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Integration of Signals from Receptor Tyrosine Kinases and G Protein-Coupled Receptors

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Key Words

G protein · Receptor · Tyrosine kinase · MAPK · Signaling

Abstract

Activation of G protein-coupled receptors (GPCRs) leads to stimulation of classical G protein signaling pathways. In addition, GPCRs can activate the mitogen-activated protein kinases (MAPKs) such as the extracellular signal-regulated kinases, c-Jun NH_2-terminal kinases (JNKs), and p38 MAPKs, and thereby influence cell proliferation, cell differentiation and mitogenesis. Cross talk between GPCRs and receptor tyrosine kinases (RTKs) is an incredibly complex process, and the exact signaling molecules involved are largely dependent on the cell type and the type of receptor that is activated. In this review we investigate recent advances that have been made in understanding the mechanisms of cross talk between GPCRs and RTKs, with a focus on GPCR-mediated activation of the Ras/MAPK pathway, GPCR-induced transactivation of RTKs, GPCR-mediated activation of JNK, and p38 MAPK, integration of signals by RhoGTPases, and activation of G protein signaling pathways by RTKs.

Introduction

Extracellular messages are transduced into intracellular signals via several classes of receptor molecules. These include the G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), cytokine receptor-activated kinases, and members of the steroid/thyroid hormone receptor superfamily. Originally these systems were thought to act in isolation, but it has become increasingly clear over the past decade that there are complex interactions between each of these pathways. Indeed, protein kinases from one signaling pathway can phosphorylate components of other signaling pathways, and some proteins can be phosphorylated by several different protein kinases. Cross talk between pathways allows cells to integrate information from many different sources, providing an incredibly intricate level of control over all the regulatory systems of the cell. Comprehending the complexities of cross talk between the different signaling pathways has been the focus of much recent research, and different aspects of this phenomenon are conversed in several other articles in this issue. Understanding these processes is of great interest since disorders of cell proliferation and differentiation are fundamental to diseases such as cancer. This paper will focus on one specific area of cellular signaling, the integration of signals from RTKs and GPCRs.

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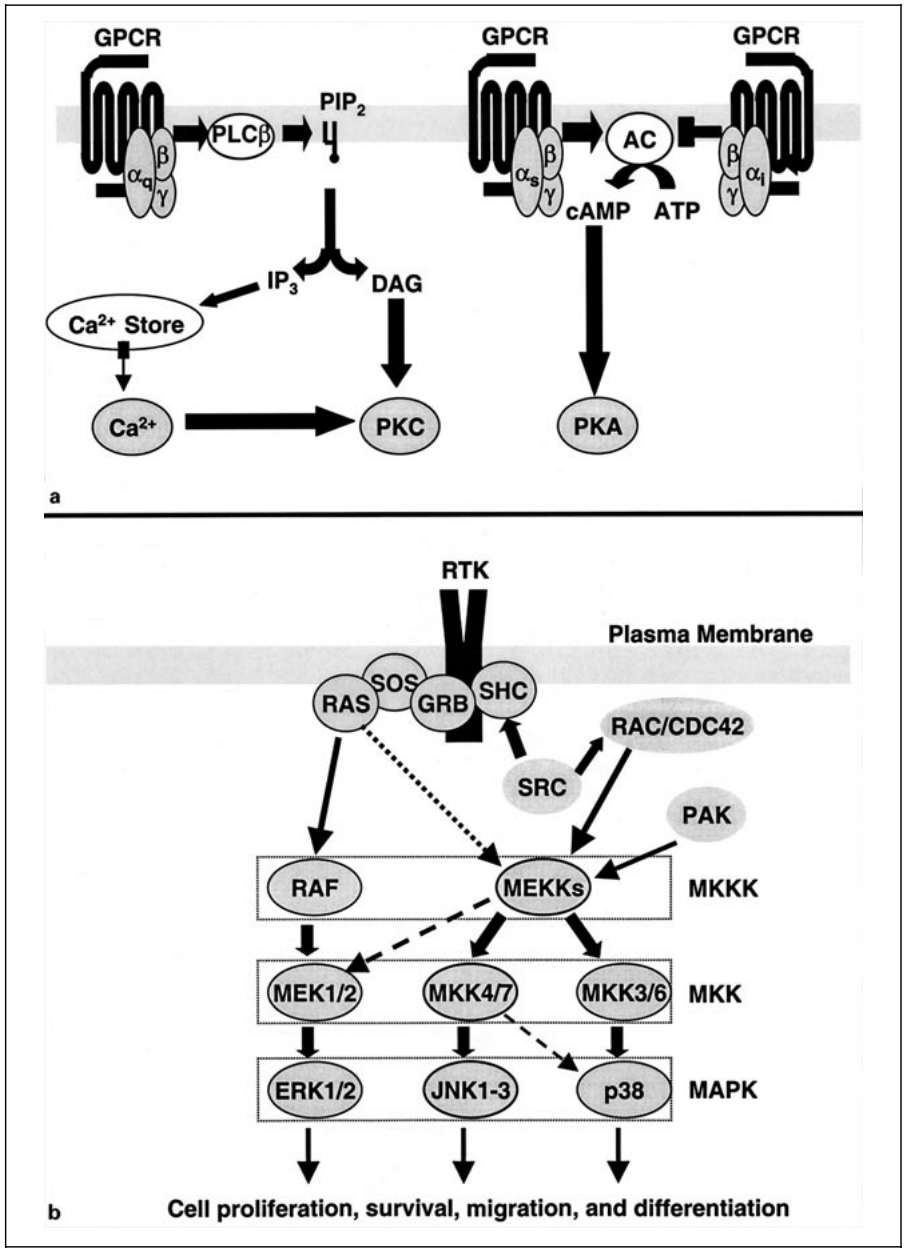

Fig. 1. Schematic diagram illustrating the main GPCR signaling pathways (**a**) and the RTK signaling network (**b**). Items shaded grey are involved in cross talk between GPCRs and RTKs. See text for further details.

We will first briefly review the basic principles of GPCR and RTK signaling, before tackling the complex issue of cross talk between these two pathways.

Signaling by GPCRs and RTKs

The heterotrimeric G proteins transmit signals from membrane-bound GPCRs to intracellular targets, such as ion channels and enzymes. G proteins are composed of three separate subunits: the Gα subunit, which binds GDP/GTP, and the Gβ and Gγ subunits, which form a tightly bound Gβγ complex. Activation of a GPCR induces a conformational change in the Gα, causing GDP to be released and GTP to bind in its place. The Gα and Gβγ subunits then dissociate from the receptor and interact with a variety of effector molecules. Hydrolysis of GTP causes the Gα subunit to return to its inactive (GDP-bound) conformation, and triggers reassembly of the Gαβγ heterotrimer. This ensures that cellular activation is

only a transient event. The G proteins are classified into four different families – G_s, G_i, G_q, and G_{12}. The primary effect of the G_s family is to stimulate the intracellular messenger adenylyl cyclase (AC), which catalyzes the conversion of cytoplasmic ATP to cyclic AMP (cAMP), whereas the G_i family inhibits this pathway. In contrast, the G_q family activates phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol-1,4,5-phosphate (IP_3) and diacylglycerol (DAG) (see fig. 1a). G proteins and their intracellular targets are involved in a multitude of biological functions, including cell growth, cell differentiation, neurotransmission, smell, taste, chemotaxis, oncogenesis, and others [as reviewed in 1, 2]. GPCRs are thus a major therapeutic target for a wide variety of diseases, and more than 1,000 GPCRs have been identified.

RTKs form part of the enzyme-linked family of receptors, and include the majority of growth factor receptors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and nerve growth factor (NGF). The intracellular domain of RTKs acts as a tyrosine protein kinase. Activation of an RTK by an external signaling molecule causes two receptor molecules to dimerize and activates their intrinsic tyrosine kinase activity, resulting in transphosphorylation of tyrosine residues present in their cytoplasmic domain. The phosphorylated tyrosine residues are then recognized by intracellular signaling molecules to propagate the signal. Many different RTKs share similar signaling pathways, and the specific biological responses to activation of different RTKs seem to be attributed to a specific cell context rather than specific signaling pathways. One example is the Trk receptors, the RTKs that are activated by the neurotrophins. Trk receptors are prominently expressed in the nervous system, and their activation by neurotrophins leads to neuronal differentiation [reviewed in 3]. However, when ectopically expressed in fibroblasts, activation of Trk receptors can enhance proliferation in a manner similar to that elicited by other RTKs such as fibroblast growth factor receptors [4]. Cellular context may also affect the ability of RTKs to respond to their nonpreferred ligands, as exemplified by the observation that the RTK TrkB can be activated by the neurotrophin NT-3 in fibroblasts but not in the neuronal cells PC12 [5].

The main signaling pathway, and by far the best understood, linking activation of RTKs to the nucleus is via Ras, a small membrane-bound monomeric GTP-binding protein. Ras is coupled to RTKs by a short series of linking proteins, a SH2-containing adaptor protein (Grb2) that is activated by the RTK, and a Ras-activating protein mSos (the mammalian equivalent of 'son of sevenless', a protein involved in photoreceptor development in *Drosophila*) that is activated and localized to the membrane by adaptor proteins such as Grb2 and Shc. Following activation, Ras stimulates a cascade of serine/threonine protein kinases, called the mitogen-activated protein kinases (MAPKs), which transmit the message into the nucleus (see fig. 1b). These include mitogen-activated protein kinase kinase kinase (MAPKKK or MKKK; also called Raf), which phosphorylates MAPK kinase (MAPKK or MKK; also called MEK), which in turn phosphorylates MAPK. MAPK then phosphorylates various gene regulatory proteins, altering their ability to regulate gene transcription and thereby influencing cell proliferation, survival, migration and/or differentiation [as reviewed in 6]. This MAPK cascade is highly conserved amongst eukaryotic species.

There are at least three different MAPKs. These include the extracellular signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs) and the p38 MAPKs. Within each of these subtypes many variants have been classified, with 12 different members of the MAPK family, 7 MAPKKs, and 14 MAPKKKs identified to date in mammalian cells [as reviewed in 7]. The specificity of MAPK interactions, and the effector molecules they stimulate, depends largely on which MAPK subtypes are stimulated. For instance, ERKs are stimulated primarily by growth factors and modulate cell growth and differentiation, whereas JNKs and p38 MAPKs are activated most commonly by stress (such as irradiation and heat shock) and are involved in cell growth, differentiation, survival, apoptosis, and cytokine production.

Signal Integration between GPCRs and RTKs

GPCRs are involved in mitogenesis [8] and cellular transformation [9], and mutations in GPCRs have been linked to the formation of tumors in many different cell types [10, 11]. Thus, G proteins and their receptors must somehow be involved in normal and abnormal cell growth. The obvious question is how do GPCRs influence cell growth? There is much evidence showing substantial integration and cooperation between the GPCR and RTK signaling pathways, and GPCRs appear to use RTKs as signaling intermediates [12] to mediate cell growth. Cross talk between the two pathways is multifaceted and extremely complex (fig. 2).

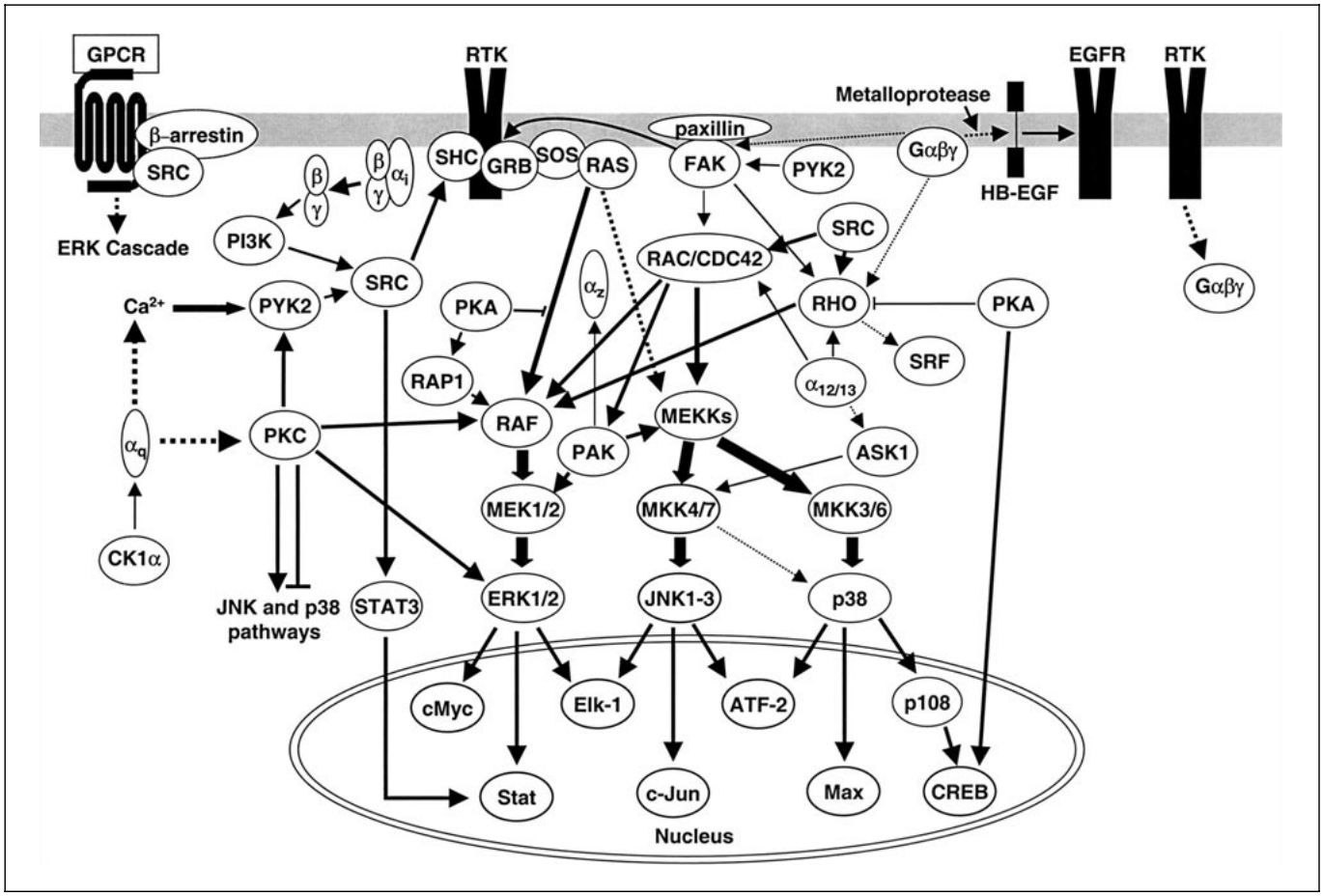

Fig. 2. Cross talk between GPCR and RTK signaling pathways. Also shown are the complex roles played by nonreceptor tyrosine kinases such as Src, Pyk2 and FAK, as well as members of the Ras and Rho family of low molecular weight GTP-binding proteins, in linking these cascades together. Solid arrows represent a direct connection, whereas dashed arrows indicate an indirect link. The thickness of the arrow is an indicator of the relative importance of the pathway. The diagram does not attempt to represent all aspects of cross talk between the GPCR and RTK signaling pathways, but rather focuses on the main topics covered in the text. For further details on the interactions between individual components refer to the text and figure 1.

GPCRs Activate the Ras/MAPK Signaling Pathway

A number of GPCRs, such as the adenosine A_1, endothelin-1, thrombin, bombesin, lysophosphatidic acid (LPA), α-adrenergic, muscarinic acetylcholine (mACh), bradykinin, thromboxane A_2 and dopamine receptors, can activate MAPKs to regulate cell proliferation and differentiation [as reviewed in 13]. How GPCRs activate MAPK appears to be a function of the cell type, as well as the receptors and G proteins available in each individual cell line.

Activation of G_i-Coupled Receptors. There is convincing evidence that activation of MAPK by G_i-coupled receptors is mediated via Gβγ subunits. Crespo et al. [14] demonstrated that ligand-induced activation of G_i-coupled M_2 muscarinic receptors in COS-7 cells increases phosphorylation of ERK2, and coexpression of the M_2 receptor with Gβγ in these same cells markedly stimulates the activity of ERK2. Such a role for Gβγ in ERK stimulation following activation of G_i-coupled receptors has been confirmed in many studies [15–17]. The process by which Gβγ stimulates MAPK is complex. Activation of several G_i-coupled receptors increases phosphorylation of the adaptor protein Shc, leading to increased Shc-Grb2-Sos complex formation and subsequent activation of Ras and the MAPK cascade [15, 16, 18]. Expression of Gβγ alone can also stimulate Shc activation, and Shc phosphorylation by G_i-coupled receptors can be inhibited by coexpression of a Gβγ binding peptide (β-adrenergic receptor

kinase-carboxy terminus or βARKct) [15, 16]. In HEK293 cells, α_{2A}-receptor-stimulated phosphorylation of ERK1/2 is blocked by βARKct, and by the expression of dominant negative mutants of Ras, mSos1, and Raf [17], further supporting a model of Gβγ-induced phosphorylation of Shc and activation of the Ras/MAPK pathway. Crespo et al. [14] proposed that the Gβγ subunits might bind to the pleckstrin homology domain of one or more Ras-regulatory proteins, and thereby influence Ras function.

In COS-7 cells inhibition of the endogenous activity of Src, a nonreceptor tyrosine kinase, by using a dominant negative mutant of c-Src, also inhibits Gβγ and G_i-coupled receptor-mediated phosphorylation of Shc, implicating a role for Src in Shc activation [19]. Furthermore, wortmannin, which inhibits phosphatidylinositol 3-kinase (PI3K), inhibits Gβγ-mediated Shc phosphorylation and MAPK activation, and expression of an inactive mutant of PI3Kγ (an isoform of PI3K that is activated by Gβγ) blocks stimulation of MAPK by Gβγ [16, 20]. The SH2 domain of c-Src can bind to phosphatidylinositol 3,4,5-triphosphate, the product of PI3K [21]. Thus, it could be that G_i-coupled receptors activate Src via Gβγ stimulation of PI3K.

Activation of G_s-Coupled Receptors. Agonist-induced activation of G_s-coupled receptors also stimulates MAPK activity [22–24]. In COS-7 cells, one report has demonstrated that increases in cAMP can increase MAPK activity [22], whereas another has reported that cAMP and protein kinase A (PKA) inhibit isoproterenol and growth-factor-stimulated MAPK activation [23]. In these same cells, expression of $G\alpha_s$ does not stimulate MAPK activity [14, 22, 23], but treatment of the cells with the Gβγ-binding βARKct, or expression of the Ras-inhibitory molecules *ras* N17 or Rap-1a, inhibits β-adrenergic receptor-mediated activation of MAPK [23]. These results suggest that $G\alpha_s$ and Gβγ have opposing effects on MAPK activation, with the Gβγ subunit activating the MAPK pathway via Ras and $G\alpha_s$ inhibiting MAPK activation through cAMP and PKA. The balance between these two pathways likely determines the final signaling response.

Quite a different role for the $G\alpha_s$ subunit has been proposed in mouse lymphoma cells. In studies using $G\alpha_s$-deficient S49 mouse lymphoma cells (*cyc⁻*), $G\alpha_s$ was found to be necessary for β-adrenergic receptor-mediated activation of MAPK, whereas Gβγ was found to play a structural but not a transducing role. Activation of MAPK via $G\alpha_s$ was dependent on PKA, and this pathway involved not Ras but rather a small G protein Rap-1. Rap-1 is thought to directly activate Raf, leading to MAPK activation [24].

In Rat-1 fibroblasts, increasing the level of cAMP by pretreatment of the cells with either forskolin (an activator of AC) or IBMX (a cAMP phosphodiesterase inhibitor) almost completely abolishes EGF-induced stimulation of Raf-1, MEK, and MAPK. cAMP has no effect, however, on EGF-induced RTK phosphorylation, association of the EGF receptor (EGFR) with Grb2 and Shc, or activation of Ras, suggesting that in these cells cAMP inhibits activation of the MAPK pathway by acting downstream of Ras, likely by inhibiting Ras-induced activation of Raf-1 [25, 26]. Such a role for cAMP is in agreement with studies showing that cAMP inhibits proliferation in Rat-1 cells [27].

Finally, in HEK293 cells, agonist-induced stimulation of the G_s-coupled β_2-adrenergic receptor activates MAPK. This MAPK activation is sensitive to pertussis toxin (PTX, which ADP-ribosylates G_i and G_o proteins) and βARKct, suggesting an involvement of the Gβγ subunit of G_i-coupled receptors. The authors suggested that in these cells PKA-mediated phosphorylation of the β_2-adrenergic receptor acts as a 'molecular switch', serving to couple the receptor to G_i proteins and leading to βγ-mediated activation of the ERK1/2 pathway [28]. In summary, the role of cAMP and PKA in MAPK activation is a function of cell type. Such diverse roles are not surprising considering that the effects of these intracellular signaling agents on cell proliferation vary incredibly, with cAMP stimulating, inhibiting, or having no effect on proliferation, depending on the tissue and cell type.

Activation of G_q-Coupled Receptors. In contrast to G_i- and G_s-coupled receptors, MAPK activation by G_q-coupled receptors involves activation of protein kinase C (PKC). Activation of G_q-coupled M_1 muscarinic receptors stimulates ERK2 activity in COS-7 cells, as does expression of $G\alpha_q$ or treatment with the PKC activator phorbol ester [14]. In HEK293 cells, stimulation of ERK1/2 by activation of the G_q-coupled α_{1B}-adrenergic receptor is blocked by coexpression of dominant negative mutants of Ras, mSos1, and Raf, but not by coexpressing the Gβγ binding peptide βARKct. ERK1/2 activation is inhibited by the PLC inhibitor U73122 or by chelating intracellular calcium with BAPTA, and stimulated in the presence of the calcium ionophore A23187, suggesting a regulatory role for IP_3 and calcium ions [17]. Changes in intracellular calcium have also been shown to be involved in ERK1/2 activation by the G_q-coupled receptors bradykinin and LPA [29]. Taken together, this evidence suggests that $G\alpha_q$ activates the MAPK pathway by stimulation of PLC, leading to the production of IP_3 and DAG. DAG activates PKC, which activates the MAPK path-

way, whereas IP_3 increases intracellular calcium levels, further contributing to ERK1/2 activation. The mechanism by which PKC activates ERK is not entirely understood. Some reports suggest that PKC activation of ERK occurs via a mechanism independent of Ras, perhaps by direct phosphorylation of Raf-1 by PKC, since expression of a dominant negative mutant of Raf-1 but not Ras blocks activation of ERK by PKC [30, 31]. Another study on COS cells, however, reported that stimulation of PKC promotes Ras activation and the formation of Ras-Raf-1 complexes. The assembly of these complexes was thought to occcur by a mechanism distinct from that initiated by RTK activation [32]. These disparities may be due to different regulatory mechanisms in the individual cell lines.

For some G_q-coupled receptors, direct activation of the MAPK pathway by PKC may not be sufficient to account for all MAPK activation, because inhibition of PKC does not completely abolish the response. Activation of the G_q-coupled thyrotropin-releasing hormone (TRH) receptor stimulates MAPK activity in rat pituitary tumor GH3 cells in a PKC-dependent manner. But, TRH also stimulates phosphorylation of Raf-1 in a PKC-independent fashion, likely by phosphorlyation of Shc, leading to increased association between Shc and Grb2 [33]. A TRH receptor-mediated, PKC-independent stimulation of MAPK activity has also been identified in COS-7 cells, but this stimulation involves Gβγ [34]. Gonadotropin-releasing hormone (GnRH) stimulation of ERK also involves multiple pathways. One recent report using αT3-1 cells described a pathway in which Raf-1 is activated directly by PKC, and a second, more minor, PKC- and Gβγ-independent pathway in which Raf-1 is activated by Src and Ras [35]. Another study using these same cells and COS-7 cells described two pathways equally involved in ERK stimulation by GnRH: PKC-dependent activation of Raf-1, and also a pathway involving activation of Ras and the MAPK cascade via PKC-dependent EGFR autophosphorlyation. Src was required for GnRH-mediated ERK and Ras activation, but not GnRH-induced Shc activation, suggesting that Src acts independently of Shc to activate Ras in this system [36]. The reason for the discrepancy between these two studies is not entirely clear, but Benard et al. [35] proposed that differences in the duration of serum starvation that the cells underwent may account for the differing results. To add an additional level of complexity to the story, Budd et al. [37] recently reported that, in addition to activation of the DAG/PKC pathway, phosphorylation of the third intracellular loop of the G_q-coupled M_3 muscarinic receptor by casein kinase 1α (in a PKC-independent manner) also contributes to ERK1/2 activation in CHO cells. These two mechanisms act in concert to elicit a full ERK1/2 response. Thus, depending on the type of receptor and cells involved, multiple mechanisms may be responsible for MAPK activation following activation of G_q-coupled receptors.

Transactivation of RTKs following GPCR Activation

Many GPCR agonists have been shown to trigger the MAPK cascade, and hence mediate cell proliferation by tyrosine phosphorylating RTKs [38–40]. Stimulation of Rat-1 fibroblasts with endothelin-1, LPA, or thrombin leads to tyrosine phosphorylation of the EGFR and ERK1/2. Expression of a dominant negative mutant of the EGFR or treatment of the cells with an EGFR antagonist can block these effects, and also inhibit the Shc activation and Shc-Grb2 association normally observed following treatment of Rat-1 cells with these GPCR agonists [38]. In both Rat-1 cells and COS-7 cells βARKct strongly inhibits LPA-stimulated ERK activation [41]. Overexpression of $G\beta_1\gamma_2$ subunits in COS-7 cells also leads to tyrosine phosphorylation of Shc and the EGFR, strongly implicating Gβγ as a mediator in this pathway. Gβγ likely mediates its effects via Src, since expression of a dominant negative mutant of Src, or expression of Csk (C-terminal Src kinase, which inactivates the Src family of kinases), blocks $G\beta_1\gamma_2$ and G_i-coupled receptor-mediated phosphorylation of the EGFR and Shc. Src can bind to the EGFR following LPA treatment, suggesting that Gβγ activates Src (through an as yet undetermined pathway), which in turn tyrosine-phosphorylates the EGFR. The activated EGFR then phosphorylates Shc and Grb2, and tirggers the MAPK cascade. In neuronal cell types, such as rat PC12 cells, the EGFR transactivation in response to the GPCR agonist bradykinin is calcium-dependent [39].

Other mechanisms for transactivation of RTKs by GPCRs have also been proposed. Prenzel et al. [42] found that transactivation of the EGFR by GPCRs such as the thrombin, muscarinic, endothelin, bombesin, and LPA receptors involves the activation of a metalloprotease. This metalloprotease cleaves proheparin-binding EGF (proHB-EGF) and causes the release of HB-EGF, a ligand for the EGFR. HB-EGF then activates the EGFR, leading to stimulation of the ERK cascade. In vascular smooth muscle cells, activation of ERK and p38 MAPK, but not JNK, by angiotensin II depends on this metalloprotease-induced EGFR transactivation [43], demonstrating the importance of this pathway in some cellular systems. At present the pathways involved in GPCR-induced metal-

loprotease activation, or the identity of the metalloprotease are not known.

Finally, Gao et al. [40] have shown that the G_q-coupled thromboxane A_2 receptor activates the EGFR by first activating G_i proteins in a PKC-dependent manner. This is followed by G_i-mediated transactivation of the EGFR via Src, and subsequent MAPK activation. Thus, it appears that not only do GPCRs cross-talk with RTKs, but different G proteins also cross-talk with each other before interacting with the RTK signaling pathway, suggesting an entirely new level of complexity that we have not even begun to understand.

Role of β-Arrestin and Dynamin in GPCR-Induced MAPK Activation

Many GPCRs undergo internalization following agonist stimulation. This process involves phosphorylation of the receptor by G protein-coupled receptor kinases, as well as the binding of proteins such as β-arrestin and dynamin. β-Arrestin binding to the receptor inhibits further G protein activation and recruits the receptor to clathrin-coated pits, leading to receptor recycling or degradation, whereas dynamin plays a role in fission of the budding vesicle from the plasma membrane. In addition to these roles in receptor sequestration, recent evidence also supports an important function for β-arrestin and dynamin in MAPK phosphorylation following activation of GPCRs. Expression of dominant negative mutants of β-arrestin or dynamin in HEK293 or COS-7 cells markedly inhibits MAPK activation following stimulation of the β_2-adrenergic receptor or serotonin $5HT1_A$ receptor [44, 45]. Inhibitors of receptor internalization, such as concanavalin A, low temperature, or monodansylcadavarine, have a similar effect, suggesting the process of endocytosis is an important event in MAPK activation by GPCRs. The dominant negative mutants of β-arrestin or dynamin do not affect LPA- or isoproterenol-stimulated Shc phosphorylation or Raf-1 activation, indicating that β-arrestin and dynamin exert their effects downstream of Raf-1 [44]. However, a recent study found that activation of ERK by GnRH requires, in part, dynamin-dependent activation of Ras [35].

β-Arrestin can function as an adaptor protein, forming multiprotein complexes at the plasma membrane that contain different components of the MAPK signaling pathway. In HEK293 and COS-7 cells β-arrestin forms a complex containing both c-Src and the agonist-occupied GPCR. β-Arrestin thereby recruits c-Src to the plasma membrane, where it activates the MAPK pathway via Ras, and targets the receptor-Src complex to clathrin-coated pits. Both of these processes are required for β_2-adrenergic receptor-mediated activation of ERK [45]. Another recent study found that agonist-induced stimulation of the G_q-coupled proteinase-activated receptor 2 (PAR2) results in the formation of a protein signaling complex containing the internalized PAR2 receptor, Raf-1, β-arrestin, and activated ERK. This complex is required for PKC-dependent ERK1/2 activation, and leads to activation of nonnuclear effectors of ERK. In contrast, a mutant of PAR2 unable to interact with β-arrestin activates ERK1/2 via a Ras-independent pathway, but activation of ERK1/2 by this alternative pathway results in nuclear translocation and increased cell proliferation. The authors propose that, since ERK has both cytosolic and nuclear effectors, it could be that β-arrestin functions to organize the components of the MAPK signaling pathway and determine the localization and specificity of action of ERK1/2 [46], providing fine-tuned control of this complex pathway.

Role of Nonreceptor Protein Tyrosine Kinases

As previously discussed, Src plays an important role in mediating MAPK activation by GPCRs. Inhibiting Src kinase activity virtually abolishes GPCR-mediated ERK1/2 phosphorylation in many cell types, confirming the pivotal role of this kinase [47]. Exactly how GPCRs activate Src is not entirely clear but, depending on the cell type or ligand used, there is increasing evidence for a role of the nonreceptor proline-rich tyrosine kinase 2 (Pyk2; also called βCAK, RAFTK, or CADTK) and focal adhesion kinase (FAK) in this process. Pyk2 is a Grb-2-binding protein that is a relative of the FAK family of protein tyrosine kinases. Both Pyk2 and FAK can be activated by many stimuli, including, amongst others, membrane depolarization, increases in intracellular calcium, activation of GPCRs, and stimulation of PKC [as reviewed in 13]. In neuronal cells, activation of the G_q-coupled bradykinin receptor tyrosine phosphorylates Pyk2 and ERK1/2 in a calcium-dependent manner [48]. Furthermore, expression of a dominant negative mutant of Pyk2 can inhibit stimulation of MAPK following activation of the G_q-coupled α_{1B}- or G_i-coupled α_{2A}-adrenergic receptors in HEK293 cells [17], or following treatment with LPA and bradykinin in neuronal PC12 cells [29]. Following stimulation by GPCRs, Pyk2 interacts with Src leading to Shc activation, recruitment of Grb2-Sos, and subsequent stimulation of the MAPK cascade. Pyk2 can also be activated by PKC, but the presence of PKC is not required for bradykinin-induced activation of Pyk2 [48], suggesting multiple pathways are involved in activation of this

kinase. An additional role for Pyk2 has been proposed in neuronal cells. In these cells, as mentioned previously, EGFR transactivation in response to the GPCR agonist bradykinin is strongly calcium-dependent [39]. Increasing the concentration of intracellular calcium also triggers phosphorylation of Pyk2 in these same cells, promoting interaction between Pyk2 and Src. Lev et al. [48] therefore proposed that association of Pyk2 with Src might be involved in calcium-dependent GPCR-induced transactivation of RTKs.

Focal adhesions are regions where cells attach to the extracellular matrix and where the cytoskeleton anchors to the plasma membrane. Once activated by β integrins, FAKs provide docking sites at the plasma membrane for signaling proteins such as c-Src and Grb-Sos, leading to activation of the MAPK cascade. FAK can also be activated by GPCRs such as LPA, bombesin, vasopressin, and endothelin [49, 50], thus it has been proposed that FAK, like Shc and Pyk2, may also be involved in recruiting Grb-Sos to the membrane following activation of GPCRs. In PC12 neuronal cells, both cytochalasin D, an agent which disrupts the integrity of the actin cytoskeleton, and H_3N^+-arginine-glycine-aspartate-serine COO^-, which inhibits integrin dimerization, block LPA- and bradykinin-induced activation of ERK1/2, suggesting a requirement for intact focal adhesions in these cells [47]. In contrast, in HEK293 cells these two agents only partially inhibit ERK1/2 activation following LPA and thrombin stimulation. In Rat-1a fibroblasts, tyrosine phosphorylation of FAK by LPA or bombesin can be inhibited by cytochalasin D [51], whereas activation of ERK1/2 by LPA or thrombin is not affected by treatment with this agent [47], suggesting that intact focal adhesion complexes are required for FAK activation but not GPCR-induced MAPK activation in these cells. Thus, it seems that there can be focal adhesion-dependent and adhesin-independent activation of ERK, and the relative involvement of FAK largely depends on the cell type being investigated. Pyk2 can be activated upon adhesion to the extracellular matrix, and inhibiting translocation of Pyk2 to focal adhesions by overexpression of a kinase-deficient mutant of Pyk2 abolishes histamine-induced activation of ERK1/2. Pyk2 may therefore provide a link between GPCRs and focal adhesion-dependent ERK activation. Finally, the Src-like nonreceptor tyrosine kinases Fyn, Lyn and Yes, as well as Syk and Btk, have also been linked to GPCR-induced MAPK activation [52, 53]. Additional links between Gβγ and MAPK include the protein tyrosine phosphatase SH-PTP1 [54] and Ras-GRF [55].

GPCR-Mediated Activation of JNK and p38 MAPK

In addition to stimulation of ERK, GPCR agonists can also activate the two other MAPK subtypes, JNK and p38. JNK and p38 MAPKs are activated most commonly by cytokines such as tumor necrosis factor and interleukin-1β, as well as cellular stress. In the case of the JNK pathway, the MAPK cascade involves activation of MEKK1–4 (MAPK/ERK kinase) or transforming growth factor-β-activated kinase 1 (TAK1), which in turn activates MKK4 (also called MEK4, SEK1, SKK1) or MKK7 (also called MEK7, SEK2, SKK4), followed by JNK activation. JNKs can then phosphorylate and activate transcription factors such as c-Jun, Elk-1 and ATF2. The p38 MAPK pathway involves activation of TAK1 as well as thousand and one acid kinase, leading to activation of MKK3 (also called MEK3, SKK2) or MKK6 (also called MEK6, SKK3), and subsequent p38 MAPK activation. p38 MAPK phosphorylates and activates transcription factors such as Elk-1, ATF2 and MEF2C [review in 7, 56]. There can also be substantial cross talk between the ERK, JNK, and p38 MAPK signaling pathways.

Although much progress has been made in understanding the pathways linking GPCRs to ERK, the mechanism by which GPCRs activate JNK and p38 MAPK is not nearly as well defined. There is accumulating evidence, however, showing that Gα and Gβγ subunits are involved, as well as activation of members of the Rho and Ras family of GTPases.

Gα versus Gβγ Subunits. The role of the Gα and Gβγ subunits in JNK and p38 MAPK activation appears to be a function of cell type and the G protein involved. In HEK293T cells, activation of the G_q-coupled α_{1B}-adrenergic receptor inhibits cell proliferation via activation of JNK and p38 MAPK. The $G\alpha_q$ subunit appears to mediate the effect of the receptor, since transfection of a constitutively active mutant of $G\alpha_q$ can also markedly inhibit cell proliferation, and this inhibition is blocked by a kinase-deficient mutant of MKK4 (which inhibits the JNK pathway) [57]. Activation of the G_q-coupled m1 muscarinic acetylcholine receptor (mAChR) in NIH3T3 [58] and Rat-1 [59] cells increases the activity of JNK. Expression of this same receptor, as well as the m2 mAChR, in COS-7 cells also leads to JNK activation but, unlike in HEK293 cells where the $G\alpha_q$ subunit is involved in the JNK response, JNK activation in these cells cannot be mimicked by expressing $G\alpha_s$, $G\alpha_{i2}$, $G\alpha_q$ or G_{13}. Rather, $G\beta_\gamma$ subunits are most likely involved since overexpression of Gβγ subunits robustly stimulates JNK activation, and βARKct can block both m1- and m2-AChR-induced NK activation [60].

In NIH3T3 cells, expression of GTPase-deficient mutants of $G\alpha_{12}$ or $G\alpha_{13}$ increases activation of JNK. This $G\alpha_{12}$ and $G\alpha_{13}$ activation is specific for JNK and not ERK, and is dependent on Ras [61]. In HEK293 cells, expression of constitutively active mutants of $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$, but not $G\alpha_o$ or $G\alpha_z$, increases the activity of JNK [62]. Overexpression of Gβγ also stimulates JNK activity to a similar level to that seen with $G\alpha_{i2}$, and this activation can be inhibited by βARKct [63]. Thus, depending on the cell type and the receptor stimulated, JNK activation can be mediated via Gα subunits, Gβγ subunits, or a combination of the two.

Rac, Cdc42 and Rho Mediate JNK Activation by GPCRs. G-protein-mediated activation of JNK can be Ras-dependent or RAS-independent, depending on the GPCR and cell type involved. There is substantial evidence to show that the Ras-independent pathway leading to JNK activation involves small GTP-binding proteins from the Rho family, Rac-1 and Cdc42 [64]. Both these proteins are able to regulate the activity of JNK independent of the Ras/MAPK cascade [65]. In HEK293 cells, expression of dominant negative mutants of Ras and Rac1 (but not RhoA) inhibit Gβγ and m1- or m2-AChR-induced JNK activation, while a dominant negative mutant of Cdc42 blocks stimulation induced by m1-AChRs [60]. Expression of dominant negative mutants of Ras in NIH3T3 cells but not HEK293 cells inhibits $G\alpha_{12}$-mediated JNK activation [66], while dominant negative Rac-1 inhibits JNK activation by $G\alpha_{12}$ to varying degrees in HEK293, NIH3T3s, COS-1, and HeLa cells. Dominant negative Cdc42 also blocks $G\alpha_{12}$-induced JNK activation in HEK293 and COS-1 cells, although to a lesser extent than dominant negative Rac-1 [66]. Likewise, dominant negative mutants of Ras and Rac can inhibit G_i-coupled opioid receptor-like (ORL1)-induced JNK activation in COS-7 cells [67].

JNK activation following expression of a constitutively active mutant of $G\alpha_i$ can be inhibited by dominant negative mutants of Rho and Cdc42, and inhibitors for the Src family of tyrosine kinases, but not dominant negative Rac or kinase-deficient mutants of MKK4 or MKK7 [62]. In contrast, Gβγ has been shown to stimulate JNK by activating MKK4 and, to a smaller degree, MKK7. The MKK4 pathway requires Rho, Cdc42, and tyrosine kinases, while the MKK7 pathway is dependent on Rac [63]. Yamauchi et al. [62] proposed that there might be two separate pathways leading from G_i-coupled receptors to JNK, one involving $G\alpha_i$ and another involving Gβγ. $G\alpha_i$ stimulates Src, Rho, and Cdc42, which in turn stimulate MKKs other than MKK4 and MKK7, leading to JNK activation. Gβγ on the other hand stimulates an as yet unknown protein tyrosine (but see below), and Rho and Cdc42, which in turn stimulate MKK4, followed by JNK. Gβγ may also stimulate MKK7 via Rac, which then stimulates JNK [62].

One potential downstream effector of Rac and Cdc42 in the pathway leading to JNK activation is p21-activated kinase (PAK), a serine/threonine kinase that binds to and is activated by Cdc42 or Rac. Expression of constitutively activated PAK3 in COS-1 cells [68], or overexpression of PAK1 or PAK2 in HEK293 cells [69], can activate JNK and, to a lesser extent, p38 kinase. A dominant negative mutant of PAK can also partially inhibit Gβγ-mediated JNK activation in COS-7 cells [70], confirming an involvement of this kinase in the G protein-linked JNK signaling cascade. $G\alpha_z$ can also be phosphorylated by PAK [71], and activation of G_z can induce JNK phosphorylation in some cell types [as reviewed in 72], Thus, $G\alpha_z$ may be involved in transducing and integrating signals from Cdc42 and Rac.

PAK may also represent a link between the GPCR and RTK signaling pathways. Hyperphosphorylation of PAK and a subsequent decrease in kinase activity can be induced by the cyclin-dependent kinase-5 (Cdk5) and its activator p35 in a Rac-dependent manner [73]. Increasing evidence indicates that the p35/Cdk5 complex is involved in mediating the biological responses to RTKs. Activation of the RTK ErbB by its ligand neuregulin results in increased Cdk5 kinase activity, and inhibition of Cdk5 activity decreases neuregulin-induced transcription of acetylcholine receptors in muscle [74]. Likewise, NGF induces neurite outgrowth from PC12 cells by activation of the RTK TrkA, which induces sustained activation of ERK and subsequent induction of p35 expression and Cdk5 kinase activity [75]. Therefore, the JNK activation triggered by GPCRs may be modulated by RTKs via the serine/threonine kinases Cdk5 and PAK.

PI3Kγ may play a role in linking GPCRs, and in particular Gβγ, to JNK activation. In COS-7 cells, overexpression of PI3Kγ stimulates JNK activity [70]. This stimulation can be blocked by βARKct, or by expression of dominant negative mutants of Ras, Rac and PAK, but not RhoA or Cdc42 [20, 70, 76]. Both Gβγ- and m2 mAChR-induced JNK activation can be blocked by inhibitors of PI3K [70], while JNK activation induced by shear stress in vascular endothelial cells requires both Gβγ subunits and PI3Kγ [77, 78]. JNK and p38 MAPK activation induced by the cannabinoid CB1 receptor is also sensitive to inhibitors of PI3K (JNK activation by this GPCR may also involve transactivation of the PDGF

receptor) [79]. PI3K is not required, however, for $G\alpha_i$-induced activation of JNK in HEK293 cells [62], for activation of JNK by the G_i-coupled ORL1 receptor in COS-7 cells [67], or for p38 MAPK activation by the gastrin/CCKB receptor in CHO cells [80]. MKK4 or MKK7 activation of $G\beta\gamma$ in HEK293 cells is also PI3K-independent [63]. Thus, PI3K is not always an essential requirement for JNK activation, but it may be an important regulator in G protein-mediated Ras-, Rac- and PAK-dependent activation of JNK. GPCR-induced activation of PI3K may provide a link between the ERK and JNK signaling pathways.

PKC has varying effects on JNK and p38 MAPK activation. In NIH3T3 cells, JNK activation by the m1-AChR is PKC-independent [58], whereas in CHO cells, Rat-1 fibroblasts, or GN4 epithelial cells, PKC inhibition actually stimulates JNK activation induced by activation of the m3-AChR [81], ET-1 receptor [82] or angiotensin II receptor [83], respectively, suggesting that PKC plays an inhibitory role in these pathways. Activation of p38 MAPK by gastrin in CHO cells [80, 84] is PKC-dependent, while JNK activation by GnRH involves sequential activation of PKC, Src, Cdc42, and MEKK1 [85].

Pyk2 has also been implicated in the activation of JNK by GPCRs [48, 86]. Phosphorylation of Pyk2 enhances the activation of JNK but not p38 MAPK by the G-protein-coupled Kaposi's sarcoma-associated herpesvirus (KSHV) receptor [87], which constitutively activates JNK and p38 MAPK. Pyk2 is also involved in the activation of the Src-related kinase Lyn by the KSHV receptor [87]. In GN4 rat liver epithelial cells, increases in intracellular calcium following activation of angiotensin II receptors stimulates a calcium-dependent tyrosine kinase that highly resembles human Pyk2, leading to JNK stimulation. This pathways leading to JNK activation is different from that activated in response to stress, confirming that multiple pathways are involved in activation of this MAPK. Tokiwa et al. [86] proposed that Pyk2 might interact with Grb2 and Sos, since Ras-dependent activation of JNK has also been reported. Pyk2 contains a binding site for PI3K, so PI3K may serve as an intermediate between Pyk2 and Rho-like GTPases.

In HEK293T cells, activation of FAK by anchoring to the cell membrane stimulates JNK [88]. Unlike FAK-mediated activation of ERK, however, FAK-induced JNK activation does not require tyrosine phosphorylation of Shc. Rather, Igishi et al. [88] demonstrated that recruitment of paxillin, a cellular protein that is a substrate of FAK, to the cell membrane to form a stable complex with FAK is sufficient to activate JNK. Coexpression of dominant negative mutants of Rho, Rac and Cdc42 can inhibit activation of JNK by paxillin, suggesting that paxillin recruitment to the plasma membrane activates Rho, Rac and Cdc42, which then in turn stimulate JNK activity.

Other proteins implicated in JNK and p38 MAPK activation are mixed lineage kinase 3, which may bind Cdc42 and Rac-1 and mediate activation of the MEKK-MKK-JNK cascade [89], and the nonreceptor tyrosine kinase Bruton's kinase (Btk). $G\alpha_{12}$ can directly interact with Btk [90], and G_q-coupled receptor-induced stimulation of p38 MAPK activity is dependent on the presence of Btk [91]. Apoptosis signal-regulating kinase 1 (ASK1, or MAPKKK5) may also be involved, since in COS-7 cells ASK1 and MEKK1 activate JNK and mediate $G\alpha_{12}$- and $G\alpha_{13}$-induced apoptosis. ASK1 is not involved in Rac1 and Cdc42-mediated JNK activation [92], suggesting that two separate pathways, one involving ASK1 and another involving MEKK1, can be activated by $G\alpha_{12}$ and $G\alpha_{13}$ and lead to apoptosis. Rho has also been shown to be involved in $G\alpha_{13}$-induced apoptosis in these same cells [93], suggesting that Rho may regulate the ASK1-JNK pathway. β-Arrestin2 may facilitate the activation of JNK by ASK1, since multiprotein complexes containing JNK3, ASK1, and MKK4 have been found in β-arrestin2 immunoprecipitates in COS-7 cells [94].

Further downstream, GPCR stimulation of the ERK, JNK, and p38 MAPK pathways leads to activation of a series of transcriptional responses. Activation of the m1 mAChR strongly stimulates serum response element (SRE)-driven reporter gene activity. Both $G\alpha_{12}$ and $G\beta\gamma$ are able to active SRE, and this response is dependent on RhoA [95]. In PC12 cells, activation of the α_{1A}-adrenergic receptor with norepinephrine (NE) promotes activation of the reporters activator protein-1 (AP-1) and SRE, and to a lesser extent cAMP response element (CRE), nuclear factor-κB (NFκB), and nuclear factor of activated T cells (NFAT) (interestingly, NGF and EGF activate only SRE and AP1, implying that GPCRs may activate a bigger array of transcriptional responses than RTKs). Inhibition of p38 MAPK partially blocks AP-1, SRE, NFAT, and CRE activation by NE, implicating this protein kinase in linking the α_{1A}-adrenergic receptor to these reporters. This p38 MAPK-dependent pathway involved in activation of AP-1, SRE, NFAT, and CRE activation by NE is PKC-dependent. MEK is also required for NE-induced AP-1, NFAT and SRE activation, but inhibits CRE and NFκB activation, while PKC, MEK, Src and p38 MAPK are all involved in NE-induced differentiation of PC12 cells [96].

Rho GTPases Also Stimulate the ERK/MAP Pathway following GPCR Activation

The Rho family of proteins, particularly Rac-1, may also be involved in the Ras/MAPK cascade. Toxin B, an inhibitor of the Rho family of GTPases, inhibits endothelin-1 induced activation of MEK1/2 and ERK1/2, but not c-Raf, in neonatal rat ventricular myocytes. A dominant negative mutant of Rac-1 has the same effect, while an activating mutant of Rac-1 interacts with constitutively activated c-Raf to increase activation of ERK and increase the expression of atrial natriuretic factor in these cells [97]. How Rac-1 exerts its effects is not clear. Rac-1 may signal through PAK to phosphorylate MEK1 in its c-Raf-binding domain, thereby promoting the association of MEK1 with c-Raf, leading to ERK activation [69, 98]. PI3K may also be involved since one of the products of PI3K, phosphatidylinositol 3,4,5-triphosphate, can activate Rac-1 [99].

The effects of cAMP on cell proliferation may involve Rho, since elevated cAMP has been found to inactivate Rho and block activation of MAPK to inhibit cell growth [100]. PKA phosphorylates RhoA, causing it to translocate from the membrane to the cytosol, thereby terminating Rho signaling [101].

In addition to the studies mentioned above, Rho has been implicated in GPCR-mediated actin cytoskeletal rearrangement, FAK phosphorylation, cell migration and adhesion, growth and gene expression, and contraction/myosin light chain phosphorylation. G proteins activate Rho by regulating the activity of Rho-guanine nucleotide exchange factors, although the mechanism by which this occurs is still poorly understood. A detailed description of these interactions is beyond the scope of this review, and the reader is referred to Seasholtz et al. [102] for a comprehensive review of this topic.

GPCR-Mediated Activation of STAT

Signal transducers and activators of transcription (STATs) can also be activated by GPCRs [103]. STATs are transcription factors that are traditionally thought to be activated by cytokines, such as ciliary neurotrophic factor (CNTF), and to be tyrosine-phosphorylated by Janus kinases [104], but recent evidence has also implicated STAT3 as a downstream effector of $G\alpha_o$ and $G\alpha_i$. Activation of $G\alpha_o$ can cause transformation of NIH3T3 cells, and this transformation may require activation of STAT3. Expression of $G\alpha_o$ in NIH3T3 cells activates STAT3, while a dominant negative mutant of STAT3 can inhibit $G\alpha_o$-mediated transformation of these cells. Src is also involved in this pathway, since Csk, which inactivates Src, can block $G\alpha_o$-induced STAT3 activation [105]. $G\alpha_{i2}$ proteins also regulate the activity of STAT3 to control cell proliferation in response to activation of the CSF-1 receptor in NIH3T3 cells, and this pathway is dependent on Src [106]. Rap-1, Rac-1, and Ral-1 may also be involved in STAT3 activation by $G\alpha_o$ and $G\alpha_{i2}$ [107]. Finally, synergistic effects of CNTF, which activates STAT, and NGF, which activate RTKs, on neuronal differentiation have been demonstrated [108], raising the possibility of yet another link between the GPCR and RTK pathways via STAT.

Activation of G Protein Signaling Pathways by RTKs

Up until now we have described the mechanisms by which GPCRs activate RTK signaling pathways. Cross talk between GPCRs and RTKs is however a two-way process, and RTKs can also modulate the activity of signaling pathways traditionally thought to be controlled exclusively by ligands that couple to GPCRs. In mouse lymphoma S49 cyc^- cells and rat cardiac membranes, EGF, which normally activates the MAPK cascade via the EGFR, can stimulate AC activity by activating $G\alpha_s$ [109]. The protein tyrosine kinase activity of the EGFR is essential for this AC stimulation, and the EGFR protein tyrosine kinase can phosphorylate $G\alpha_s$, leading to an improved ability to stimulate AC [110]. Thus, the EGFR may act in concert with $G\alpha_s$ to activate AC.

In Rat-1 fibroblasts the insulin-like growth factor-I (IGF-I) receptor tyrosine kinase, which is involved in cell growth, activates a G protein that is sensitive to PTX, and activation of this G protein leads to $G\beta\gamma$-mediated stimulation of MAPK [111]. In human intestinal smooth muscle cells stimulation of IGF-I also activates MAPK, and this response can be partially inhibited by treatment of the cells with PTX. IGF-I can stimulate $G\alpha_{i2}$, but not $G\alpha_{i1}$, $G\alpha_{i3}$, or $G\alpha_{q/11}$, and the stimulation of $G\alpha_{i2}$ is blocked by an inhibitor of IGF-I receptor tyrosine kinase. The $G\beta\gamma$ subunits of G_{i2} are likely responsible for IGF-I-induced activation of MAPK, as a $G\beta\gamma$-neutralizing antibody inhibits the increase in MAPK activity. IGF-I-induced stimulation of $G\alpha_{i2}$ leads to an inhibition of AC and cAMP activity in these cells. Since cAMP is a growth inhibitor, this inhibitory effect of $G\alpha_{i2}$ may help to further promote the growth-stimulatory effects of IGF-I. Taken together these results suggest that IGF-I activates G_{i2}, leading to $G\beta\gamma$-mediated stimulation of MAPK and $G\alpha_{i2}$-mediated inhibition of cAMP [112].

Besides direct activation of G proteins, RTKs may also modulate signaling pathways of GPCRs by regulating the expression of G proteins or GPCRs. The expression of

$G\beta_1$ and the GPCR RDC-1 in cultured myotubes is upregulated by neuregulin, which activates the RTK ErbB receptors [113]. Regulating the expression of G proteins and GPCRs may represent a more long-term modulation of GPCR pathways by RTKs.

Conclusions

It is clear that regulation of cellular events occurs by the integration of a number of highly complex signaling networks (fig. 2), rather than by isolated linear pathways. GPCRs acting through the G_s, G_q, G_i and G_{12} families of G proteins have been shown to activate the MAPK cascade, although the mechanisms involved are largely dependent on cell type and the receptor involved. GPCRs can activate MAPKs via intracellular intermediates such as calcium, PKC, cAMP, or PI3K, or via Gα and Gβγ subunits. These mediators may act directly at RTKs or at any one of a number of downstream effects. RTKs can also interact with components of the G protein signaling cascade, creating an intricate network that provides remarkable control over cellular regulation. There are still many unanswered questions, in particular how does the cell know to respond one way to some extracellular stimuli and in a different manner to other seemingly similar stimuli? It is likely that the balance between the different pathways is crucial to determining the final outcome.

Despite the incredible progress that has been made in understanding the complexities of cross talk between GPCR and RTK signaling pathways, the story is likely to become much more complicated with the discovery of new MAPK signaling pathways. Indeed, in addition to the ERK1/2, JNK, and p38 MAPK pathways, four p38-like MAPKs ERK6, Mxi2, SAPK3, and p38β, as well as ERK5 and ERK3 have been identified. A JNK-independent pathway linking activation of the m1 mAChR to c-*jun* promoter activity has recently been described that involves activation of the novel MAPK ERK5, as well as p38α, p38γ, and p38δ [114]. Future studies will help to determine the relative importance of this pathway.

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