Chapter 15
Arrestin-Dependent ERK Activation and Its Disruption

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Abstract Regulation of the ERK1/2 cascade is one of the most studied functions of arrestins and illustrates many of the features that enable them to function as GPCR-regulated scaffolds. While all four arrestins can bind the component kinases of the ERK cascade; c-Raf1, MEK1/2 and ERK1/2, their binding is dependent on arrestin conformation, such that inactive ERK1/2 can be sequestered by a microtubule-bound pool of arrestin, while activated ERK1/2 binds with high affinity only to the GPCR-bound arrestin conformation. The result is both a dampening of basal pathway activity, and the arrestin-dependent activation of a spatially and temporally constrained pool of ERK1/2 that differs in function from ERK1/2 activated by G protein-dependent mechanisms or classical receptor tyrosine kinase growth factor receptors. Arrestin-bound ERK1/2 performs numerous functions in cells, among them contributing to the regulation of GPCR internalization and trafficking, control of cell proliferation and non-proliferative cell growth, and regulation of cytoskeletal dynamics involved in cell migration and chemotaxis. The finding that arrestin binding of c-Raf1 and MEK1/2 can be disrupted by point mutations that eliminate its ability to activate ERK1/2 without disrupting its other functions indicates that the two major functions of arrestins, GPCR desensitization and signaling, are dissociable, and offers tools to probe arrestin's diverse cellular functions.

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Introduction

The sheer complexity of cell signaling has necessitated the evolution of scaffold proteins whose roles are to increase the efficiency of information transfer between successive enzymes in a signaling cascade; to enhance fidelity by dampening cross talk between parallel cascades; and to target effectors to specific subcellular locations. The arrestins, which were originally discovered for their role in G protein-coupled receptor (GPCR) desensitization and internalization, are now recognized to also serve as signaling scaffolds, linking GPCRs to numerous intracellular signaling pathways independent of heterotrimeric G protein signaling (Gurevich and Gurevich 2006; Luttrell and Gesty-Palmer 2010). Indeed, arrestins perform roles in GPCR signaling that are analogous to the functions of other scaffolds, such as insulin receptor substrate-1, which organizes proteins into signaling complexes around activated insulin receptors (White 1998).

The visual/β-arrestins[1] are semi-bisymmetric soluble proteins composed of β-strand sandwich structures organized into N- and C-domains, that form two concave baskets connected by a short hinge (Vishnivetskiy et al. 2002; Aubry et al. 2009). Despite their relatively small 48–50 kDa size, arrestins are uniquely suited to function as GPCR-regulated scaffolds. First, they have the ability to recognize activated GPCRs and translocate from the cytosol to the plasma membrane upon receptor activation. Second, they have the flexibility to bind multiple cargo proteins on the convex side of the N- and C-domains away from the sites of GPCR interaction, a property that allows them to recruit signaling proteins into GPCR-based 'signalsome' complexes. Third, they can exist in different intracellular pools wherein they adopt different conformations. This is critical to their scaffolding function in that many arrestin-bound signaling proteins exhibit a preference for the cytosolic, microtubule-bound, or GPCR-bound arrestin conformation. Thus, arrestin binding can constrain signaling proteins to one cellular compartment until an external GPCR-mediated stimulus prompts a conformational change that causes them to release some cargos and associate with others.

Among the first reported and most studied arrestin-mediated signals is activation of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) mitogen-activated protein (MAP) kinase cascade (DeFea et al. 2000; Luttrell et al. 2001). The ubiquitous MAP kinases play critical roles in cell cycle regulation/proliferation and survival/apoptotic signaling by controlling phosphorylation of nuclear transcription

[1]Various names of arrestin proteins are used: Arrestin-1 is also called S-antigen, 48 kDa protein, visual or rod arrestin; β-arrestin or β-arrestin1 is also called arrestin-2; β-arrestin2 is also called arrestin-3 and hTHY-ARRX, and arrestin-4 is also called cone or X-arrestin (its gene is called "arrestin 3" in the HUGO database).

factors, e.g. Elk1 and c-Jun, as well as diverse regulatory functions mediated through phosphorylation of cytosolic substrates (Davis 2000; Pearson et al. 2001; Kyriakis and Avruch 2012). Eukaryotic cells possess several different MAP kinases that fall into three groups; the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAP kinases. Each MAP kinase module consists of three component kinases; a MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK) that must phosphorylate one another in succession. Not surprisingly, MAP kinase regulation commonly involves scaffold proteins that aid in assembling the correct MAPKKK-MAPKK-MAPK sets and targeting activated MAP kinases to their relevant substrates (Burack and Shaw 2000). In this chapter, we discuss the roles that arrestins play in balancing G protein- and arrestin-dependent ERK1/2 activation, the mechanism of arrestin-dependent scaffolding of the ERK1/2 cascade, and how the assembly of GPCR-arrestin-ERK1/2 'signalsomes' control ERK1/2 function by activating a spatially and temporally distinct ERK1/2 pool.

G Protein- and Arrestin-Dependent ERK1/2 Regulation

Among its many functions, ERK1/2 is involved in phosphorylating nuclear transcription factors necessary for G0–G1 cell cycle transition and the passage of cells through mitosis or meiosis (Pearson et al. 2001). Not surprisingly then, it is subject to extensive convergent regulation, including Ras-dependent proliferative signals originating from GPCRs and receptor tyrosine kinases, protein kinase (PK)A and PKC mediated signals downstream of heterotrimeric G proteins, and signals transmitted via arrestin scaffolds (Luttrell and Miller 2013). While receptor and cell type variability is more the rule than the exception, it is clear that most heterotrimeric G protein families signal to ERK1/2. Stimulation of the Gq/11-phospholipase Cβ-PKC pathway can activate ERK1/2 through direct phosphorylation of the MAPKKK c-Raf1 by PKCα (Kolch et al. 1993; Hawes et al. 1995). The consequences of stimulating of cAMP production by Gs-adenylyl cyclase are complex and determined primarily by which Raf isoform, c-Raf1 or B-Raf, is predominantly expressed. Unlike PKC, phosphorylation of cRaf-1 by PKA inhibits its activation (Wu et al. 1993). As a result, activation of Gs-coupled receptors in some cells inhibits, rather than activates, ERK1/2 (Lefkowitz et al. 2002). On the other hand, B-Raf is activated both by PKA-dependent phosphorylation of the Ras-family GTPase, Rap-1 (Vossler et al. 1997) and by cAMP binding to the Rap-1 guanine nucleotide exchange factor, Epac (DeRooij et al. 1998), permitting direct cAMP mediated activation of a B-Raf-MEK1/2-ERK1/2 module. Pertussis toxin-sensitive G protein-dependent activation of ERK1/2 by Gi/o-coupled GPCRs is mediated primarily by Gβγ subunits and typically involves activation of receptor or non-receptor tyrosine kinases leading to Ras-dependent activation of c-Raf1 (van Biesen et al. 1995; Luttrell et al. 1996, 1997, 1999).

ERK1/2 activated by any of these mechanisms is free to translocate into the cell nucleus and regulate transcription by phosphorylating nuclear transcription factors.

Since they are first and foremost negative regulators of G protein signaling, arrestins are positioned to regulate the balance between G protein-mediated signals that activate ERK1/2 dependent transcription, and arrestin-dependent signals that direct the kinase to perform other functions. Because nuclear ERK1/2 is rapidly inactivated by dephosphorylation, the time course of ERK1/2 activation by G protein-regulated effectors parallels the level of heterotrimeric G protein activity, and arrestin-dependent GPCR desensitization limits the duration of G protein-mediated ERK1/2 activation. In β-arrestin1/2 null murine embryo fibroblasts, for example, ERK1/2 activation by lysophosphatidic acid (LPA) receptors results primarily from Gi/o-dependent transactivation of epidermal growth factor (EGF) receptors (Gesty-Palmer et al. 2005). Because LPA receptor desensitization is impaired in the absence of arrestins, the EGF receptor-dependent ERK1/2 signal is persistent, lasting for several hours in the continued presence of LPA. Reintroducing β-arrestin2, which restores desensitization, makes the transactivation-dependent signal transient, such that it contributes significantly to ERK1/2 activation only during the first few minutes of stimulation. But as G protein-dependent signaling wanes, the arrestin-dependent scaffolding of the c-Raf1-MEK1/2-ERK1/2 module provides an alternative mechanism for sustaining ERK1/2 activity.

Arrestin Scaffolding of the ERK1/2 Cascade

The arrestins have been reported to engage all three major MAP kinase modules, but with different effects in terms of the direction of regulation. This likely reflects the differing functions of MAP kinase modules, and a consistent role of arrestins to favor activation of growth/survival pathways and suppression of apoptotic signals (Gurevich and Gurevich 2013). All four visual/β-arrestins can bind the component kinases of the JNK cascade, ASK1-MKK4/7-JNK1/2/3 (McDonald et al. 2000; Song et al. 2009; Kook et al. 2013), but only β-arrestin2 is able to orient the kinases so as to support efficient phosphorylation (Seo et al. 2011; Zhan et al. 2011). Moreover, the JNK cascade binds tightly to the 'inactive' cytosolic arrestin conformation such that GPCR activation is neither required nor apparently involved (Song et al. 2006). The net result is that arrestin expression tends to suppress JNK signaling, either by sequestering pathway elements bound to β-arrestin1 in non-productive 'silent scaffolds', or in the case of β-arrestin2 keeping active JNK out of the nucleus and away from its transcription factor targets by virtue of its intrinsic Nuclear Export Signal (NES) (Scott et al. 2002; Breitman et al. 2012). In contrast, ERK1/2 binding to arrestins is dependent upon the conformational changes that occur upon receptor binding, and thus dependent upon GPCR stimulation.

Assembly of the Arrestin-Bound ERK Activation Complex

One obvious question is how relatively small globular proteins like arrestins can simultaneously engage the three kinases of the ERK1/2 module, each of which is roughly equal in size to the arrestin itself (Gurevich and Gurevich 2006). The earliest observation, obtained by co-immunoprecipitation of overexpressed pathway components from transfected cells, was that c-Raf1 and ERK2 bound independently to β-arrestin2, while binding of MEK1 was enhanced by the presence of either c-Raf1 or ERK2 in the complex (Luttrell et al. 2001). This suggested that MEK1 binding was at least in part indirect. Subsequent work, however, indicates that MEK1 can bind β-arrestin1 directly, both as immobilized peptides and in cells (Meng et al. 2009). In cells, all three kinases interact directly with either the isolated N- or C-terminal domains of β-arrestin1 and β-arrestin2 (Song et al. 2009), suggesting a broad interaction surface involving the cytoplasmic surface of both domains. The same applies to ASK1-MKK4-JNK3, the three components of the JNK3 cascade. ERK2 binding has even been reconstituted using pure proteins in vitro, where ERK2 directly binds free β-arrestin1 and β-arrestin 2, as well as receptor-associated visual arrestin, β-arrestin1 and β-arrestin2 (Coffa et al. 2011a).

Unlike JNK3, the binding of ERK2 to β-arrestins is enhanced by conformational changes that occur upon GPCR binding. Early reports suggested that GPCR stimulation promoted assembly of the complex (Luttrell et al. 2001). This is supported by comparisons of the binding of ERK pathway components to wild type 'cytosolic' β-arrestin versus either 'active' or 'inactive' β-arrestin mutants. Mutations that destabilize the polar core generate 'pre-activated' arrestins whose ability to recognize GPCRs is independent of receptor phosphorylation (Gurevich and Benovic 1997; Kovoor et al. 1999). Conversely, arrestin mutants with deletions in the hinge region that restrict interdomain flexibility exhibit reduced receptor affinity and enhanced microtubule binding (Vishnivetskiy et al. 2002; Hanson et al. 2007). In cells, ERK2 binds preferentially to the 'active' and 'microtubule-bound' β-arrestin conformations, while exhibiting virtually no binding to the 'inactive' cytosolic conformation (Coffa et al. 2011a). Like ERK2, c-Raf1 prefers the receptor-bound conformation, although the difference is less dramatic, while MEK1 binds equivalently to both free arrestin (Meng et al. 2009; Coffa et al. 2011b) and the mutationally constrained conformations (Coffa et al. 2011a).

Kinetics of Arrestin-Dependent ERK Activation

One property of visual/β-arrestins that contributes to their effectiveness as scaffolds is that they are relatively abundant in relation to the GPCRs they desensitize and catalytically active signaling proteins they carry as cargos. The ability to shuttle between abundant but low-affinity microtubule binding sites and smaller numbers of high affinity binding sites on activated GPCR permits them to sequester ERK1/2

away from its site of activation and dampen basal pathway activity until called to respond to an extracellular stimulus (Fig. 15.1). Thus, cRaf1 and ERK1/2 binding to the microtubule-associated pool of arrestin provides a mechanism for dampening basal ERK1/2 activity in the absence of receptor stimulation. In cells, overexpressing visual arrestin, β-arrestin1 and β-arrestin2, but not arrestin4, recruits

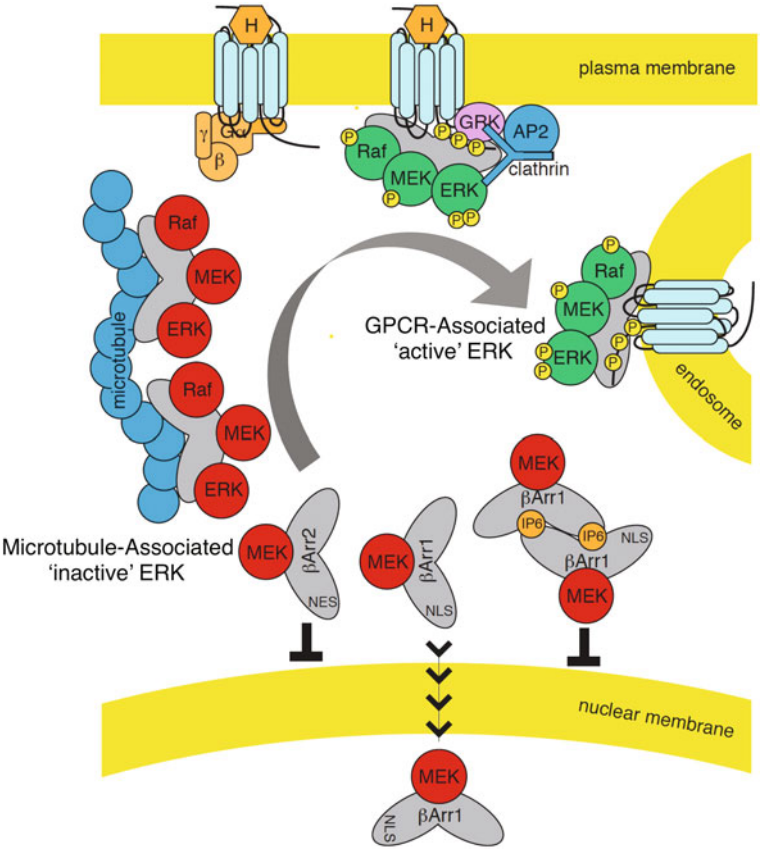

Fig. 15.1 β-Arrestins function as GPCR regulated scaffolds for the cRaf-1–MEK1/2–ERK1/2 MAP kinase module. In quiescent cells, arrestins reside in a either microtubule-bound pool or free in the cytosol, where they are excluded from the cell nucleus either by the intrinsic nuclear export sequence (NES) of β-arrestin2 or through inositol hexakisphosphate (IP6) promoted β-arrestin1 self-association (Hanson et al. 2008). Upon stimulation, the arrestins trade low affinity microtubule and IP6 interactions for high affinity binding sites on GRK phosphorylated receptors. Two of the ERK1/2 cascade components, cRaf-1 and ERK1/2, bind preferentially to the GPCR- and microtubule-bound β-arrestin conformations, while MEK1/2 binds equivalently to all three pools. Thus, inactive ERK1/2 can be sequestered in a microtubule-associated pool until a GPCR stimulus promotes assembly of the ERK1/2 activation complex on the plasma membrane. Since ERK1/2 has very low affinity for the cytosolic β-arrestin conformation, ERK1/2 remains active only while in GPCR-arrestin 'signalsome' complexes

ERK1/2 to microtubules and quenches its activity (Hanson et al. 2007), as does the β-arrestin2 mutant that mimics the microtuble-associated conformation (Coffa et al. 2011a). On the other hand, arrestin-dependent activation of ERK1/2 appears to be dependent upon binding to GPCRs as originally proposed (Coffa et al. 2011a, b). In fact, expressing a neurokinin 1 receptor–β-arrestin1 chimera is sufficient to activate ERK1/2 in the absence of G protein signaling, probably by bringing cRaf-1 into contact with membrane-delimited activators, e.g. Ras, which initiate the ERK1/2 phosphorylation cascade (Jafri et al. 2006).

When a GPCR-activating stimulus is received, the initial burst of heterotrimeric G protein signaling activates ERK1/2 via second messenger-dependent protein kinases, e.g. PKC, a process that is rapidly desensitized by GPCR kinase (GRK)-dependent arrestin recruitment. But the waning of G protein-mediated signaling coincides with the onset of arrestin-dependent ERK1/2 activation. Thus, ERK1/2 activity does not cease as the receptor switches from a G protein-coupled to an arrestin-coupled signaling mode. The contribution of G protein-dependent and arrestin-dependent signals to angiotensin AT_{1A} receptor-stimulated ERK1/2 activation has been dissected using isoform-selective arrestin RNA interference, pharmacologic inhibitors, G protein-uncoupled receptor mutants, and arrestin pathway-selective ligands (Wei et al. 2003; Ahn et al. 2004). When β-arrestin2 expression is silenced in HEK293 cells, AT_{1A} receptor-stimulated ERK1/2 activation becomes transient and sensitive to PKC inhibition, indicating that it occurs via a Gq/11-PLCβ-PKC pathway. In a reciprocal manner, inhibiting PKC in the presence of β-arrestin2 blocks the initial spike in ERK1/2 activity, but does not prevent the persistent late phase response. Exposing the G protein-uncoupled DRY-AAY AT_{1A} receptor mutant to angiotensin II, or the wild type AT_{1A} receptor to the arrestin-selective biased ligand, [Sar^1-Ile^4-Ile^8]-AngII, generates only the sustained signal, which is insensitive to PKC inhibition but abolished by RNA interference targeting β-arrestin2. Studies performed on the β2 adrenergic and type 1 parathyroid hormone (PTH) receptors have produced analogous results (Shenoy et al. 2006; Gesty-Palmer et al. 2006). Thus, arrestin binding 'switches' the receptor from G protein-dependent to arrestin-dependent ERK1/2 activation, which proceed with different time courses (Fig. 15.2a).

Subcellular Targeting of ERK

The majority of GPCRs fall into one of two classes based on their selectivity for the two β-arrestin isoforms and the longevity of the receptor-arrestin interaction (Oakley et al. 2000, 2001). One, termed 'class A', exhibits higher affinity for β-arrestin2 than β-arrestin1 and forms transient receptor-arrestin complexes that dissociate as the receptor internalizes. These receptors are rapidly resensitized and recycled back to the plasma membrane. The other, 'class B' group exhibits equivalent affinities for β-arrestin1 and β-arrestin2 and forms long lasting

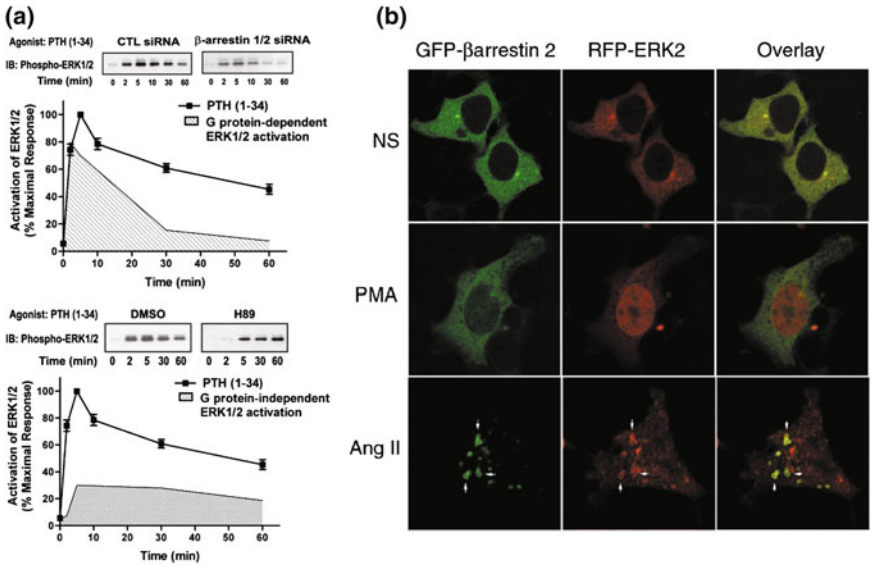

Fig. 15.2 Arrestin scaffolding produces a temporally and spatially discrete pool of active ERK1/2. **a** G protein and arrestin signaling activate ERK1/2 with distinct time courses. In HEK293 cells stimulation of PTH_1 receptors with PTH(1-34) activates ERK1/2 via both PKA-dependent and arrestin-dependent mechanisms. As shown in the *upper panel*, silencing β-arrestin1/2 expression with small interfering RNA (siRNA) inhibits the late phase of ERK1/2 activation, revealing the early component (*shaded area*) to be G protein-mediated. Conversely, as shown in the *lower panel*, pharmacologic inhibition of PKA with H89 blocks the early phase of ERK1/2 activation without affecting the late sustained phase of arrestin-dependent signal (*shaded area*). Data originally published in: Gesty-Palmer et al. (2006). **b** Arrestin scaffolding localizes active ERK1/2 to GPCR-arrestin 'signalsomes'. In non-stimulated (NS) HEK293 cells, both *green* fluorescent protein (GFP)-tagged β-arrestin2 and *red* fluorescent protein (RFP)-tagged ERK2 are diffusely cytosolic. Phorbol ester (PMA) stimulates PKC-dependent activation of ERK and promotes nuclear translocation of RFP-ERK2. In contrast, stimulation of angiotensin AT_{1A} receptors by angiotensin II (Ang II) recruits both GFP-β-arrestin2 and RFP-ERK2 to the receptor where they traffic with it into endosomes (*white arrows*). Data originally published in: Luttrell et al. (2001)

receptor-arrestin complexes that remain intact as the receptor undergoes endosomal sorting. These receptors tend to be sequestered in endosomes and either recycle slowly or are degraded.

Because activated ERK1/2 has little affinity for free cytosolic arrestin and would dissociate soon after the receptor and arrestin disengage, arrestin-bound active ERK1/2 is constrained within receptor-arrestin 'signalsome' complexes (Fig. 15.2b). Thus, ERK1/2 activated by arrestin scaffolds remains part the signalsome complex, at least when activated by 'class B' GPCRs like the angiotensin AT_{1A} and vasopressin V_2 receptors (Tohgo et al. 2003). Because the GPCR-arrestin complex is stable, activated ERK1/2 is targeted to the plasma membrane and early endosomes (DeFea et al. 2000; Luttrell et al. 2001). In fact, an estimated 75–80% of

the active ERK1/2 produced in response to short term stimulation of protease activated receptor (PAR)2 is associated with the GPCR-arrestin signalsome (DeFea et al. 2000). As a result, nuclear translocation of active ERK1/2 is retarded and its kinase activity is directed away from nuclear, and toward cytosolic, targets.

Functions of Arrestin-Dependent ERK Activation

While ERK1/2 activated by classical receptor tyrosine kinases and heterotrimeric G protein-mediated pathways is free to translocate to the cell nucleus and gain access to nuclear transcription factors, arrestin-dependent ERK1/2 activation produces a spatially-constrained ERK1/2 pool that is unable to produce a transcriptional response (Tohgo et al. 2003; Wei et al. 2004; Lee et al. 2008). Rather than transcriptional regulation, the arrestin-bound ERK1/2 activity appears to be directed predominantly toward membrane and cytosolic substrates, where it plays a key role in the regulation of vesicle trafficking, cytoskeletal rearrangement, and protein translation.

Regulation of GPCR Internalization and Trafficking

The most conserved arrestin function is the negative regulation of G protein signaling through direct steric hindrance of the GPCR-G protein interaction, and in the case of β-arrestins, mediation of clathrin-dependent GPCR endocytosis by linking GPCRs to clathrin and AP-2 (Goodman et al. 1996; Laporte et al. 1999). Besides these functions, several β-arrestin enzymatic cargos have been reported either to accelerate second messenger degradation or modulate the endocytic process. These include the cAMP phosphodiesterases and diacylglycerol kinase that accelerate second messenger degradation (Perry et al. 2002; Nelson et al. 2007), E3 ubiquitin ligases and de-ubiquitinases that modulate the stability of the GPCR-arrestin interaction (Shenoy and Lefkowitz 2003, 2005; Bhandari et al. 2007; Shenoy et al. 2008, 2009), and c-Src, which regulates GRK2 degradation (Penela et al. 2001), promotes dynamin self assembly (Ahn et al. 1999, 2002), and controls the stability of the β-arrestin-AP2 complex (Fessart et al. 2005, 2007; Zimmerman et al. 2009). Arrestin-bound ERK1/2 also appears to modulate the interaction between β-arrestin1 and the clathrin-dependent endocytic machinery. ERK1/2 phosphorylates Ser^{412} in the C-terminus of β-arrestin1, limiting its ability to bind clathrin (Lin et al. 1997, 1999). β-arrestin1 in the cytosol is almost stoichiometrically phosphorylated on Ser^{412}, and dephosphorylation of Ser^{412} upon receptor binding promotes receptor internalization and ERK1/2 activation. Re-phosphorylation by ERK1/2 in the signalsome complex probably provides either negative feedback regulation of receptor endocytosis or facilitates receptor internalization by promoting dissociation of arrestin and clathrin, allowing the receptor to exit

clathrin-coated vesicles. In rodent, but not human, β-arrestin2, ERK1/2 phosphorylation of S^{178} stabilizes the GPCR-arrestin complex in endosomes and delays recycling (Khoury et al. 2014).

Cell Proliferation

Owing in part to their tripartite functions of dampening basal ERK1/2 activity, desensitizing G protein-mediated pathways involved in generating a transcriptionally-active pool of ERK1/2, and activating ERK1/2 in signalsomes, the role of arrestins in the regulation of proliferative ERK1/2 signaling is complex and context-dependent. Primary calvarial preosteoblasts from β-arrestin2 null mice proliferate faster that wild type, and treatment with an arrestin pathway-selective 'biased' agonist of the PTH_1 receptor, [D-Trp^{12},Tyr^{34}]-bPTH(7-34), slows proliferation in a β-arrestin2-dependent manner, suggesting that arrestins restrain preosteoblast proliferation (Gesty-Palmer et al. 2013). Such effects are consistent β-arrestin-dependent sequestration of inactive ERK1/2 in a microtubule-bound pool (Hanson et al. 2007; Coffa et al. 2011b) and cytosolic retention of active ERK1/2 bound to stable GPCR-arrestin complexes (DeFea et al. 2000; Tohgo et al. 2002; Ahn et al. 2004). On the other hand, neointimal hyperplasia following carotid endothelial injury is reduced in β-arrestin2 null mice, where its loss is associated with decreased GPCR-stimulated vascular smooth muscle cell proliferation and migration, consistent with a stimulatory role for β-arrestin2 signaling in the proliferative response (Kim et al. 2008). In this system, knockout of β-arrestin1 has the opposite effect, suggesting that β-arrestin1 and 2 play opposing roles.

The proliferative effects of β-arrestin2 probably reflect its role in 'transactivation' of EGF receptors via another arrestin-bound cargo, c-Src, rather than direct scaffolding of the ERK1/2 cascade. Many GPCRs stimulate cell proliferation through crosstalk with the EGF receptor family of tyrosine kinase kinases, which in turn promote Ras-dependent mitogenic ERK1/2 signaling. EGF receptors are activated by matrix metalloprotease-dependent shedding of EGF family growth factors, e.g. heparin-binding EGF, which exist as preformed membrane associated precursors (Carpenter 2000), and GPCRs possess both G protein-dependent and G protein-independent mechanisms of promoting their release. EGF receptor transactivation is the major mechanism underlying ERK1/2 activation by endogenous LPA receptors in β-arrestin1/2 null murine embryo fibroblasts, clearly indicating that arrestin signaling is not essential for GPCR-EGF receptor cross talk (Gesty-Palmer et al. 2005). On the other hand, in HEK293 cells β1-Adrenergic receptor-mediated EGF receptor transactivation and ERK1/2 activation are inhibited by downregulating β-arrestin1/2 or GRK5/6, inhibiting Src or MMP activity, or exposing cells to a heparin-binding EGF neutralizing antibody, suggesting that β1 receptor-mediated EGF receptor transactivation is β-arrestin-dependent (Noma et al. 2007). Consistent with this, a GRK site mutant β1 receptor that cannot undergo arrestin-dependent desensitization fails to transactivate EGF receptors

despite exaggerated G protein signaling. In human coronary smooth muscle cells, the arrestin pathway-selective angiotensin AT_{1A} receptor agonist, [Sar^1-Ile^4-Ile^8]-AngII, induces ERK1/2 activation and proliferation by promoting EGF receptor transactivation (Miura et al. 2004), while in rat vascular smooth muscle both angiotensin II and [Sar^1-Ile^4-Ile^8]-AngII stimulate Src-dependent EGF receptor phosphorylation on Tyr^{845}, an effect that is lost when β-arrestin2 is downregulated by RNA interference (Kim et al. 2009). Similarly, the LH receptor activates c-Fyn in an β-arrestin2-dependent manner (Galet and Ascoli 2008). Downregulating arrestin expression reduces the rate of LH receptor internalization and inhibits LH-mediated activation of c-Fyn, phosphorylation of the anti-apoptotic focal adhesion kinase, and the release of EGF-like growth factors.

Non-proliferative Cell Growth

β-Arrestin scaffolding of the ERK1/2 cascade allows it to preferentially target cytosolic substrates involved in the control of protein translation including the ribosomal S6 kinase, p90RSK (Aplin et al. 2007) and the MAP kinase-interacting kinase, MNK1, a regulator of the ribosomal protein translation initiation complex. β-Arrestin2-dependent ERK1/2 activation by the angiotensin AT_{1A} receptor increases phosphorylation of MNK1 and eukaryotic translation initiation factor 4E, increasing rates of mRNA translation (DeWire et al. 2008). In addition, arrestin-dependent assembly of another signaling complex composed of protein phosphatase 2A (PP2A), AKT and glycogen synthase kinase 3β (GSK3β), is involved in regulation of mammalian target of rapamycin (mTOR)-dependent protein translation (Kendall et al. 2014). Increased rates of protein translation in response to angiotensin II [Sar^1-Ile^4-Ile^8]-AngII involves activation of arrestin-bound pools of both ERK1/2 and AKT, AKT-mediated phosphorylation of mTOR and its downstream effector p70/p85 ribosomal S6 kinase, and ERK1/2 phosphorylation of p90RSK.

Cell Migration and Chemotaxis

Several arrestin cargos have been implicated in GPCR-mediated actin cytoskeletal rearrangement leading to enhanced cell migration and chemotaxis, and promoting cancer metastasis. GPCR-stimulated chemotaxis is dependent upon two factors; the ability to sense a chemoattractant gradient and to establish cell polarity through cytoskeletal rearrangement at the leading edge (DeFea 2013). Arrestin-dependent GPCR desensitization and recycling are critical to the former (Tomhave et al. 1994; Aragay et al. 1998), while the coordinated regulation of several processes by β-arrestin scaffolds, including ERK1/2 and p38 MAPK activation, filamin A recruitment, cofilin dephosphorylation, and regulation of small GTPases contribute to the cellular shape changes needed for the latter. During PAR2-induced

chemotaxis, PAR2-arrestin-ERK1/2 complexes localize to the leading edge of the cell where ERK1/2 activity is required for actin cytoskeletal reorganization (Ge et al. 2003, 2004). Similarly, assembly of a complex containing the angiotensin AT_{1A} receptor, β-arrestin, ERK1/2, and the actin filament bundling protein, filamin A, coordinates the formation of membrane ruffles in Hep2 cells (Scott et al. 2006). In HeLa and HEK293 cells, overexpression/downregulation of β-arrestin2 reciprocally enhances/attenuates activation of both p38 MAPK and ERK1/2 by the chemokine receptor CXCR4, and inhibition of p38 MAPK blocks β-arrestin2 dependent chemotaxis, (Sun et al. 2002). Similarly, both β-arrestin2 and p38 MAPK have been implicated in AT_{1A} and LPA receptor-mediated chemotaxis in HEK293 cells (Hunton et al. 2005), and arrestin-dependent scaffolding of p38 MAPK is involved in control of cell polarization, actin bundle formation and internalization of platelet-activating factor receptors in polymorphonuclear neutrophils (McLaughlin et al. 2006).

Along with activating localized pools of ERK1/2 and p38 MAPK, β-arrestin scaffolds coordinate the activity of other cargos that drive actin assembly and regulate small GTPase activity. β-Arrestin scaffolding of a cofilin-chronophin-LIM kinase complex is necessary for the localized generation of free barbed ends on actin filaments that supports filament extension and generates membrane protrusions (Zoudilova et al. 2007, 2010). As a result, downregulation of either β-arrestin1 or 2 in MDA-MB-468 cells inhibits PAR2 stimulated cofilin dephosphorylation and chemotaxis. Arrestin-dependent regulation of the small GTPase, RalA, through its interaction with the Ral-guanine nucleotide dissociation stimulator, Ral-GDS, has been implicated in formyl-Met-Leu-Phe receptor stimulated membrane ruffling (Bhattacharya et al. 2002), Ral-dependent activation of PLCδ1 by the angiotensin AT_{1A} receptor (Godin et al. 2010), and LPA receptor mediated proliferation and migration of breast cancer cells (Li et al. 2009a). Regulation of RhoA via the interaction between β-arrestin1 and ARFGAP21 promotes angiotensin AT_{1A} receptor dependent RhoA activation and membrane ruffling (Anthony et al. 2011), while β-arrestin1 and Gq/11 coordinately activate RhoA to promote stress fiber formation in AT_{1A} receptor-expressing HEK293 cells (Barnes et al. 2005).

Disrupting Arrestin-Dependent ERK Regulation

Because arrestins function both as negative regulators of heterotrimeric G proteins and as G protein-independent signaling scaffolds, arrestin knockouts exhibit both prolonged/exaggerated G protein signaling (Kohout et al. 2001) and loss of arrestin-dependent signals (Wei et al. 2003; Ahn et al. 2004). Thus, it is often difficult to attribute phenotypes associated with arrestin loss-of-function to one role or the other (Luttrell and Gesty-Palmer 2010). Dissociating the desensitizing and signaling functions of arrestins, such that desensitization/sequestration are preserved while arrestin signaling is disrupted, is one approach that might help shed light onto the physiological roles of arrestin scaffolds.

Despite the fact that c-Raf1-MEK1/2-ERK1/2 and ASK1-MKK4-JNK3 engage a broad interface involving both the N- and C-domains of β-arrestin1 and β-arrestin2 (Song et al. 2009), binding of ERK1/2 and JNK3 pathway components can be affected by point mutations that disrupt arrestin-dependent scaffolding while leaving the rest of the protein, and its other functions, intact. Peptide array analysis of the binding of JNK3 pathway components to β-arrestin1 and β-arrestin2 has identified conserved ASK1 binding motifs located in the N- and C-domains of both proteins, such that a β-arrestin1 $D^{26,29}A$ mutation in the N-domain eliminates ASK1 binding in vitro and in cells, as does a $A^{391,392}D$ mutation in the C-domain (Li et al. 2009b). Similarly, mutating E^{66}, D^{67}, and D^{69} of β-arrestin1 to alanine ablates its binding to MKK4. Applying a similar approach to β-arrestin-ERK1/2 pathway interactions identified D^{26} and D^{29} as also critical for the β-arrestin1-MEK1 interaction, such that the $D^{26,29}A$ mutant fails to interact with MEK1 in cells and fails to undergo ERK1/2-dependent S^{412} phosphorylation (Meng et al. 2009). Interestingly, a cell permeable 25 amino acid β-arrestin1 peptide encompassing the β-arrestin1-MEK1 binding site prevents MEK1 binding to wild type β-arrestin1 and S^{412} phosphorylation, while promoting β-arrestin1 binding to other cargos, including c-Src and clathrin, and enhancing β2 adrenergic receptor internalization.

Both ERK1/2 and cRaf-1 binding are also sensitive to point mutations. $K^{285}A/R^{286}A$ and $K^{295}A$ mutants of β-arrestin2 exhibit impaired ERK1/2 binding and fail to support β2 adrenergic receptor-mediated ERK1/2 activation in cells (Xu et al. 2008). However, these mutations, which involve the polar core of the protein, also interfere with β-arrestin2 self-association and β2-adrenergic receptor binding. Greater specificity has been obtained by targeting cRaf-1 binding. Alanine-scanning mutagenesis of conserved residues on the non-receptor binding surface of β-arrestin1 and β-arrestin2 identified R^{307} in the C-domain of β-arrestin1 as important for c-Raf1 binding, such that an $R^{307}A$ mutant of β-arrestin1 exhibits significantly reduced c-Raf1 binding with no effect on binding to active phosphorylated rhodopsin or to MEK1 and ERK2 (Coffa et al. 2011b). In β-arrestin1/2 double null cells, the $R^{307}A$ mutant fails to rescue β2 adrenergic receptor-stimulated ERK1/2 activation by the arrestin-biased β2 receptor agonist, ICI118551, indicating that it can function as a dominant negative inhibitor of arrestin-dependent ERK1/2 activation while not impairing receptor desensitization. Interestingly, the analogous $K^{308}A$ mutation in β-arrestin2 does not disrupt c-Raf1 binding or its ability to rescue arrestin-dependent ERK1/2 activation in the null background.

Conclusions

ERK1/2 is a nodal kinase that is critical to many cellular functions, from cell cycle progression and survival to cell growth, migration and metastasis. As such, it is subject to multiple forms of convergent regulation that determine both where it is active and for how long (Stork 2002; Luttrell 2003). By binding components of the

ERK1/2 cascade in a conformation-sensitive manner, arrestins are critical regulators of ERK1/2 pathway activity, both in the presence and absence of GPCR stimulation. Association of cRaf-1-MEK1/2-ERK1/2 with the microtubule-associated storage pool of arrestins keeps ERK1/2 pathway components away from its membrane delimited activators and contributes to dampening basal pathway activity. Stimulus-dependent recruitment of arrestins to high affinity GPCR binding sites has two effects. Arrestin-dependent desensitization limits the duration of G protein-dependent signals that activate a transcriptionally competent pool of ERK1/2 via second messenger-dependent protein kinases and EGF receptor cross talk. At the same time, arrestin scaffolding of the ERK1/2 cascade produces sustained and spatially discrete pool of active ERK1/2 that is preferentially targeted to membrane and cytosolic substrates involved in GPCR trafficking, cytoskeletal rearrangement, and protein translation. Thus, arrestins fulfill every criterion of a GPCR-regulated signaling scaffold.

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