Regulation of Mitogen-Activated Protein Kinase Signaling Networks by G Protein-Coupled Receptors

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The family of receptors that transmit signals through the activation of heterotrimeric GTP-binding proteins (G proteins) constitutes the largest group of cell surface proteins involved in signal transduction. These receptors participate in a broad range of important biological functions and are implicated in a number of disease states. More than half of all drugs currently available influence G protein-coupled receptors (GPCRs). These receptors affect the generation of small molecules that act as intracellular mediators or second messengers, and can regulate a highly interconnected network of biochemical routes controlling the activity of several members of the mitogen-activated protein kinase (MAPK) superfamily. They include extracellular signal-regulated kinase 1 (ERK1) and ERK2 (or p44MAPK and p42MAPK), c-Jun NH2-terminal kinases (JNKs), ERK5 (or BMK), and p38 MAPKs, including p38α (or CSBP-1), p38β, p38γ (or SAPK3 or ERK6), and p38δ (or SAPK4). This review will focus on the molecular mechanisms by which GPCRs signal to the nucleus through this intricate network of second messenger-generating systems and MAPK signaling pathways, thereby affecting the expression of genes whose products influence many biological processes, including normal and aberrant cell growth.

The G Protein-Coupled Receptor Signaling System

With more than 1000 members, the family of receptors that transmit signals through the activation of heterotrimeric GTP-binding proteins (G proteins) represents the largest group of cell surface receptors encoded by the mammalian genome (>1% of human proteins). About 20 mammalian G protein α subunits have been identified, which can be divided into four families based on their primary sequence similarity: Gαs, Gαi, Gαq, and Gα13 (15). These G protein α subunits regulate the activity of several second messenger-generating systems. For example, the Gαi family controls the activity of phosphatidylinositolspecific phospholipases, such as phospholipase C-β (PLC-β), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (16). IP3 and DAG in turn lead to an increase in the intracellular concentrations of free calcium ([Ca2+]i) and the activation of a number of protein kinases, including protein kinase C (PKC) (17-20) (Fig. 1). The members of the Gαi family activate adenylyl cyclases, whereas Gα family members can inhibit a subset of these enzymes, thereby controlling the intracellular concentrations of adenosine 3’,5’-monophosphate (cAMP). Indeed, nine distinct adenylyl cyclases have been cloned so far, and each appears to be distinctly regulated by Gαi and Gai, as well as by βγ subunits, [Ca2+]i, and PKCs (21-23). Thus, the impact on the intracellular concentrations of cAMP by agonists acting on GPCRs will be highly dependent on which adenylyl cyclases are expressed in each cell type. Gα subunits of the Gαi family, which includes Gαi1, Gαi2, Gαi3, Gαo, transducin (Gtαi), and gustducin (Ggαi), also activate a variety of phospholipases and phosphodiesterases, and promote the opening of several ion channels (12). The nature of the signaling pathways controlled by the Gαi1 family has just begun to be elucidated. Members of this enigmatic Gα family, which includes Gtα12 and Gtα13, provide links between GPCRs and the activation of the small GTP-binding protein Rho (24-28).

In addition to the many Gα subunits, 12 G protein γ subunits and 6 G protein β subunits have been cloned. Gβγ dimers, when released from the heterotrimeric complex upon Gα activation, can themselves regulate the activity of many signaling molecules, including ion channels, phosphatidylinositol 3-kinases (PI3Ks), phospholipases, adenylyl cyclases, and receptor kinases (13). The possibility exists that distinct pools of Gβγ subunits may play different roles in signal transmission (12). Taken together, this emerging body of information shows the tremendous complexity of the G protein-GPCR signal-transducing system, whose biochemical and biological consequences have just begun to be appreciated.

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G Protein-Coupled Receptors and Cell Growth Control

GPCRs are involved in many important biological functions, such as photo- and chemoreception, neurotransmission, regulation of secretion from endocrine and exocrine glands, exocytosis, chemotaxis, blood pressure control, platelet function, as well as embryogenesis, angiogenesis, tissue regeneration, and control of normal and aberrant cell growth. The ability to transduce proliferative signals has been frequently associated with the activation of polypeptide growth factor receptors that have an intrinsic protein tyrosine kinase activity (29). However, the availability of bacterial toxins, such as pertussis toxin that inhibits the function of G protein α subunits of the G_i family (29), helped to establish that GPCRs mediate proliferative responses to many mitogens, including thrombin (31, 32), lysophosphatidic acid (LPA) (33), and many other substances that are normally found in serum (34). In addition, pharmacological and biochemical evidence indicates that a large number of mitogens, including bombesin, vasopressin, bradykinin, substance K, acetylcholine receptor agonists, angiotensin II, and many others (35) stimulate cell proliferation by acting on receptors coupled to G proteins that are insensitive to bacterial toxins. If persistently activated, some of these GPCRs contribute to malignant transformation (36–39), and ultimately to human cancer (40–48). Furthermore, recent data suggest that certain DNA viruses also encode functional GPCRs, including human cytomegalovirus (HCMV) (49, 50), Herpesvirus saimiri (HVS) (51), and the Kapoşi’s sarcoma-associated herpesvirus (KSHV) (52). The latter is likely to represent a constitutively active chemokine receptor, which harbors transforming potential (52) and might actively participate in the angiogenic process that characterizes this typically AIDS-associated malignancy (53).

The ability of GPCRs to affect cell growth prompted several groups to examine whether their immediate downstream targets, the G proteins, also have transforming potential if mutationally activated. GTPase-deficient mutants of Gα13, Gα16, Gα11, Gα12, and Gα13 were found to display oncogenic properties when expressed in several cellular systems; and naturally occurring activated mutants of certain G proteins were also identified in various disease states, including cancer (54). This led to the designation of certain activated Gα mutants as oncogenes, including Gα13, Gα12, and Gα12, referred to as the gsp (55), gip2 (56), and gep oncogenes (57, 58), respectively.

The nature of the intracellular signaling pathways mediating the proliferative effects of G proteins and their coupled receptors is still poorly understood. Whereas conventional second messenger-generating systems, such as adenyl cyclases, ion channels, and phospholipases, were the focus of the early research efforts addressing this issue (35, 59), an emerging body of information indicates that additional effector pathways participate in proliferative signaling by GPCRs (48). In particular, GPCRs have been shown to activate members of the extracellular signal-regulated kinases (ERKs) or mitogen-activated protein kinases (MAPks) family, which are key components of intracellular signaling pathways that control cell proliferation (60, 61). The elucidation of the molecular mechanisms whereby GPCRs activate MAPks is believed to be central to understanding how these receptors regulate cell growth and has recently become one of the most exciting areas of investigation in the G protein field.

The Classical MAPK Pathway

The initial search for candidate molecules mediating the mitogenic effects of growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which stimulate receptor tyrosine kinases, led to the identification of a tyrosine-phosphorylated protein of 42 kD (62, 63), which exhibited similar electrophoretic characteristics to those of a phototyrosine-containing molecule detected upon activation of PKc with phorbol esters (64) or in virally transformed cells (65). Soon after, this molecule was identified as p42MAPK and was shown to require a dual phosphorylation of both threonine and tyrosine for its full activation (66, 67). Molecular cloning of this molecule and a highly related protein, p44MAPK, revealed that they belong to a family of serine-threonine kinases related to the kinases Fus3 (a yeast MAPK involved in the pheromone response) and Kss1 (kinase suppressor of SST2, a yeast MAPK involved in filamentous growth) from the yeast Saccharomyces cerevisiae (68, 69), which act as the final step in a kinase cascade that participates in the pheromone-induced mating response (70). In mammalian cells, p44MAPK and p42MAPK, also known as ERK1 (extracellular signal-regulated kinase 1) and ERK2, respectively, referred to herein as MAPks, are believed to be central components of proliferative pathways. Their enzymatic activity increases in response to mitogenic stimulation, impeding their function prevents cell proliferation in response to many growth factors (71), and constitutive activation of molecules acting upstream of MAPks is sufficient for tumorigenesis (72, 73).

How growth factor receptors of the tyrosine kinase class activate MAPks appears now to be relatively well understood. For example, binding of EGF to its cognate receptors leads to the tyrosine phosphorylation of several substrates including the EGF receptor (EGFR) itself. Such phosphorylation sites serve as docking sites for the binding of adapter proteins that contain structural motifs involved in protein-protein interaction. These adapter proteins include Grb2 and SEM5 (73), which possess a Src homology 2 (SH2) domain and two SH3 domains, and Shc (an adapter protein in the Ras pathway), which possesses a phototyrosine binding (PTB) domain, a SH2 domain, and a SH3 domain (74). Both PTB and SH2 domains bind phototyrosine-containing polypeptides (75, 76). Shc is also a substrate for the EGFR, and upon tyrosine phosphorylation binds to the SH2 domain of Grb2 (77). Thus, activation of EGFRs results in the association of Grb2 to the EGFR, which also results in the recruitment of SOS, which binds to the SH3 domains of Grb2 (78) (Fig. 1). SOS stimulates the exchange of GDP bound to Ras for GTP (78) and initiates a protein kinase cascade that includes, sequentially, a MAPK kinase kinase (MAPKKK), which phosphorylates and activates a MAPK kinase (MAPKK), which in turn phosphorylates and activates a MAPK. MAPks then phosphorylate and regulate the activity of key enzymes and nuclear proteins, which can ultimately regulate the expression of genes essential for cell proliferation (60). A-Raf, B-Raf, and Raf-1 are examples of MAPKKKs; MEK1 and MEK2 are examples of MAPKks.

Signaling by G Protein-Coupled Receptors to the MAPK Pathway

The activation of MAPks in response to agonists acting on GPCRs is well documented. These agonists include a variety of GPCR ligands of very diverse structure, including polypeptides, such as bombesin, endothelin-1, somatostatin, interleukin-8, luteinizing hormone-releasing hormone (LHRH), thyrotropin-releasing hor-
Fig. 1. Multiple pathways linking G protein-coupled receptors to the MAPKs. Biochemical routes stimulating Ras, as well as novel pathways acting on Rap1, are depicted (see text for details). Arrows, positive stimulation; blocked lines, inhibition.

Several laboratories have explored whether βγ heterodimers participate in signaling to the MAPK pathway. Indeed, overexpression of βγ subunits was found to be sufficient to stimulate MAPKs (81, 82). Furthermore, stimulation of MAPK activity by coexpressed βγ dimers did not require PKC activation, but involved the activation of Ras (82, 83). Taken together, these findings suggested that signaling from GPCRs to MAPK involves βγ heterodimers acting on a Ras-dependent pathway, thus providing the first indication that βγ subunits link G proteins to small GTPases of the Ras superfamily. Furthermore, these findings established that the signaling pathway linking GPCRs to MAPK converges with that used by receptor tyrosine kinases at the level of Ras (Fig. 1).

A role for nonreceptor tyrosine kinases.

The observation that genistein, a relatively nonspecific tyrosine kinase inhibitor, diminishes the activation of MAPK by LPA provided the first indication that tyrosine kinases have a role in the activation of MAPKs by GPCRs (86). Soon after, it was shown that stimulation of GPCRs could promote the rapid phosphorylation of Shc on tyrosine residues and the consequent formation of Shc-Grb2 complexes (87, 88). Several nonreceptor tyrosine kinases have been proposed to initiate this response. For example, Src or Src-like kinases have been found to mediate the phosphorylation of Shc by GPCRs and βγ subunits (89), and inhibitors of Src-like kinases diminish the activation of MAPK by Gαζ- and Gζ-coupled receptors (90). However, how Src is activated in response to GPCR agonists is not fully understood. Interestingly, studies on the mechanism by which β-adrenergic receptors stimulate MAPK may provide some clues (91). In this case, binding of β-adrenergic agonists to their cognate G protein-linked receptors results in the rapid phosphorylation of the agonist-occupied receptor by the G protein-coupled receptor kinase (GRK) and the consequent recruitment of a protein known as arrestin to the GRK-phosphorylated receptor. In turn, arrestin functions as an adapter protein, binding both Src and the agonist-occupied receptor, thereby promoting the recruitment of the Src kinase to the membrane and its consequent activation (91). The subsequent internalization of β-adrenergic receptor-arrestin-Src complexes and their accumulation into clathrin-coated vesicles appear to be required for the activation of MAPK (91). The process of receptor internalization (and signaling to MAPK) involves the phosphorylation of dynamin I, a GTPase that participates in the formation of clathrin-coated vesicles, by Src (92). However, receptor endocytosis may not be necessary for the stimulation of MAPK by other GPCRs (93, 94). Pertur-
bation of the clathrin-mediated endocytic pathway may affect the ability to stimulate MAPK even when provoked by receptors that do not undergo endocytosis (95). Furthermore, dominant negative mutants of dynamin I may prevent the activation of MAPKs by additional mechanisms that are independent of its ability to inhibit the formation of endocytic vesicle and GPCR internalization (96, 97). Thus, components of the endocytic machinery may play multiple roles in signal transmission. Their relative contribution to the activation of MAPKs by GPCRs and other cell surface receptors in each cellular system will certainly be the focus of further exploration.

Signaling from GPCRs to MAPK may not require the activation of Src and the phosphorylation of Shc and dynamin I in certain cell lines (98, 99). For example, in some cellular systems the tyrosine phosphorylation of another dynamin isoform, dynamin II, by a yet to be identified kinase may play a key role in signaling from LPA receptors to MAPK (100). Furthermore, in lymphoid cells deletion of Csk (COOH-terminal Src family kinase) or the Src-like tyrosine kinase Lyn prevents the activation of MAPKs by Gq- but not Gi-coupled receptors; however, in cells lacking the Bruton’s tyrosine kinase (Btk), only the Gq-dependent response is affected (101, 102). In contrast, in lymphoid cells lacking the nonreceptor tyrosine kinase Syk, both Gq- and Gi-dependent responses were inhibited (101, 102). As Btk and Syk exhibit a very restricted tissue distribution, these observations suggest that GPCRs might use highly specialized pathways in certain cell types. Indeed, whereas activation of Syk by GPCRs has been reported in some cells (103), this kinase or the highly related kinase Zap70 are activated in mast cells and T cells, respectively, after stimulation of multimeric cell surface receptors but not in response to GPCRs (104, 105).

Another nonreceptor tyrosine kinase implicated in the activation of MAPK by GPCRs is Pyk2, a Ca2+- and PKC-dependent kinase, which can mediate the activation of MAPK by both Gq- and Gi-coupled receptors when [Ca2+]i is increased as a consequence of PLC activation by βγ subunits or by Gαq (106–108). This appears to be particularly important in certain frequently studied neuronal-like cell lines, such as PC12 cells (106, 107, 109). Pyk2 is closely related to FAK (focal adhesion kinase), which is part of the focal adhesion complex. In this case, integrin engagement leads to the recruitment of a multimeric intracellular complex that includes FAK and Src, and leads to the phosphorylation of FAK in multiple sites, thereby creating docking sites for many adapter proteins, including Grb2, paxillin, p130Cas, and Src (110, 111). FAK was also shown to be activated by Gq- and Gi-coupled receptors (112, 114), and thus may represent an additional candidate to mediate in GPCR signaling to MAPK (109).

A role for receptor tyrosine kinases. Certain growth factor receptor tyrosine kinases have been shown also to participate in GPCR signaling. For example, PDGF receptors (PDGFR) and EGF receptors (EGFR) become tyrosine-phosphorylated upon GPCR stimulation (115-118) and participate in MAPK activation by GPCRs. In this case, the phosphorylation of tyrosine kinase receptors would be expected to provide docking sites for the recruitment of signaling complexes leading to Ras activation. How these growth factor receptors are activated in response to GPCR stimulation is still unclear. One such possibility involves the activation of Src and the phosphorylation of EGFR by this nonreceptor tyrosine kinase (119). Another interesting possibility is that activation of GPCRs may lead to the proteolytic cleavage of latent agonist for the receptor tyrosine kinase. For example, transforming growth factor-α (TGF-α), amphiregulin, heparin-binding-epidermal growth factor (HB-EGF), and others are produced as inactive, membrane-spanning prohormones that are processed and released through regulated proteolysis (120) by metalloproteases, most likely of the ADAM (a disintegrin-like and metalloprotease domain-containing protein) family (121) (Fig. 1). The activation of such prohormones might stimulate receptor tyrosine kinases in an autocrine manner and also act on receptor tyrosine kinases expressed by neighboring cells. Such a mechanism of “inside-out” communication between GPCRs has been recently demonstrated (122). In this case, EGFR transactivation upon GPCR stimulation was shown to involve the cleavage of pro HB-EGF by a metalloproteinase activity that is rapidly induced upon GPCR-ligand interaction. Activation of MAPK itself can result in the shedding of HB-EGF (123). Thus, whether the activation of EGFR is the cause or the consequence of MAPK stimulation by GPCRs warrants further investigation.

We conclude that GPCRs make extensive use of nonreceptor and receptor tyrosine kinases that, upon autophosphorylation or phosphorylation of membrane-bound substrates, create docking sites where proteins containing phosphotyrosine-binding domains (PTB and SH2 domains) can assemble in multimeric signaling complexes leading to the recruitment of SOS and the consequent activation of Ras. It is not, therefore, surprising that the nature of the endogenous tyrosine kinase(s) used in this biochemical route is frequently dictated by their availability or the availability of yet to be identified accessory molecules in each cellular setting. For example, detailed analysis of some of the candidate tyrosine kinases (Src, Pyk2, and EGFR) revealed their distinct contribution in signaling to MAPK in a set of commonly used cell lines (109). In this case, EGFR and Pyk2 were found to be critical for MAPK activation by Gq- and Gi-coupled receptors in Rat 1 and PC12 cells, respectively, whereas both played partial roles in HEK 293 cells; and Src activation was important in all cases (109). These data illustrate how the relative contribution of tyrosine kinases and their downstream targets can be strictly tissue and cell type specific. This concept emerges from what, at first glance, would appear to represent a collection of seemingly conflicting reports (124).

Signaling through Ras-GRF. Although the complexity of tyrosine kinase pathways activated by GPCRs appears to be perplexing, in-depth analysis of signaling molecules used by GPCRs to stimulate MAPK in a variety of cellular systems is now providing the first glimpse of the existence of additional biochemical routes that might cooperate with, or even act instead of, those described above to connect GPCRs to MAPKs. Regarding the pathway linking GPCRs and Gβγ to Ras and MAPKs, additional molecules include the protein tyrosine phosphatase SH-PTP1 (125), Ras-guanine-nucleotide releasing factor (Ras-GRF) (126), kinase suppressor of Ras-1 (KSR-1) (127), and PI3Kγ (128). Ras-GRF is a distinct Ras-guanine-nucleotide exchange factor expressed in neuronal cells, and its activity is enhanced in response to GPCR stimulation, or upon coexpression of Gβγ by a mechanism involving calcium and the direct phosphorylation of Ras-GRF by a yet to be identified kinase (126). A Ras-GRF-related protein, Ras-GRF2, is also expressed in nonneuronal-derived tissues (129) and might represent a good candidate to mediate the GPCR-initiated signaling in nonneuronal cells.

Signaling through PI 3-kinase. The early observation that
wortmannin, a PI3K inhibitor, diminishes MAPK activation by GPCRs (130) provided the first indication of a role for this lipid kinase in GPCR signaling to MAPK. A likely candidate to signal from GPCR to MAPK was the PI3K isoform PI3Kγ (131), which does not bind the p85 PI3K noncatalytic subunit and is not stimulated by tyrosine phosphorylation, but is activated upon physical interaction with Gβγ complexes (131). PI3Kγ was found to act downstream from Gβγ and upstream of Src-like kinases and Shc, Grb2, SOS, and Ras, suggesting a potential mechanism by which G proteins can regulate nonreceptor tyrosine kinases and, in turn, control the MAPK pathway (128). Interestingly, recent evidence suggests that the ability to stimulate MAPK by PI3Kγ can be dissociated from its lipid-kinase activity (132), which is consistent with the observation that PI3Kα cannot replace PI3Kγ function in the MAPK pathway, although PI3Kγ does increase endogenous levels of phosphatidylinositol trisphosphate (PIP3) (128). The ability to act as a protein kinase or as a scaffold for signaling molecules may explain the unique properties of PI3Kγ (133). Because this PI3K isoform exhibits a restricted tissue distribution, additional PI3K isotypes may participate in signaling through GPCRs, and accumulating evidence suggests that PI3Kβ can also be stimulated by GPCRs (134). The molecular dissection of the contribution of PI3Ks and their enzymatic products in signaling MAPK activation by GPCRs warrants further investigation.

**Signaling through phospholipase C.** The fact that Gq-coupled receptors can potently stimulate PLC-β suggests that the consequent stimulation of PKC may contribute to MAPK activation by this class of GPCRs. However, this is not a straightforward issue, as the mechanism of activation of MAPK by PKC is still unclear. PKCs can phosphorylate Raf (135, 136), but this alone does not result in an increased ability of Raf to phosphorylate MEK (MAPK or ERK kinase) (137). On the other hand, although DAG can stimulate the Ras exchange factor, Ras-GRP, expressed in lymphocytes (138, 139), PKC activation does not enhance the accumulation of Ras-GTP in the vast majority of cell types (140). Nonetheless, PKC stimulation activates MAPK in a Ras-dependent manner in several cellular settings (141-143). Furthermore, recently available data suggest that PKCs stimulate Ras and MAPKs by a mechanism distinct from those known to mediate Ras activation by receptor tyrosine kinases (144). Evidence for an increasing number of molecules that aid in the fine tuning of the Ras-Raf signaling system comes from the facts that (i) Ras alone is not sufficient to activate Raf fully in vitro (137), and (ii) Ras does not fully activate Raf when both are expressed in Sf9 cells (145). Thus, PKCs might act directly on Raf to facilitate full activation of Raf upon binding to Ras (143), as well as acting on the molecules involved in fine tuning the Ras-Raf interaction.

Gq-coupled receptors can activate MAPK in a PKC-dependent (142, 146), fully PKC-independent (147, 148), or partially PKC-dependent (149) manner. The requirement for PKC for MAPK activation might be highly dependent on the level of receptor expression and on the diversity of PKC isotypes and signaling molecules expressed in each cell type. Another possible explanation for this discrepancy lies in the observation that PLC-β, a downstream target for Gq, can also exert a potent GTPase-activating protein (GAP) activity on G proteins of the Gq family (150). At low receptor density or high PLC-β expression, GPCR stimulation would activate PLC-β through Gq, the PLC-β would enhance the rate of GTP hydrolysis by Gq, which would limit the availability of the βγ complex because of the reassociation of GDP-bound Gq with βγ complexes and allow only the PKC-dependent pathway to proceed. Thus, the PLC-β to PKC pathway would be active, but the βγ to Ras pathway would have insufficient time to occur under those conditions, resulting in only the PKC-dependent pathway of MAPK activation.

**Signaling through Rap.** Another mechanism by which G protein α subunits may also activate MAPK involves the regulation of the Ras-like GTPase Rap1. This molecule, originally identified as a biological antagonist of Ras (151), has received much attention, because it can block MAPK activation by competing with Ras for binding to Raf-1 and A-Raf, or it can stimulate MAPK through the activation of B-Raf (152, 153) (Fig. 1). A guanine-nucleotide exchange factor for Rap1, named Epac, has been identified and shown to be activated by cAMP in a PKA (cAMP-dependent protein kinase)-independent manner (154). Gαo, Gαq, and Gα13 can bind directly to Rap1 GTPases (155-157). In the case of Gα13, its GDP-bound inactive form binds Rap1GAP, thus preventing its activity and enhancing the accumulation of Rap1-GTP, which can stimulate MAPK in certain neuronal-derived cells (155). Gαo, on the other hand, binds an NH2-terminal extended form of Rap1GAP, termed Rap1GAPII, resulting in its enhanced activity and the consequent decreased levels of Rap1-GTP (156). In the latter case, this effect of Gαo was shown to contribute to the Gβγ-dependent activation of MAPK by decreasing the levels of Rap1-GTP that was acting as a Ras competitor. The activated form of Gαo also binds Rap1GAP, and forms stable molecular complexes with Rap1 (157), suggesting that Rap1 can participate in the regulation of MAPK by Gαo. These findings exemplify the complexity of the regulatory pathways used by GPCRs; these pathways act on molecules whose final function, inhibitory or stimulatory, on MAPK may be highly dependent on the cell type or tissue under investigation (Fig. 1).

**Signaling by G Protein-Coupled Receptors to Other MAPK-Related Pathways**

Activation of MAPKs frequently results in their rapid translocation to the nucleus, where they phosphorylate and regulate the functional activity of various transcription factors (158). Because GPCRs and tyrosine kinases converge at the level of Ras to activate MAPKs, activation of both classes of cell surface receptors was expected to elicit a similar response by nuclear transcription factors. However, this was found not to be the case (159).

**Signaling through the JNK pathway.** Expression of c-Jun (a transcription factor proto-oncogene) in NIH 3T3 cells was induced only by GPCR stimulation but not by receptor tyrosine kinases, and this response did not correlate with MAPK activation (159), suggesting that GPCRs control a distinct biochemical route regulating gene expression. A family of enzymes structurally related to MAPKs, termed c-Jun NH2-terminal kinases (JNKs) (160) or stress-activated protein kinases (SAPKs) (161), selectively phosphorylate c-Jun, thereby enhancing its transcriptional activity. Consistent with this finding and the earlier observation that c-Jun enhances c-jun expression (162), it was observed that GPCRs but not tyrosine kinase receptors potently activate JNK in murine fibroblasts (159).

These findings indicated that the GPCR signaling routes might diverge at the level of JNK from those used by tyrosine kinase receptors. An unexpected prediction from these studies was that a distinct set of upstream signaling molecules might regulate
JNK and MAPK. Two findings prompted several labs to examine whether the Rho family of GTPases participates in GPCR signaling to the JNK pathway: (i) signaling from GPCRs to Jun was MAPK independent, and (ii) Rac and Cdc42, two small GTPases of the Rho family, could control the activity of PAK1, a serine-threonine kinase, in a manner similar to that of Ras acting on Raf (163). Indeed, Rac1 and Cdc42 were found to initiate an independent kinase cascade regulating JNK activity (164, 165). Other components of this pathway include several MAPKKKs: MAPK or ERK kinase kinases (MEKK1, MEKK2, MEKK3, MEKK4, and MEKK5), ASK1 (apoptosis-stimulated kinase 1), MLK3 (mixed-lineage kinase 3), TPL2 (tumor progression locus 2, also known as Cot), TAK (TGF-β-activated kinase), MUK (MAPK upstream kinase), GCK (germlinal center kinase), and PAK, which can all contribute to activation of JNKs, apparently through two MAPKKKs, MKK4 (also called Sek or JNKK1) and MKK7 (166-169). So far, however, there is limited information regarding the precise architecture of the signaling pathways in which each of these kinases act. Substrates for JNK include transcription factors of the c-jun family, as well as other transcription factors, including Elk-1 and Elk-2, Sap-1 (serum response factor accessory protein-1), NFA4 (nuclear factor of activated T cells 4), and ATF2 (activating transcription factor 2) (169).

How GPCRs are linked to JNK is still unclear. Detailed examination of the pathway linking GPCRs to JNK provided evidence that free βγ dimers (170) and Gα12 or Gα13 (171-174) convey signals from this class of receptors to JNK. Furthermore, in all cases JNK activation was blocked in cells expressing dominant negative mutants of Rac1 or Cdc42 or both, suggesting a role for these GTPases in the activation of JNK by GPCRs. Regulation of GEFs may link βγ and Gα12 or Gα13 to Rac1 or Cdc42. Cumulative work of several laboratories, however, suggests that a number of molecules might play this role: (i) Ras-GRF1, which upon βγ binding, can act as a Ras GEF and induce guanine-nucleotide exchange on Rac1 through its Dbl homology domain (DH domain) (175); (ii) Ras-GRF2, which is highly related to Ras-GRF1 and can transmit calcium-initiated signals to Ras and Rac1 (176); (iii) Dbl, a GEF for Cdc42 and Rho that binds βγ subunits, although that does not result in a demonstrable increase in its GEF activity (177); and (iv) Tiam1, a Rac-specific GEF that is phosphorylated on threonine residues in a PKC-dependent manner in cells stimulated with LPA, bombesin, or bradykinin. Efforts to knock out the genes for these GEFs should provide valuable information regarding their relative contribution in signaling to small GTPases by GPCRs.

Recent studies suggest that βγ subunits activate JNK preferentially through MKK4 by stimulating Rho and Cdc42, and to a lesser extent through MKK7 acting downstream from Rac1 (178), whereas Gα12 uses Src-like kinases to stimulate JNK (179). In line with the latter observations, it has been recently shown that Pyk2 and FAK can also participate in signaling to JNK through the recruitment of an adapter protein Crk (180, 181) or paxillin (182), both of which are expected to activate GEFs for small GTPases of the Rho family (183, 184). Although a preliminary model of how GPCRs can stimulate small GTPases of the Rho family to activate JNK is depicted in Fig. 2, this model is likely to be redefined in the foreseeable future, when more biological and biochemical data become available.

**Signaling through the p38 and ERK5 pathways.** Much less is known about the molecular mechanisms connecting GPCRs to each member of the p38 family of kinases related to MAPKs and to ERK5. In particular, the family of p38 kinases has grown rapidly over the past few years. To date, four p38 kinases have been described and named p38α (or CSBP-1), p38β, p38γ (also known as SAPK3 or ERK6), and p38δ (or SAPK4), some of which also have splice variants (169). This family of proteins exhibits a common Thr-Gly-Tyr phosphorylation motif and shows a pattern of activation by stress insults and cell surface receptors very reminiscent of that displayed by JNKs (185). Although the upstream activators for these kinases are not yet well defined, potential p38 MAPKKKs include ASK1 and TAK1 (169). MKK6 seems to be a very general stimulator for p38s, whereas MKK3 and MKK4 preferentially phosphorylate p38α (186). The specific transcription factors that can be regulated by p38s include cAMP responsive element binding protein (CREB), ATF1 (activating transcription factor 1), ATF2, Max, CHOP (C/EBP homologous protein), and MEF2C (169). In addition, these MAPKs can also trigger the activation of other serine-threonine kinases such as Mnk1 (MAPK-interacting kinase 1) and Mnk2 and MAPAPKs (MAPK-activated protein kinases) (169).

Although the activation of p38 by GPCRs has been documented in primary cells, immortalized cell lines, perfused organs, and even after in vivo manipulations (186-189), there is very limited knowledge on the mechanism of activation of this particular family of MAPKs. The p38α enzyme is activated by both Gαq and Gβγ (197), and a role for the tyrosine kinase Btk in Gβγ-mediated stimulation has been suggested from observations in lymphocytic cells (198). In addition, it has been shown that Gα12 and Gα13 can activate the putative MAPKKK, ASK1, although the activation of p38s was not examined (199). Electrophysiological studies have implicated p38s downstream from Gα13 (200). Gβγ-coupled receptors can activate not only p38α and p38β, but also the most distantly related p38γ and p38δ enzymes (201). We can conclude that how GPCRs activate p38 MAPKs is still far from being fully understood, thus warranting further investigation.

A MAPK distantly related to p42MAPK and p44MAPK has recently been characterized and termed BMK1 (big mitogen-activated protein kinase 1) or ERK5. It contains a Thr-Glu-Tyr motif in its activation loop, similar to that of MAPKs (202). This kinase is larger than any other known MAPK (~80 kD) and is selectively activated by MEK5 (203). ERK5 can be stimulated by oxidative stress, and may also play a role in early gene expression triggered by serum by directly phosphorylating the transcription factors MEF2C (203) or c-Myc (204). Interestingly, the search for the mechanism whereby GPCRs regulate c-jun expression revealed a key role for transcription factors of the MEF2 family in the transcriptional response from the c-jun promoter (205). Furthermore, it has been recently observed that GPCRs can potently activate ERK5, and that this MAPK links GPCRs to the transcriptional activation of MEF2A and MEF2C, and to c-jun expression (207). Current work suggests that GPCRs activate ERK5 through Gαq and Gα13 by a still unknown pathway (206).

**Scaffolding Proteins Provide Signal Specificity: A Lesson from Yeast**

Given the increasing complexity of the biochemical routes regulating MAPKs, it is difficult to envision how the selectivity and specificity in signal transduction can be achieved. However, the recent identification of molecules capable of binding components of MAPK cascades suggests a potential role for scaffold-
ing proteins as the physical basis for the specificity of signal transmission in mammalian cells. This is highly reminiscent of that in the yeast MAPK cascades, where specificity is achieved by the assembling of hierarchical protein modules. For example, in *Saccharomyces cerevisiae*, mating factors (α or a) activate G protein-linked pheromone receptors which, in turn, induce the dissociation of a G protein into α (GPA1) and βγ (Ste4 and Ste18) subunits (70). Free βγ dimers then activate a serine-threonine kinase, Ste20, a process that involves a Cdc42 GEF, Cdc24 (207), and Cdc42 (208), thereby stimulating the activity of a linear cascade of kinases including, sequentially, Ste11 (a yeast MAPKKK) and Ste7 (a yeast MAPKK), which phosphorylate and activate the yeast MAPK homologs Fus3 and Kss1. The connection of Gβγ to small GTPases represents a biologically relevant example of a pathway extraordinarily conserved throughout evolution. Available data indicate that additional molecules might be also involved to organize these kinase cascades, including a protein designated Ste5, which binds yeast βγ and plays a role as a platform or scaffold recruiting Ste11, Ste7, and Kss1 and Fus3 (209, 210). The Ste5, βγ, Ste11, Ste7, Kss1, Fus3 complex represents a prototypic MAPKKK-MAPKK-MAPK module. For another yeast MAPK pathway, the p38 osmosensor system, the scaffolding capability lies within Pbs2, a MAPKK that is able to link together the osmosensitive receptor, the most upstream MAPKKK, Ste11p, and the p38 MAPK, HOG1p (high-osmolarity glycerol response kinase) (211).

In the mammalian MAPK pathway, Ksr (212) and MP1 (213) can bind both MAPK and its upstream activator MEK1, and have been proposed to act as scaffold polypeptides in this cascade. For the JNK pathway, two proteins, JIP (JNK inhibitory protein) and JSAP (a novel JNK inhibitory protein), perform this function, bridging together MLKs, M KK7, and JNK (214, 215), and MEKK1, SEK (stress-activated protein kinase kinase or ERK kinase), and JNK (214, 216), respectively. We can expect that additional scaffolding molecules will soon be identified, thus providing the molecular basis for how the selectivity and specificity can be maintained when transmitting signals from the membrane to the nucleus.

**Conclusion: G Protein-Coupled Receptors and MAPKs: Signal Transduction or Signal Integration?**

Over the last few years we have begun to appreciate the complexity of the signaling pathways by which cell surface GPCRs transmit signals to the nucleus. In the simplest possible model, GPCRs activate G proteins, thereby causing their dissociation into α-GTP and βγ subunits. Each G protein subunit can then activate a specif-
fic set of small GTP-binding proteins of the Ras and Rho families which, in turn, control the activity of parallel kinase cascades, resulting in the phosphorylation of key nuclear transcription factors.

However, the stimulation of GPCRs leads to the activation of a number of second messenger-generating systems and can even affect the activity of other cell surface molecules, including integrins and growth factor tyrosine kinase receptors. This can have a remarkable impact on signal transmission, as these receptors and their associated intracellular molecules can provide docking sites for the assembly of distinct multimeric signaling complexes. Furthermore, classical second messengers can also affect the duration and even the final biochemical or biological response. For example, changes in intracellular [Ca\textsuperscript{2+}] can affect the duration and even the final biochemical or biological response. For example, changes in intracellular [Ca\textsuperscript{2+}], caused either by G\textsubscript{q} or by G\textsubscript{12} subunits, might exert a profound effect on many aspects of signal transmission. Similarly, many GPCRs can stimulate adenyl cyclases (even if not coupled to G\textsubscript{s}), and the accumulation of cAMP can have a remarkable impact on signal transmission, as these molecules expressed in each particular cellular setting (81, 224-229). Thus, changes in the intracellular concentrations of cAMP are expected to initiate a complex series of events that influence MAPK cascades.

While evidence of a possible cross-talk among MAPK cascades is already emerging (230), we now also begin to understand the complex interactions among molecules that bind to regulatory elements within the promoter region of growth-regulating genes. Activation of the Rac- or Cdc42-JNK pathway by GPCRs through G\textalpha\textsubscript{12} or G\textalpha\textsubscript{13} and by dimers would be expected to regulate the c-jun promoter through an AP1 (activating protein 1) or ATF2 sites (231); however, the data suggest that JNK, p38\alpha, p38\gamma, and ERK5 all cooperate through the activation of the AP1 and ATF2 sites and an adjacent MEF2 site to activate the c-jun promoter (201). Thus, we can conclude that in this case GPCRs control the expression of c-jun through several independent MAPK cascades that converge in the nucleus to stimulate transcription factors acting on its promoter (Fig. 2).

Similarly, the c-fos (a transcription factor proto-oncogene) promoter contains a serum response element (SRE) that can be stimulated by either MAPK or JNK, which stimulates the ternary complex factor (TCF) p62\textsuperscript{TCF} (167, 232-234). The p62\textsuperscript{TCF} protein binds the SRE in cooperation with the serum response factor (SRF). SRF can also be stimulated by a yet to be identified pathway initiated by Rho, thereby affecting the expression of genes containing SRE elements within their promoter region (235). However, the c-fos promoter also contains many other sites adjacent to the SRE (236-238), suggesting that the regulation of the c-fos promoter may be more complex than anticipated, involving multiple interactions among its binding proteins. Taken together, the emerging picture is that activation or repression of gene expression results from the activity of a number of interlinked regulatory molecules and pathways, rather than from a single linear series of signaling events.

We conclude that the activation of GPCRs can affect the activity of a highly interconnected network of cytoplasmic signaling pathways. Depending on the G protein-coupling specificity and the repertoire of available signaling molecules, this can lead to a temporally distinct pattern of activation of each member of the MAPK superfamily. Ultimately, these kinases can modify key cytoplasmic molecules as well as translocate to the nucleus where they phosphorylate nuclear proteins, including transcription factors, thereby affecting an intricate balance of regulatory molecules controlling gene expression. The recent explosion of knowledge on the basic mechanisms regulating signal transduction now affords the unique opportunity to begin unraveling the intricacies of how these signals are integrated in space and time to elicit a final biological response. This knowledge may also help us to understand how subtle perturbation of this signaling network can result in pathological situations, thus providing golden opportunities to identify novel molecular targets for pharmacological intervention in a variety of diseases.


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