hypothesis, a ‘biased’ ligand for a GPCR modulates specific phosphorylation patterns and might activate only a selective set of signalling pathways in contrast to a ‘balanced’ ligand, which non-selectively activates both G protein-mediated and phosphorylation/arrestin-regulated physiological 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 signalling and should further contribute to barcode-based novel drug design.

Conclusions

GPCR phosphorylation plays important roles in regulating GPCR function. The phospho-barcode concept developed over the past decade explains the multidimensional nature of the signalling 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-barcording further expands our knowledge and preliminary data reveal that arrestins precisely
recognize and transduce the phospho-message from the receptor by reading the message through its N-terminal phospho-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 signalling networks. Since the phosphorylation patterns of a given receptor are ligand-specific, the phospho-barcoding machinery has great potential to be utilized in pharmaceutical development. These novel drugs are expected to selectively elicit signalling 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 physiological responses. Moreover, with more than 220 downstream effectors, the molecular mechanism underlying arrestin conformational signalling 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


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

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Footnotes

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Zhao Yang and Fan Yang contributed equally to this work.

The authors declare no conflict of interest.
Figure Legends

**Figure 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.

**Figure 2 The flute model for the phospho-barcoding mechanism of arrestin signalling.** The 10 phosphate-binding sites located in the N-terminal region of arrestin function as sensors of the phospho-message in the receptor C-tail or the intracellular loops. A phospho-barcode of 1-4-6-7 directs clathrin recruitment and the endocytic function of arrestin, whereas a phospho-barcode of 1-5 directs SRC recruitment and signalling. There are theoretically more than 1000 phospho-patterns that produce many arrestin conformations, dictating numerous downstream effector interactions.
**Table 1.** Examples of receptor phosphorylation at C-terminus and corresponding functions

<table>
<thead>
<tr>
<th>GPCR</th>
<th>Phosphorylation sites</th>
<th>Kinase</th>
<th>Method</th>
<th>Cell type</th>
<th>Function</th>
<th>References</th>
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<tr>
<td>Angiotensin II receptor type I (AT1αR)</td>
<td>T332, S335, T336, S338</td>
<td>PKC/GRK</td>
<td>Mut</td>
<td>CHO</td>
<td>Internalization</td>
<td>(Thomas et al., 1998)</td>
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<td></td>
<td>S331, S338, S348</td>
<td>PKC</td>
<td>Mut</td>
<td>CHO</td>
<td>ND</td>
<td>(Qian et al., 1999)</td>
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<td></td>
<td>S345, S346</td>
<td>PKA</td>
<td>Mut</td>
<td>Mouse L cells</td>
<td>Desensitization</td>
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<tr>
<td></td>
<td>S355, S356, S364</td>
<td>GRK</td>
<td>Mut</td>
<td>HEK293</td>
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<td>MS and P-SA</td>
<td>HEK293</td>
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<td>(Nobles et al., 2011)</td>
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<td></td>
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<td>GRK2</td>
<td>MS and P-SA</td>
<td>HEK293</td>
<td>In vitro β-arrestin-1 mediated SRC activation and internalization</td>
<td>(Yang et al., 2015)</td>
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<tr>
<td></td>
<td>T384, S396, S401, and S407</td>
<td>GRK2</td>
<td>PAA</td>
<td>In vitro</td>
<td>ND</td>
<td>(Fredericks et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>T384, T393, S396, S401, S407, S411</td>
<td>GRK5</td>
<td>PAA</td>
<td>In vitro</td>
<td>ND</td>
<td>(Fredericks et al., 1996)</td>
</tr>
<tr>
<td>CXC hemokine receptor type 4 (CXCR-4)</td>
<td>S324, S325</td>
<td>PKC/GRK6</td>
<td>MS and P-SA</td>
<td>HEK293</td>
<td>Receptor degradation</td>
<td>(Busillo et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>S330/339, cluster S346-S352</td>
<td>GRK2/6</td>
<td>MS and P-SA</td>
<td>HEK293</td>
<td>β-arrestin-2 recruitment and calcium mobilization</td>
<td>(Busillo et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>S330/339, cluster S346-S352</td>
<td>GRK3/6</td>
<td>MS and P-SA</td>
<td>HEK293</td>
<td>β-arrestin-1 mediated ERK1/2 activation</td>
<td>(Busillo et al., 2010)</td>
</tr>
<tr>
<td>Dopamine receptor D1 (DRD1)</td>
<td>T347</td>
<td>ND</td>
<td>Mut</td>
<td>HEK293</td>
<td>Promote secondary phosphorylation of residues within the ICL3</td>
<td>(Kim et al., 2004)</td>
</tr>
</tbody>
</table>
| Follicle-stimulating hormone receptor (FSHR) | Cluster T638-T644 | GRK2 | Mut | HEK293 | β-arrestin-mediated desensitization and internalization | (Kara et al., 2006)
|---|---|---|---|---|---|---|
| S783 | AMPK | MS and EDA | Rat hippocampal neurons | Receptor recycling | (Terunuma et al., 2010)
| S867 | CaMKII | MS | Rat hippocampal neurons | Internalization | (Guetg et al., 2010)
| S892 | PKA | Mut | HEK293 | Internalization | (Couve et al., 2002)
| Ghrelin receptor type 1a (GHSR1a) | S349, T350 | ND | MS | HEK293 | Stabilization of the receptor-β-arrestin1/2 complex and binding and internalization | (Bouzo-Lorenzo et al., 2016)
| | S362, S363, T366 | ND | MS | HEK293 | | (Bouzo-Lorenzo et al., 2016)
| G protein-coupled receptor 120 (GPR120) | T347, S350, S357 | PKC/GRK6 | Mut | HEK293 | β-arrestin-2 recruitment and calcium mobilization | (Burns et al., 2014)
| | T347, T349, S350 | ND | MS | CHO Flp-In™ cells | β-arrestin-2 recruitment and Akt activation | (Prihandoko et al., 2016)
| | S357, S361 | ND | MS | CHO Flp-In™ cells | β-arrestin-2 recruitment and internalization | (Prihandoko et al., 2016)
| μ-opioid receptor (MOR) | S356, S357, S363 | PKC | MS | HEK293 | Desensitization | (Chen et al., 2013; Wang et al., 2002)
| | T370 | CaMKII | MS | HEK293 | ND | (Chen et al., 2013)
<table>
<thead>
<tr>
<th>Source</th>
<th>Target</th>
<th>Modification</th>
<th>Cell Line</th>
<th>Assay</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Thyrotropin-releasing hormone receptor (TRHR)</td>
<td>S355, S360, S364, T365</td>
<td>GRK2</td>
<td>CHO</td>
<td>β-arrestin-2 recruitment and desensitization</td>
<td>(Chen et al., 2013; Lowe et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>T365, T371, S383</td>
<td>CK2</td>
<td>COS-1</td>
<td>β-arrestin-2-mediated desensitization and internalization</td>
<td>(Jones et al., 2007)</td>
</tr>
<tr>
<td>Orexin-2 receptor (OX2R)</td>
<td>Cluster T399-S403</td>
<td>GRK</td>
<td>HEK293FT</td>
<td>ND</td>
<td>(Jaeger et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Cluster S406-T409, Cluster T427-T431</td>
<td>GRK</td>
<td>HEK293FT</td>
<td>Receptor-β-arrestin1/2-ubiquitin complex formation</td>
<td>(Jaeger et al., 2014)</td>
</tr>
</tbody>
</table>

Abbreviations: CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; CK2, casein kinase II; EDA, Edman degradation analysis; Mut, mutagenesis; MS, mass spectrometry; ND, not determined; P-SA, phospho-specific antibody; PAA, phospho-amino acid analysis.
Table 2. Examples of receptor phosphorylation on intracellular loops and corresponding functions

<table>
<thead>
<tr>
<th>GPCR</th>
<th>Phosphorylation sites</th>
<th>Region</th>
<th>Kinase</th>
<th>Method</th>
<th>Cell type</th>
<th>Function</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>β2 adrenergic receptor (β2AR)</td>
<td>S261, S262</td>
<td>ICL3</td>
<td>PKA</td>
<td>Mut</td>
<td>Mouse L cells</td>
<td>Desensitization</td>
<td>(Clark et al., 1989)</td>
</tr>
<tr>
<td>Dopamine receptor D1 (DRD1)</td>
<td>T268</td>
<td>ICL3</td>
<td>PKA</td>
<td>Mut</td>
<td>Rat C6 Glioma cells</td>
<td>Desensitization</td>
<td>(Jiang and Sibley, 1999)</td>
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<tr>
<td></td>
<td>S256, S258, S259</td>
<td>ND</td>
<td>Mut</td>
<td>HEK293T</td>
<td>β-arrestin-2 recruitment and desensitization</td>
<td>(Kim et al., 2004)</td>
<td></td>
</tr>
<tr>
<td>Dopamine receptor D2 (DRD2)</td>
<td>S228, S229, T352, T354, S355</td>
<td>ICL3</td>
<td>PKC</td>
<td>Mut</td>
<td>HEK293T</td>
<td>β-arrestin-1-dependent internalization</td>
<td>(Namkung and Sibley, 2004)</td>
</tr>
<tr>
<td></td>
<td>S285, S286, T287, S288, T293, S311, S317, S321</td>
<td>GRK2/3</td>
<td>Mut</td>
<td>HEK293T</td>
<td>Regulation of receptor trafficking and recycling</td>
<td>(Namkung et al., 2009)</td>
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</tr>
<tr>
<td>Dopamine receptor D3 (DRD3)</td>
<td>S229</td>
<td>CaMKII</td>
<td>Mut</td>
<td>HEK293</td>
<td>Inhibit ERK1/2 activation</td>
<td>(Liu et al., 2009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S229, S257</td>
<td>ICL3</td>
<td>PKC</td>
<td>Mut</td>
<td>HEK293</td>
<td>β-arrestin-independent desensitization and internalization</td>
<td>(Cho et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>GRK4</td>
<td>ND</td>
<td>HEK293</td>
<td>Human renal proximal tubule cells</td>
<td>ERK1/2 activation</td>
<td>(Villar et al., 2009)</td>
</tr>
<tr>
<td>Follicle-stimulating hormone receptor (FSHR)</td>
<td>T369, S371, T376</td>
<td>ICL1</td>
<td>ND</td>
<td>Mut</td>
<td>HEK293</td>
<td>Desensitization and internalization</td>
<td>(Nakamura et al., 1998)</td>
</tr>
<tr>
<td>Muscarinic acetylcholine receptor M2 (M2-mAChR)</td>
<td>Cluster S286-S290</td>
<td>ICL3</td>
<td>GRK2</td>
<td>Mut</td>
<td>HEK293T</td>
<td>Internalization</td>
<td>(Pals-Rylaarsdam and Hosey, 1997; Pals-Rylaarsdam et al., 1995)</td>
</tr>
</tbody>
</table>
|                                              | Cluster T307-S311     | GRK2   | Mut    | HEK293T| Desensitization and internalization | (Pals-Rylaarsdam and Hosey, 1997; Pals-
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cluster</th>
<th>Kinase</th>
<th>Mutagenesis</th>
<th>Cell Line</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>M3 mAChR</td>
<td>S331-S333, S348-S351</td>
<td>GRK2</td>
<td>Mut</td>
<td>COS7/CHO</td>
<td>Internalization</td>
</tr>
<tr>
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<td>Cluster S351-Asp356</td>
<td>ICL3</td>
<td>CK2</td>
<td>CPM</td>
<td>CHO</td>
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<tr>
<td></td>
<td>Cluster Lys370-S425</td>
<td>CK1(\alpha)</td>
<td>Mut</td>
<td>CHO</td>
<td>ERK1/2 activation</td>
</tr>
<tr>
<td>V2R</td>
<td>S255</td>
<td>ICL3</td>
<td>PKA</td>
<td>MS</td>
<td>HEK293</td>
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</tbody>
</table>

Abbreviations: CPM, chymotryptic phosphopeptide mapping; ICL, intracellular loop. See the footnotes to Table 1 for details of the other abbreviations.
**Figure 1**

![Diagram showing the signaling pathways involving various proteins and enzymes.](molpharm.aspetjournals.org/content/52/6/1444/F1.large.jpg)
Figure 2

Agonist

GPCR

Phospho-barcode

A music of the receptor

>1000 patterns

β-arrestin 1

Clathrin

SRC

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