G PROTEIN-COUPLED RECEPTOR SIGNALLING IN NEUROENDOCRINE SYSTEMS

‘Location, location, location’: activation and targeting of MAP kinases by G protein-coupled receptors

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Abstract

A growing body of data supports the conclusion that G protein-coupled receptors can regulate cellular growth and differentiation by controlling the activity of MAP kinases. The activation of heterotrimeric G protein pools initiates a complex network of signals leading to MAP kinase activation that frequently involves cross-talk between G protein-coupled receptors and receptor tyrosine kinases or focal adhesions. The dominant mechanism of MAP kinase activation varies significantly between receptor and cell type. Moreover, the mechanism of MAP kinase activation has a substantial impact on MAP kinase function. Some signals lead to the targeting of activated MAP kinase to specific extranuclear locations, while others activate a MAP kinase pool that is free to translocate to the nucleus and contribute to a mitogenic response.

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Introduction

The G protein-coupled receptors (GPCRs) make up the largest superfamily of cell surface receptors in the human genome, where they are represented by at least 600 distinct genes. The mammalian GPCRs are divided on the basis of sequence homology into three broad classes. While all share a common core architecture of seven membrane-spanning α -helices, each class differs extensively in both primary sequence and the size and complexity of the intracellular and extracellular loops that connect the transmembrane domains. This diversity permits GPCRs to detect an extraordinary array of extracellular stimuli, from neurotransmitters and peptide hormones to odorants and photons of light. The binding of ligand to a GPCR stabilizes the receptor in an ‘active’ conformation, thereby accelerating the normally slow basal rate of receptor-catalyzed G protein turnover. Contact

between intracellular receptor domains and the GDP-bound $G\alpha$ subunit of a heterotrimeric G protein triggers GTP for GDP exchange on the $G\alpha$ subunit and dissociation of the GTP-bound $G\alpha$ subunit from the $G\beta\gamma$ heterodimer. Once dissociated, both $G\alpha$ -GTP and $G\beta\gamma$ subunits regulate the activity of enzymatic effectors, such as adenylyl cyclases, phospholipase C (PLC) isoforms, and ion channels.

While the central role of GPCRs in neurotransmission and in the regulation of intermediary metabolism has been long established, appreciation of their involvement in the control of cell proliferation and differentiation is little more than a decade old. The discovery that activating mutations in heptahelical receptors or $G\alpha$ subunits underlie certain relatively rare forms of endocrine neoplasia, including hyperfunctioning thyroid adenomas and growth hormone-secreting pituitary adenomas, first established the oncogenic potential of GPCRs

(Dhanasekaran *et al.* 1995). It is now recognized that GPCRs convey signals that influence cell growth or differentiation in a host of common clinical disorders, among them pressure overload cardiac hypertrophy, neointimal hyperplasia of vascular smooth muscle, and prostate hypertrophy. In many cases, these effects are exerted through the control of MAP kinase cascades (van Biesen *et al.* 1996a, Gutkind 1998, Pierce *et al.* 2001a).

The modular organization of MAP kinase pathways

The MAP kinases are a family of evolutionarily conserved serine/threonine kinases that transmit externally derived signals regulating cell growth, division, differentiation and apoptosis. Mammalian cells contain three major classes of MAP kinase, the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK) and p38/HOG1 MAP kinases. The ERK pathway is important for control of the G0–G1 cell cycle transition and the passage of cells through mitosis or meiosis. In contrast, the JNK/SAPK and p38/HOG1 MAP kinases are involved in regulation of growth arrest, apoptosis, and activation of immune and reticuloendothelial cells in response to a variety of environmental and hormonal stresses (Kryiakos & Avruch 1996, Pearson *et al.* 2001).

MAP kinase activity is regulated through a series of parallel kinase cascades, each of which is composed of three kinases that successively phosphorylate and activate the downstream component. As shown in Fig. 1, hormonal stimulation results in the activation of the most upstream kinase in the cascade, a MAP kinase kinase kinase, e.g. c-Raf-1 or B-Raf, that phosphorylates a MAP kinase kinase, e.g. MEK1 or MEK2, that in turn phosphorylates and activates the MAP kinase, e.g. ERK1 or ERK2. Once activated, MAP kinases phosphorylate a variety of membrane, cytoplasmic, nuclear and cytoskeletal substrates. Upon activation, MAP kinases may translocate to the nucleus and phosphorylate nuclear transcription factors, such as Elk-1, that are involved in DNA synthesis and cell division (Pearson *et al.* 2001).

It is increasingly evident that MAP kinase activation is controlled by additional, non-enzymatic proteins that function as ‘scaffolds’ for two or more of the component kinases of a MAP kinase cascade

(Burack & Shaw 2000, Pearson *et al.* 2001). These scaffolds serve at least three functions in cells: to increase the efficiency of signaling between successive kinases in a phosphorylation cascade, to ensure signaling fidelity by dampening cross-talk between parallel MAP kinase cascades, and to target MAP kinases to specific subcellular locations.

The *Saccharomyces cerevisiae* protein Ste5 is the prototypic MAP kinase scaffold. In the yeast pheromone mating pathway, Ste5 binds to each of the three components of the yeast MAP kinase pathway, Ste11, Ste7p, and either Fus3 or Kss1 (Choi *et al.* 1994). Binding of mating factor to the pheromone receptor, a GPCR, leads to translocation of Ste5 to the plasma membrane and activation of the Fus3/Kss1 cascade.

While no structural homologues of Ste5 have been isolated from mammalian cells, several mammalian proteins have been identified that can bind to two or more components of a MAP kinase module. For example, the JNK-interacting proteins JIP1 and JIP2 act as scaffolds for the JNK/SAPK pathway by binding to Ask1, MKK4 or MKK7, and JNK1 or JNK2 (Whitmarsh *et al.* 1998, Yasuda *et al.* 1999). Other proteins bind directly to only two of the three members of a MAP kinase module, but nonetheless appear to increase the efficiency of MAP kinase activation. MEK partner 1, MP1, specifically binds to MEK1 and ERK1 and enhances ERK1 activation when overexpressed (Schaeffer *et al.* 1998). β -Arrestins, GPCR-binding proteins that were originally characterized for their role in mediating homologous GPCR desensitization, can also function as scaffolds for some MAP kinase modules (Ferguson 2001, Luttrell & Lefkowitz 2002). β -Arrestin 2 binds to Ask1 and the neuronal JNK/SAPK isoform, JNK3 (McDonald *et al.* 2000). Ask1 binds the β -arrestin 2 N-terminus, whereas JNK3 binding is conferred by an RRSLHL motif in the C-terminal half of β -arrestin 2 (Miller *et al.* 2001). When overexpressed in cells, β -arrestin 2 forms complexes with Ask1, MKK4, and JNK3, but not JNK1 or JNK2, and dramatically increases Ask1-dependent phosphorylation of JNK3. Similarly, both β -arrestin 1 and 2 can bind to c-Raf-1 and ERK2, and enhance ERK activation in response to stimulation of protease-activated receptor 2 (PAR2), and angiotensin II type 1a (AT1a) receptors (DeFea *et al.* 2000a, Luttrell *et al.* 2001, Tohgo *et al.* 2002). Indeed, stimulation of PAR2 and AT1a receptors induces

Figure 1 The modular organization of MAP kinase cascades. The generic MAP kinase cascade is composed of a MAP kinase kinase kinase (MAPKKK) e.g. Ste11, RAF, Ask1, MAP kinase kinase (MAPKK) e.g. Ste7, MEK1/2, MKK4/7, MKK3/6 and MAP kinase (MAPK) e.g. Fus3/Kss1, ERK1/2, JNK1-3, p38 α , β , δ , γ , supported by a scaffolding protein (e.g. Ste5, β -Arrestin1/2, MP-1, JIP1/2) that facilitates the sequential phosphorylation events. For comparison, the *S. cerevisiae* pheromone mating pathway, and representative mammalian ERK, JNK and p38 MAP kinase pathway components, along with their known scaffolding proteins, are depicted in a similar orientation.

the assembly of protein complex containing the internalized receptor, β -arrestin, c-Raf-1 and activated ERK1/2. In this respect, the β -arrestins appear to be unique among the mammalian MAP kinase scaffolds described thus far, in that, like Ste5, their function is directly under the control of a cell surface receptor.

A complex network of GPCR-derived signals controls cellular MAP kinase activity

In early studies performed using transfected COS-7 cells, it was found that transient expression of constitutively active mutants of $G_{\alpha s}$ and $G_{\alpha q}$, or of

$G_{\beta\gamma}$ heterodimers, resulted in sustained activation of the ERK MAP kinase cascade (Faure *et al.* 1994, Hawes *et al.* 1995). Interestingly, expression of activated $G_{\alpha i2}$ did not, despite its presence as a somatic mutation in some ovarian and adrenal cortical tumors, and its ability to stimulate the growth of Rat1 fibroblasts *in vitro* (Dhanasekaran *et al.* 1995). It is now clear, for the ERK cascade at least, that a complex set of signals derived from different G protein pools converge to determine the activity of the MAP kinase module. The extent to which each of these inputs, some of which are depicted schematically in Fig. 2, contributes to MAP kinase activation varies widely between different receptors and cell types.

Figure 2 A complex network of GPCR-derived signals regulates the activity of the ERK MAP kinase cascade. GPCR stimulation results in the activation of several heterotrimeric G protein pools that affect ERK activity. Signals generated by second messenger-dependent protein kinases, such as PKA and PKC, and through cross-talk between GPCRs and EGF receptors or integrin heterodimers clustered in focal adhesions, converge on the Raf isoforms c-Raf-1 and B-Raf at the apex of the ERK module. The particular signaling mechanisms that predominate varies with receptor and between cell types.

Gai/o

Pertussis toxin-sensitive Gi/o family G proteins mediate ERK activation in response to many stimuli. The major effects of Gi activation on the ERK cascade appear to be mediated via its G $\beta\gamma$ subunits, although the inhibition of adenylate cyclase activity by G α_i subunits may modulate ERK activation through relief of the inhibitory effects of protein kinase A (PKA) on the c-Raf-1 isoform (see below). In contrast, the predominantly brain-specific G α_o subunit does contribute to GPCR-mediated ERK activation in some cell types. In CHO cells, for example, which express both G α_i and G α_o subunits and the B-Raf isoform, pertussis toxin-sensitive activation of ERK via endogenous lysophosphatidic acid (LPA) or over-expressed platelet-activating factor receptors can be restored through expression of a pertussis toxin-insensitive mutant of G α_o , but not of G α_i2 (van Biesen *et al.* 1996b). This signal is independent of tyrosine kinase or Ras activity, but is sensitive to inhibitors of protein kinase C (PKC). Similarly, expression of an activated mutant of G α_o in CHO cells leads to activation of B-Raf, but not c-Raf-1, via a Ras-independent mechanism that requires PKC and phosphatidylinositol 3-kinase activity, and that enhances the ability of epidermal growth factor (EGF) to stimulate ERK (Antonelli *et al.* 2000).

G $\beta\gamma$ subunits

The expression of proteins that sequester free G $\beta\gamma$ subunits, such as the G α subunit of transducin (Faure *et al.* 1994) or a polypeptide derived from the C-terminus of the GPCR kinase GRK2 (Koch *et al.* 1994), inhibits GPCR-stimulated ERK activation in many systems. Both Gi-coupled receptors, such as the LPA, α_2a adrenergic, and M2 muscarinic receptors, and Gq/11-coupled receptors, such as the M1 muscarinic receptor, can activate ERK via a G $\beta\gamma$ subunit-dependent pathway that requires tyrosine protein kinase activity and the low molecular weight G protein Ras (Crespo *et al.* 1994, Hawes *et al.* 1995, van Biesen *et al.* 1995).

The best understood mechanism whereby G $\beta\gamma$ subunits stimulate ERK is through the 'trans-activation' of classical receptor tyrosine kinases,

such as the EGF and platelet-derived growth factor (PDGF) receptors (Carpenter 2000, Gschwind *et al.* 2001). In this pathway, stimulation of the GPCR triggers activation of a receptor tyrosine kinase, which in turn activates ERK. In the case of the EGF receptor, transactivation often results from GPCR-stimulated production of an EGF receptor ligand at the cell surface (Prenzel *et al.* 1999). Each of the known ligands for the EGF receptor, EGF, transforming growth factor- α , heparin-binding (HB)-EGF, amphiregulin, beta-cellulin and epiregulin, is synthesized as a transmembrane precursor that is proteolyzed to produce a soluble growth factor (Riese & Stern 1998). At least one of these, HB-EGF, can undergo cleavage in response to GPCR stimulation. Release of G $\beta\gamma$ subunits causes activation of a matrix metalloprotease (MMP) that releases the growth factor (Prenzel *et al.* 1999). Once bound to HB-EGF, monomeric EGF receptors dimerize, transphosphorylate on tyrosine residues within their intracellular domains, and recruit SH2 domain-containing adapter proteins, such as Shc and Gab1. These adapters provide docking sites for a number of additional signaling proteins, among them a complex between the adapter protein Grb2 and the Ras guanine nucleotide exchange factor mSos. Subsequent Ras activation initiates the Raf-MEK-ERK cascade by promoting the membrane translocation and activation of c-Raf-1 (Fig. 2).

The G $\beta\gamma$ subunit protein effectors that regulate HB-EGF release remain undefined, although phosphatidylinositol-3' kinases (Hawes *et al.* 1996, Lopez-Illasaca *et al.* 1998, Yart *et al.* 2002) and Src family non-receptor tyrosine kinases have each been proposed as early intermediates in the pathway (Luttrell *et al.* 1997, Pierce *et al.* 2001b). Proteolysis of the HB-EGF precursor is thought to be mediated by members of the ADAM family of MMPs (Schlondorff & Blobel 1999), one of which, ADAM-12, has recently been implicated in GPCR-mediated HB-EGF shedding in the heart (Asakura *et al.* 2002). Several of the ADAMs, notably ADAM-9, -10, -12, -15, -17 and -19, possess consensus SH3 domain binding motifs within their short intracellular domains that might mediate interaction with Src kinases. G $\beta\gamma$ subunit-mediated ERK activation does not typically appear to involve activation of either PLC β isoforms or ion channels.

The molecular mechanisms underlying GPCR-mediated transactivation of PDGF receptors are less well understood. In L cells, which do not express endogenous EGF receptors, LPA-mediated ERK activation requires G $\beta\gamma$ subunit-dependent activation of the PDGF receptor via a mechanism that does not involve PKC (Herrlich *et al.* 1998). Interestingly, when EGF receptors are introduced into these cells, LPA receptors no longer utilize the PDGF receptor to support ERK activation, suggesting that cross-talk with the EGF receptor is a preferred signaling mechanism.

G α q/11

The activation of Gq/11 family G proteins triggers the activation of PLC β isoforms and subsequent increases in intracellular calcium and PKC activity. Several signals downstream of Gq/11 activation have been implicated in control of the ERK cascade. PKC activation leads to ERK activation through both Ras-dependent and Ras-independent mechanisms. PKC α can apparently activate Raf-1 by direct phosphorylation (Kolch *et al.* 1993), and stimulation of ERK by Gq/11-coupled α 1B adrenergic and M1 muscarinic receptors in fibroblasts can occur through a Raf-dependent, but Ras-independent, mechanism that is blocked by PKC downregulation and mimicked by overexpression of PLC β 2 (Faure *et al.* 1994, Hawes *et al.* 1995).

In many cases, however, Gq/11 activation causes ERK activation that is unaffected by PKC inhibition (Crespo *et al.* 1994, Daub *et al.* 1997). At least two additional signaling mechanisms appear to be responsible for these PKC-independent effects. In fibroblasts, HB-EGF shedding in response to stimulation of Gq/11-coupled receptors, such as the endothelin-1 and α -thrombin receptors, is mediated by G α q/11 subunits. Despite the fact that the MMP ADAM-9 mediates PKC-dependent HB-EGF cleavage in response to phorbol esters, the G α q/11 effect, like that mediated by G $\beta\gamma$ subunits, apparently involves neither PKC nor ADAM-9 (Prenzel *et al.* 1999). In cultured vascular smooth muscle, transactivation of PDGF-B receptors apparently accounts for ERK activation in response to stimulation of the Gq/11-coupled angiotensin

AT1a receptor (Linseman *et al.* 1995, Heeneman *et al.* 2000).

An additional, calcium-dependent mechanism may contribute to G α q/11-mediated ERK activation, particularly in cells of neuronal origin. G α q/11-dependent activation of the calcium- and cell adhesion-dependent focal adhesion kinase (FAK)-family member, Pyk2, leads to Ras-dependent ERK activation (Lev *et al.* 1995). In this system, intracellular calcium, released as a result of PLC β -mediated inositol trisphosphate production, triggers Pyk2 autophosphorylation, recruitment of the non-receptor tyrosine kinase c-Src, tyrosine phosphorylation of Shc, and Ras-dependent ERK activation (Dikic *et al.* 1996, Della Rocca *et al.* 1999).

G α s

The role of Gs proteins in GPCR-mediated ERK activation is complex. The earliest findings were that the stimulation of adenylate cyclase through Gs-coupled receptors and subsequent PKA-mediated phosphorylation of c-Raf-1 inhibited c-Raf-1 and attenuated growth factor-stimulated ERK activation (Wu *et al.* 1993). It is now clear, however, that in some cell types cAMP and PKA can directly trigger ERK activation. In cells of neuronal and hematopoietic origin, Gs activation leads to PKA-dependent phosphorylation of the Ras-family GTPase, Rap-1, and activation of the B-Raf isoform (Vossler *et al.* 1997, Grewal *et al.* 2000). This pathway may involve the activation of Src family kinases downstream of PKA (Schmitt & Stork 2002). In addition, direct binding of cAMP to the Rap-1 guanine nucleotide exchange factor Epac provides a direct mechanism for cAMP-dependent stimulation of Rap-1 (DeRoos *et al.* 1998).

A further wrinkle is that PKA phosphorylation of certain Gs-coupled receptors, notably the β 2 adrenergic (Daaka *et al.* 1997) and the murine prostacyclin receptor (Lawler *et al.* 2001) simultaneously decreases receptor coupling to Gs and increases receptor coupling to Gi. As a result, classically Gs-coupled receptors activate the ERK cascade through a Ras-dependent pathway mediated through G $\beta\gamma$ subunits derived from pertussis toxin-sensitive G proteins in several cell types (Lefkowitz *et al.* 2002).

Figure 3 Functional specialization of different ERK activation mechanisms used by GPCRs. Cross-talk between GPCRs and transactivated EGF receptors produces a pool of activated ERK that can translocate to the cell nucleus and promote a mitogenic response. In contrast, activation of ERK via β -arrestin scaffolds and focal adhesions may favor the formation of localized extranuclear ERK pools that contribute to the regulation of receptor activity, cell adhesion and cytoskeletal reorganization.

The effect of scaffolding proteins on MAP kinase distribution and function

The extensive heterogeneity of signaling mechanisms connecting GPCRs to MAP kinases inevitably raises the question of whether these pathways are functionally redundant. Experimental data are beginning to suggest that this is not the case. Rather, different mechanisms of MAP kinase activation appear to favor the formation of functionally distinct pools of active kinase. For the ERK cascade, three mechanisms in particular may have specialized roles, EGF receptor transactivation, activation of ERK bound to β -arrestin scaffolds, and focal adhesion-based ERK activation through Pyk2. The functional specialization of

these mechanisms is shown schematically in Fig. 3.

Because they can be activated in response to a wide range of heterologous signals, from GPCR and cytokine receptor stimulation to phorbol esters and ionizing radiation, EGF receptors represent a point of convergence for diverse mitogenic stimuli (Carpenter 2000). In some cases, the mitogenic response to GPCR stimulation has been attributed to Ras-dependent signals arising from transactivated EGF receptors. In cardiac fibroblasts, for example, angiotensin II-stimulated ERK activation and DNA synthesis are both EGF receptor-dependent (Murasawa *et al.* 1998). Similarly, neurokinin-1 (NK1) receptor-mediated ERK activation and DNA synthesis in U-373 MG cells is

blocked by either pharmacological inhibition of the EGF receptor or expression of a dominant-negative EGF receptor mutant (Castagliuolo *et al.* 2000).

In contrast, β -arrestin-dependent ERK activation does not appear to provide a mitogenic stimulus. As previously noted, β -arrestins can function as scaffolds for some MAP kinase modules. Activation of ERK by PAR2, NK1 and AT1a receptors occurs coincidently with the assembly of multiprotein complexes containing the receptor, β -arrestin, and activated ERK1/2 (DeFea *et al.* 2000a,b, Luttrell *et al.* 2001). In the case of the PAR2 receptor, it has been estimated that as much as 80% of the active ERK pool is associated with the β -arrestin-bound receptor (DeFea *et al.* 2000a). These GPCR- β -arrestin-ERK complexes appear not to dissociate upon ERK activation, and can be isolated either by co-immunoprecipitation or upon gel filtration of whole-cell detergent lysates. Since the GPCR- β -arrestin complexes are internalized and targeted to endosomes, β -arrestin-dependent ERK activation leads to the formation of a discrete cytosolic pool of ERK that can be visualized in association with early endosomes (DeFea *et al.* 2000a, Luttrell *et al.* 2001).

The functional relevance of these internalized β -arrestin-ERK complexes is not well understood. Several proteins involved in heptahelical receptor signaling, such as β -arrestin-1 (Lin *et al.* 1999) and GRK2 (Pitcher *et al.* 1999, Elorza *et al.* 2000) and GAIP (Ogier-Denis *et al.* 2000) are ERK substrates, so one function of β -arrestin-bound ERK might be to regulate GPCR function. Overexpression of β -arrestin predictably leads to the cytosolic retention of activated ERK and a reduction in AT1a receptor-stimulated Elk-1-driven transcription (Tohgo *et al.* 2002). Similarly, overexpression of β -arrestin 2 leads to cytosolic retention of JNK3 and co-localization of β -arrestin 2 and active JNK3 in intracellular vesicles following stimulation of AT1a receptors (McDonald *et al.* 2000). Probably the most compelling evidence that β -arrestin-dependent ERK activation creates a functionally distinct pool of ERK is the finding that the wild type PAR2 receptor does not stimulate a mitogenic response in KNRK cells, whereas a mutant PAR2 receptor that cannot bind β -arrestin, but that still activates ERK1/2 through an alternative calcium-dependent pathway, does stimulate nuclear translocation of ERK and causes cell proliferation (DeFea *et al.* 2000a).

Integrin engagement of the extracellular matrix leads to formation of focal adhesions. Recruitment and activation of FAK in focal adhesions as they form results in recruitment of Grb2-mSos and Ras-dependent ERK activation. In rat embryo fibroblasts, this active ERK is localized specifically within the focal adhesion, where it participates in the regulation of cell adhesion and organization of the cytoskeletal network (Fincham *et al.* 2000). A similar role may be performed by Pyk2, which mediates conditional, calcium-dependent activation of ERK in adherent cells in response to GPCR activation.

Conclusions

The predominant mechanism used by any given GPCR to activate MAP kinases varies with cell type and is determined by the cellular context in which the receptor is expressed. Accumulating data suggest that, rather than reflecting redundancy, the mechanism of GPCR-mediated ERK activation determines to a significant degree the function of the activated kinase. ERK substrates have been identified in the plasma membrane, cytoplasm, cytoskeleton and nucleus. By activating spatially localized pools of ERK, whether in the nucleus, on the plasma membrane in focal adhesions, or in endosomal vesicles, different signaling mechanisms may determine whether ERK serves predominantly to regulate transcription, influence receptor regulation, or control cytoskeletal rearrangement and cell motility.

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