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**COMMENTARY**

# Signaling from G-Protein-coupled Receptors to Mitogen-activated Protein (MAP)-Kinase Cascades

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**ABSTRACT.** Heterotrimeric GTP-binding protein (G-protein)-coupled receptors are able to induce a variety of responses including cell proliferation, differentiation, and activation of several intracellular kinase cascades. Prominent among these kinases are the activation of mitogen-activated protein (MAP) kinase, including the extracellular signal-regulated kinases (ERKs), ERK1 and ERK2 ($p44^{mapk}$ and $p42^{mapk}$, respectively); stress-activated protein kinases (SAPKs/JNKs); and p38 kinase. These receptors signal through G-proteins. Recent data have shown that the activation of mitogen-activated protein/ERK kinase induced by G-protein-coupled receptors is mediated by both Gα and Gβγ subunits involving a common signaling pathway with receptor-tyrosine-kinases. Gβγ-mediated mitogen-activated protein kinase activation is mediated by activation of phosphoinositide 3-kinase, followed by a tyrosine phosphorylation event, and proceeds in a sequence of events that involve functional association among the adaptor proteins Shc, Grb2, and Sos. SAPKs/JNKs and p38 are able to be activated by Gβγ proteins in a pathway involving Rho family proteins including RhoA, Rac1, and Cdc42. BIOCHEM PHARMACOL **56**;3:269–277, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** G-proteins; MAP kinase; SAPK/JNK; tyrosine kinases; PI 3-kinase; growth factors

Stimulation of a variety of cell surface receptors elicits a remarkably diverse array of cellular responses, with the precise response depending upon the identity of the stimulated receptor and the nature of the stimulated cell. Activation of receptors can induce cells to proliferate and migrate, to differentiate, to activate several metabolic pathways, or even to undergo apoptosis. In addition, overexpression or constitutive activation of cell surface receptors can contribute to cell proliferation and cancer, and loss of function in these receptors can result in disorders such as insulin-resistant diabetes [1]. The mechanisms by which cell surface receptors activate intracellular signaling pathways are, therefore, of great interest, both for understanding the control of normal cell growth, metabolism, and development, and for defining the alterations in signal transduction that occur in cells with altered receptor activity.

Within this area of study, considerable attention has focused upon the intracellular signaling pathways that initiate early biochemical events and that are associated with mitogenesis. These early events include systems in which specific kinases are activated following the generation of second messengers, such as diacylglycerol and cyclic AMP. Recently, major advances have been achieved in the elucidation of cellular signaling pathways that involve the activation of proteins or enzymes by phosphorylation on tyrosine residues. In this type of pathway, cellular signals are generated through a series of protein–protein interactions and a cascade of phosphorylation events rather than by the immediate generation of second messengers. Historically, this type of pathway has been associated with growth factor receptors that display intrinsic tyrosine kinase activity [2]; however, a number of seven-transmembrane spanning receptors (G-protein†-coupled receptors) have also been found to activate similar kinase pathways [3]. In this commentary, I will outline the regulation and function of the major signal transduction pathways activated in response to both classes of receptors, with special emphasis on the G-protein-coupled receptor signaling pathways.

## ACTIVATION OF MAP KINASE PATHWAYS

Stimulation of a variety of tyrosine kinase receptors leads to a rapid elevation of the enzymatic activity of a family of closely related serine-threonine kinases, known as MAP

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† *Abbreviations:* EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; Gα, α subunits of GTP-binding proteins; Gβγ, βγ subunits of GTP-binding proteins; G-proteins, heterotrimeric GTP-binding proteins; GAP, GTPase-activating protein; JNKs, c-jun N-terminus kinases; LPA, lysophosphatidic acid; MAP, mitogen-activated protein; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI 3-kinase, phosphoinositide 3-kinase; $PIP_3$, phosphatidylinositol 3,4,5-triphosphate; PKC, protein kinase C; PMA, phorbol-myristate acetate; PLC, phospholipase C; PTP, protein-tyrosine phosphatase; SAPKs, stress-activated protein kinase; SH2, Src-homology domain 2; SH3, Src-homology domain 3; SRF, serum response factor; TCF, ternary complex factor; and Vps, Vacuole protein sorting.

kinases. These kinases are able to convert extracellular stimuli to intracellular signals that control gene expression and, eventually, cell proliferation and differentiation [4, 5].

MAP kinases have been classified into three subfamilies: ERKs, including ERK1 and ERK2 (also known as $p44^{mapk}$ and $p42^{mapk}$, respectively); SAPKs, also called c-jun N-terminus kinases (JNKs); and p38 kinase, the homologue of the *Saccharomyces cerevisiae HOG1* gene.

Growth factors, phorbol esters, and hormones regulate MAP kinases through a series of phosphorylation events. The pathway linking cell surface receptors to these serine-threonine kinases has been the focus of intensive efforts. Whereas the connection between tyrosine kinase receptors and MAP kinase activation has been elucidated, the mechanism of activation of MAP kinase by G-protein-coupled receptors, and the activation of SAPKs/JNKs by cell surface receptors remain poorly understood. However, it has become increasingly clear recently that both tyrosine kinase receptors and G-protein-coupled receptors share common biochemical components that lead to the activation of MAP kinase cascades.

In the case of tyrosine kinase receptors (such as receptors for EGF or PDGF), it has become evident that after activation of tyrosine kinase activity, dimerization, and autophosphorylation of the receptor, there follows a cascade of intermolecular interactions involving docking proteins such as Shc and Grb2; exchange factor Sos; GTPases such as Ras; and kinases such as Raf and MEK that finally lead to the activation of MAP kinase (see Fig. 1). On the other hand, it has become clear that the activation of SAPK/JNK involves a signaling route analogous to but different from that of the Ras-MAP kinase pathway. In this regard, GTP-bound forms of Rho-related proteins, Rac1 and Cdc42, can potently stimulate SAPK/JNK but not MAP kinase activity, in a pathway apparently involving some serine-threonine kinases such as PAK or MLK3/DLK, MEKK, SKK/SEK1, and finally SAPK1/JNK1. SAPK2/p38 apparently is activated upon phosphorylation by SKK2, but the remaining components of the pathway remain to be identified. The outline of several parallel pathways leading to the activation of different subsets of MAP kinases is taking shape (Fig. 1), although the extent to which there is cross-talk among these pathways is unknown. In general, multiple elements are activated by a given stimulus or receptor, generating parallel signaling tracks that may or may not be self-reinforcing, amplify, or even down-regulate the signal. The extent to which the signal is regulated at each step is unclear.

In the case of the docking proteins involved in these cascades, the coupling of Grb2/Sos plays a central role. Sos is a cytoplasmic Ras-guanine nucleotide exchange factor (Ras-GEF) that is constitutively associated with the adaptor protein Grb2 and can be stimulated to activate Ras. When Grb2 interacts with a tyrosine-phosphorylated membrane receptor, it positions Sos at the plasma membrane where it can promote activation of Ras. After complex formation of Grb2/Sos with Ras, the nucleotide exchange activity of Ras is activated, generating the form Ras-GTP, which enables signal transduction. Hydrolysis of the bound GTP by an intrinsic GTPase activity relaxes the conformation and terminates the signal. GAPs enhance the GTPase activity of normal Ras and thus shorten the lifetime of the signaling form [6].

Alternatively, instead of Grb2 binding directly to a tyrosine-phosphorylated receptor such as the activated EGF receptor, the Sos–Grb2 complex may interact with a phosphotyrosine on Shc. The Shc gene encodes several variant proteins (p66, p52, and p46) that become tyrosine phosphorylated when various receptors are activated. It appears that Shc first binds to a tyrosine-phosphorylated receptor, which, in turn, phosphorylates a tyrosine in Shc that can then serve as bait for Grb2 docking and consequent Sos activation, as illustrated for the NGF receptor TrkA and ErbB2 (p185 or HER-2/neu) [7]. Alternatively, Shc can become phosphorylated by non-receptor tyrosine kinases such as Src.

Src is a protein tyrosine kinase that is attached to the plasma membrane by its N-terminal myristate modification and is essential for the mitogenic action of both tyrosine kinase and G-protein-coupled receptors. When activated, Src phosphorylates a number of cytoskeleton-associated proteins (e.g. focal adhesion protein tyrosine kinase and paxillin) and probably contributes to the activation of both Raf-1 and PI 3-kinase [8]. v-Src has been found to activate Ras, possibly by phosphorylation of Shc with subsequent activation of Grb2/Sos. v-Src enhances both the MEKK/JNK and Raf-1/ERK pathways.

Membrane-bound Ras-GTP is capable of interacting with and activating a number of proteins, namely p120 ras-GAP, the p110 subunit of PI 3-kinase, Ral-GDS, PKC, Raf-1, A-Raf, B-Raf, and possibly certain MEKKs. Each of these proteins is likely to be activated by Ras-GTP via a unique interaction with a particular site on the p21 protein, and each contributes to the overall consequence of Ras activation [6].

The MAPKKs (MEKs) are typically activated by serine-threonine phosphorylation catalyzed by one of the MAPKKKs (Raf, MEKK). One subgroup of the MAPKKs includes MEK1a, MEK1b, and MEK2, which phosphorylate different ERKs but nevertheless appear to elicit similar transcriptional and morphological responses. A second subgroup of these dual-specificity kinases includes SKK1/SEK1. These are preferentially activated by MEKK1 and 2, but not by the Raf proteins, and their target proteins are the SAPKs/JNKs [5].

Phosphorylation of both tyrosine and threonine residues in MAP kinases, which are found in the activation segment of the kinase domain, is essential for full kinase activity of the MAP kinases. In turn, the MAP kinases target only serine and threonine residues that are closely followed by one or more prolines in a motif recognized preferentially by a particular MAP kinase. Translocation of activated MAP kinase to the nucleus and subsequent phosphorylation of a variety of transcription factors including c-Myc, Elk-1, and

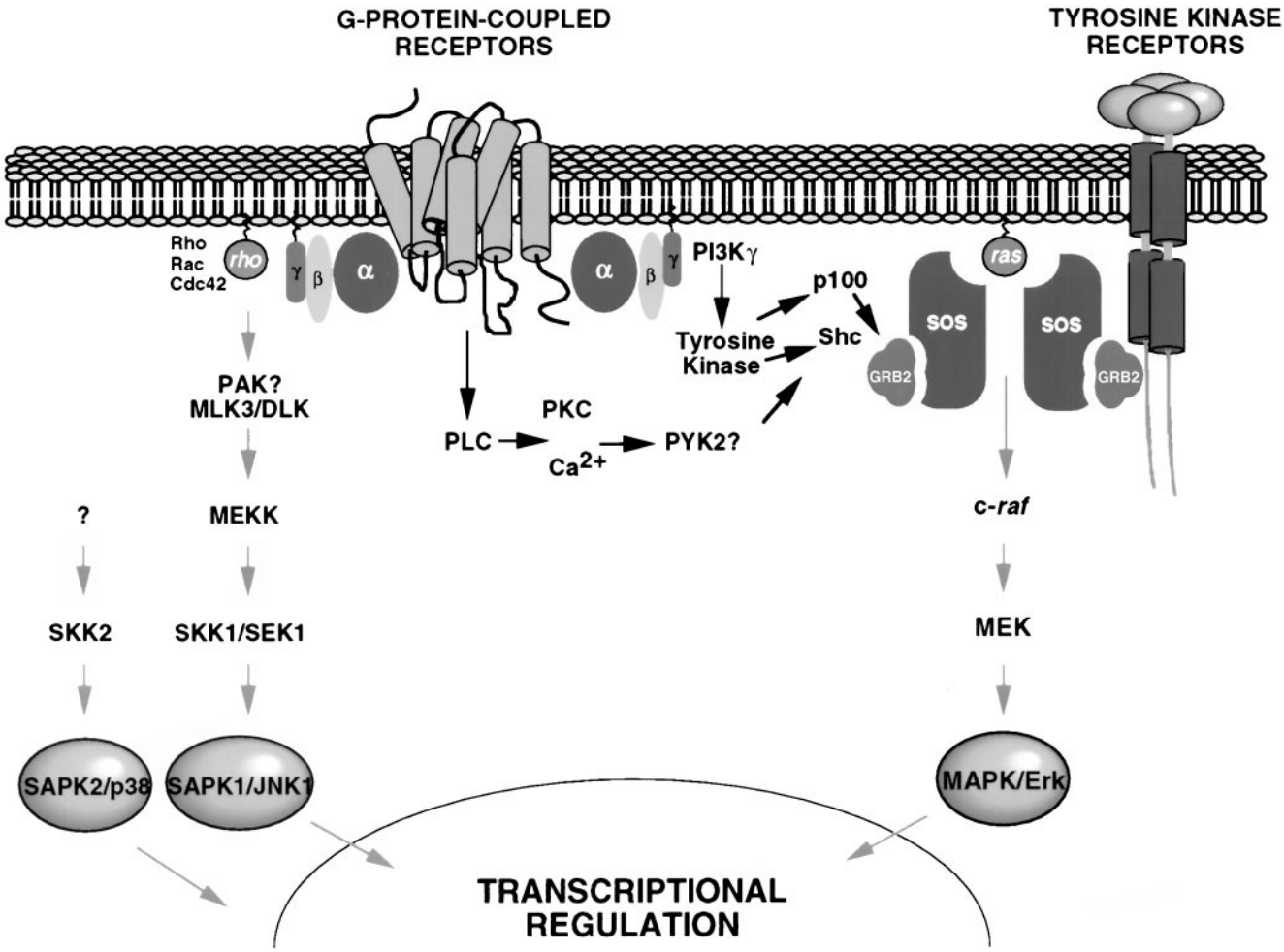


**FIG. 1. Signal-transduction pathways initiated by cell surface receptors in mammalian cells. Following the activation of G-protein-coupled receptors or tyrosine kinase receptors, the activation of discrete protein phosphorylation cascades occurs, with some cross-talk and variable activation relative to each other, depending upon the stimulus. See text for details.**

ATF2 support the involvement of MAP kinases in transducing cytoplasmic signals to nuclear responses. Specifically, ERK1 targets Elk-1, a TCF that associates with the SRF on the serum response element, whereas ERK2 exhibits a preference for c-Myc; SAPK1/JNK is able to target c-jun, ATF2, and Elk; and p38 targets ATF2, Elk, and Max.

## G-PROTEIN-COUPLED RECEPTORS AND MAP KINASE ACTIVATION

Hormones that interact with seven-transmembrane spanning receptors also induce long-term effects on gene expression and cell growth; these receptors signal through G-proteins [3].

G-proteins are heterotrimeric in nature and are composed of α, β, and γ components encoded by distinct genes. Each gene type represents a family exhibiting a varying degree of complexity, with the most diverse encoding α-subunits. Molecular cloning has revealed the existence of at least 17 Gα genes, divided into four subfamilies: $G_s$, $G_i$, $G_q$, and $G_{12}$ [9]. In addition to diversity among α-chains, there are also multiple genes encoding at least four β- and six γ-subunits.

Under basal conditions, G-proteins exist as heterotrimers with GDP bound to the α-chain. Activated receptor promotes the release of GDP, the binding of GTP, and the dissociation of GTP-Gα from the Gβγ complex. GTP-Gα and Gβγ can now interact with their effectors and propagate the signal. Endogenous GTPase activity terminates its ability to regulate effector activity and leads to Gα-GDP reassociation with Gβγ. Recently, the protein RGS, a GTPase-activating protein, has been identified; this protein stimulates the GTPase activity of the $G\alpha_i$ subunit, resulting in the accumulation of GDP-Gα, which re-forms the stable heterotrimer. Free Gβγ leads to dissociation of Gβγ from effector, thus terminating the signal propagation [10, 11].

Different types of heterotrimeric G-proteins can couple seven-transmembrane spanning receptors to growth responses. Experiments using pertussis toxin (which ribosylates G-protein α-chains of $G\alpha_i/G\alpha_o$ in a αβγ heterotrimeric state-dependent fashion) and micro-injected G-pro-

tein antibodies indicate that, depending on the cell system, mitogenic responses can be mediated by $G_i/G_o$, $G_{q/11}$, $G_s$, or $G_{12}$ subtypes of G-proteins. Furthermore, studies using expression of constitutively active G-protein α subunits have demonstrated that induction of cellular transformation is not restricted to a specific type of G-protein [12].

A variety of agonists acting on G-protein-coupled receptors are able to activate the MAP kinase pathway. At the beginning, it was found that some G-protein-coupled receptor agonists strongly stimulated polyphosphoinositide hydrolysis, and the activation of PKC was considered to be implicated in the activation of MAP kinase and sufficient to initiate cell cycle progression. Consistent with these findings is the observation that PKCα can phosphorylate Raf-1 upon serine residues *in vitro* [13].

More recently, agonists such as thrombin and the mitogenic phospholipid LPA have been shown to activate the MAP kinase pathway by a mechanism independent of changes in PKC activation. In these cases MAP kinase activation has been shown to be pertussis toxin sensitive, as well as dependent on nucleotide exchange on $p21^{ras}$ [14]. Overexpression of c-Raf-1 potentiates LPA-stimulated MAP kinase activation, while overexpression of dominant negative Ras inhibits the activation of MAP kinase induced by LPA, demonstrating the involvement of both Ras and Raf in the LPA-activated MAP kinase cascade.

Evidence coming from the laboratories of Bourne [15] and Gutkind [16] suggested a role for G-protein βγ subunits in the activation of Ras by G-protein-coupled receptors. In COS-7 cells, the transfection of βγ subunits, particularly $\beta_1\gamma_2$, was found to stimulate MAP kinase. It has been proposed that the βγ subunits may transduce the signal through the interaction with proteins that contain a PH domain (well known as a lipid binding domain) [17]. A number of proteins known to regulate Ras activity also express a PH domain [18]. Thus, free βγ subunits may affect the function of Ras by binding the PH domain of one or more Ras-regulatory proteins, since membrane localization of βγ subunits is an absolute requirement to induce the activation of MAP kinase. This suggests that the targeting of proteins containing PH domains to the plasma membrane may be a critical event in Ras activation, and this recruitment of additional intermediates to the plasma membrane can be used for further activation by some other mechanism.

Several reports have shown that the activation of MAP kinase induced by Gβγ is mediated by a common signaling pathway with receptor-tyrosine-kinases [19–21]. The data suggest that activation of MAP kinase by $G_i$-coupled receptors is preceded by the Gβγ-mediated tyrosine phosphorylation of Shc, leading to an increased functional association between Shc, Grb2, and Sos. Moreover, disruption of the Shc–Grb2–Sos complex blocks Gβγ-mediated MAP kinase activation, indicating that Gβγ does not mediate MAP kinase activation by a direct interaction with Sos. In agreement with these data, it has been demonstrated previously that LPA- and thrombin-induced activation of MAP kinase is inhibited by the tyrosine kinase inhibitor genistein [22]. Taken together, these findings suggest that additionally tyrosine kinase intermediaries may be required for the activation of $p21^{ras}$ by G-protein-coupled receptor agonists.

The tyrosine phosphorylation of Shc is induced by both tyrosine kinase receptors and some G-protein-coupled receptors such as those for endothelin [23], thyrotropin releasing hormone [24], and thrombin [25]. Although these receptors are thought to couple to pertussis toxin-insensitive G-proteins, perhaps through $G\alpha_q$ or $G\alpha_{11}$, it remains possible that tyrosine phosphorylation of Shc in these cases is also modulated by Gβγ released upon receptor activation; in particular, in the case of thrombin activation, the Shc phosphorylation has been shown to be pertussis toxin sensitive.

Some G-protein-coupled receptors such as the receptor for thrombin are known to stimulate $pp60^{src}$ activity [25]. Since an increase in tyrosine phosphorylation of Shc has been observed in $pp60^{v\text{-}src}$-transformed cells, it is possible that $pp60^{src}$ is activated by a mechanism involving Gβγ subunits. Furthermore, additional evidence demonstrates that Gβγ-mediated Shc phosphorylation is sensitive to tyrosine kinase and PI 3-kinase inhibitors (see below) [26]. Pertussis toxin-sensitive activation of the Src family kinases Src, Fyn, Yes, and Lyn in various cell types has been reported [27, 28], suggesting that these kinases may also function in $G_i$-coupled receptor signaling.

The role of Src-family kinases in this pathway has been directly evaluated recently. Luttrell *et al.* [29] have shown that recruitment and activation of c-Src is involved in LPA and Gβγ stimulation of the MAP kinase pathway. Furthermore, expression of c-Src kinase (Csk), which inactivates Src kinases, inhibits both LPA receptor-mediated Shc tyrosine phosphorylation and MAP kinase activation. It is likely that different isoforms of Src, such as Lyn, Fyn, and Yes can be the mediators of this pathway in specific cell types. Activation of Src-like proteins via G-protein-coupled receptors may also provide a direct link between this class of receptor and other receptor pathways, as c-Src is very well established as a key intermediate in tyrosine kinase receptor signaling.

One additional connection with other kinases of the Src family comes from the observation that Bruton's tyrosine kinase (Btk), reported to interact with c-Src, and its closely related kinases (Itk, Tsk, and TecA), contain a PH domain, and are activated by Gβγ subunits [30]. These findings raise the possibility that Gβγ subunits, possibly together with the products of PI 3-kinase, may regulate a class of tyrosine protein kinases and provide the initial signaling events leading to activation of Src family kinase proteins and the subsequent downstream cascades. In this regard, an intriguing observation is that phosphotyrosine phosphatase SHP2 has been shown to be essential in thrombin-mediated fibroblast proliferation [31]; moreover, phosphorylation and stimulation of SHP2 have been shown to be coupled to the platelet thrombin receptor via a pertussis toxin-sensitive

G-protein [32]. Thus, phosphotyrosine phosphatase may link the tyrosine kinase activity to Gβγ. Alternatively, a PH domain-containing tyrosine kinase may be directly activated by Gβγ by a translocation mechanism in a way similar to that of βARK.

One additional common player proposed is the EGF receptor itself (see Refs. 20 and 33). The EGF receptor becomes phosphorylated shortly after stimulation of G-protein-coupled receptors by LPA and bradykinin through transactivation of the receptor in an EGF-independent manner; the activated receptor could then serve as a scaffold for recruitment of the Grb2/Sos complex via tyrosine phosphorylation of Shc. The rapid response (within seconds of stimulation) rules out a post-transcriptional pathway, and the involvement of a putative phosphatase has been proposed to be responsible for this transactivation. The identity of such a phosphatase remains obscure.

Bombesin, vasopressin, endothelin, thrombin, and LPA receptors stimulate tyrosine phosphorylation of FAK (a focal adhesion kinase, implicated in the control of cytoskeletal reorganization). Src is also known to redistribute into a cytoskeletal compartment upon activation, where it associates with integrin-dependent cytoskeletal complexes. In addition to focal adhesion kinase and Src, integrin signaling complexes contain Csk, the phosphatase PTP1B, PI 3-kinase, and Grb2/Sos, suggesting that these complexes may regulate intracellular signal transduction pathways as well as integrin-mediated cell adhesive interactions [34].

Another tyrosine kinase, related to FAK, and regulated by $G_q$-coupled receptors (Pyk2), has been reported recently. This kinase also activates MAP kinase and SAPK/JNK and is able to bind to c-Src [35, 36]. Recent data suggest that in HEK-293 cells Pyk2 is able to attenuate MAP kinase activation after stimulation of both α1B and α2A-adrenergic receptors (activators of $G_i$/Gβγ and $G_{q/11}$ pathways respectively), in a point downstream of PLCβ and calcium/calmodulin, but upstream of Src/Shc/Sos [37]. These results suggest that in some systems both pathways converge at the level of PLC and proceed in a way involving tyrosine phosphorylation dependent on calcium. Whether Pyk2 is involved in the proposed EGF receptor transactivation warrants further investigation.

## G-PROTEIN-COUPLED RECEPTORS AND SAPK/JNK PATHWAYS

The role of Rho family proteins (including RhoA, Rac1, and Cdc42) in the activation of SAPK/JNK has been demonstrated independently by Coso *et al.* [38] and Minden *et al.* [39]. In contrast to Rac1 and Cdc42, the small G-protein Rho apparently is unable to activate SAPK/JNK in some systems, suggesting their selective involvement in some additional pathways. G-protein-coupled receptors are able to activate SAPK/JNK in a Gβγ-dependent manner [40], but the identity of molecules enabling the receptor to communicate with the small $G_s$ is poorly characterized. It has been reported recently that SAPK2/p38 is activated upon stimulation of muscarinic and β-adrenergic receptors, suggesting that the signal from the m2 and β-adrenergic receptors to SAPK2/p38 MAP kinase is mediated by Gβγ, whereas the signal from the m1 receptor is mediated by both Gβγ and $G\alpha_{q/11}$ [41].

The connection between Rho and transcriptional activation has been evaluated recently. Using NIH 3T3 cells, Fromm *et al.* [42] have shown that stimulation of m1 muscarinic receptors induced SRE-driven gene activity mediated only by the $G\alpha_{12}$ and RhoA proteins, providing evidence that these proteins are components of a novel signal transduction pathway that leads to the TCF-independent transcriptional activation of the SRE and to cellular transformation. However, the biochemical route(s) connecting G-protein-coupled receptors and $G_{12}$ to Rho, and Rho to the SRE, remains largely unknown. Furthermore, there is evidence that Rho regulates a number of signaling molecules, including critical enzymes involved in phospholipid metabolism, and several novel kinases such as Rho-kinase and protein kinase N [43] that can be candidates for downstream targets of the $G\alpha_{12}$ and RhoA pathway.

## PI 3-KINASE IN SIGNAL TRANSDUCTION BY G-PROTEIN-COUPLED RECEPTORS

Another player involved in the activation of MAP kinase cascades by G-protein-coupled receptors is PI 3-kinase. Activation of a range of receptors also results in stimulation of PI 3-kinase, which catalyzes the phosphorylation of the 3 position of the inositol ring of phosphoinositides, leading to the generation of the phosphoinositides Ptdlns(3)P, Ptdlns(3,4)$P_2$, and Ptdlns(3,4,5)$P_3$ in a variety of cell types. This activity is found in virtually all cell types and has an effect on a wide variety of cellular processes, including the oxidative burst response in neutrophils, chemotaxis, formation of membrane ruffles, mitogenesis, and activation of intracellular signaling molecules, such as $p70^{S6\text{-kinase}}$ and Akt [44].

Several PI 3-kinases have now been identified from a wide range of sources, and these share sequence homology within their lipid-kinase domains [45]. The classical form of PI 3-kinase is a dimeric enzyme (subunits of 110 and 85 kDa); p85 possesses one SH3 group, two SH2 groups, and a domain that may have GAP activity towards an unidentified target.

Two isoforms, p85α and p85β, have been isolated. p85α is ubiquitously distributed, whereas p85β is detected predominantly in brain and lymphoid tissues. These isoforms exhibit extensive amino acid homology to each other. The role of individual p85 isoforms in the regulation of PI 3-kinase activity is not known. Several mammalian isoforms of the catalytic subunit have been cloned, e.g. a bovine clone called p110α, and a human clone named p110β [45]. Both isoforms of the catalytic subunit have PI 3-kinase activity and associate with p85. A further clone with very high homology to Vps34p was cloned recently

[46]. In addition, a PI 3-kinase responsive to Gβγ proteins has been cloned [47, 48]. The protein termed PI 3-kinase γ (or p110γ) has a predicted molecular mass of 120 kDa. There is no similarity between PI 3-kinase γ and either PI 3-kinase α or PI 3-kinase β in the $NH_2$-terminal region through which PI 3-kinase α and PI 3-kinase β bind to p85. A potential PH domain is located near its amino terminus. PI 3-kinase γ is very well expressed in myeloid (U937 and K562) and lymphoid cells. PI 3-kinase γ forms a complex with p101, an adaptor protein specific for PI 3-kinase γ, which is distinct from p85 and markedly stimulates the Gβγ responsiveness of PI 3-kinase γ. The isolation of a novel class of *Drosophila* and murine PI 3-kinase, named Cpk and Cpk-m (mmp170), respectively, and a PI 3-kinase δ regulated by p85 and well expressed in lymphoid cells have also been reported [45].

$p21^{ras}$ can bind directly to the catalytic subunit of PI 3-kinase α and PI 3-kinase γ in a GTP-dependent manner [49, 50]. A dominant-negative Ras mutant is able to inhibit the NGF- and EGF-mediated elevation of 3′-phosphorylated inositol lipids in PC12 cells, suggesting that Ras acts upstream of PI 3-kinase. As Ras is central for mitogenesis in many cells, it is very important to understand how Ras affects PI 3-kinase activity and what effects PI 3-kinase products have in the regulation of cell growth.

Roche *et al.* [51] have demonstrated that inhibitory antibodies to the p110 subunit of PI 3-kinase block DNA synthesis in response to PDGF and EGF, but not that induced by colony-stimulating factor (CSF)-1, bombesin, or LPA. Other members of the Src kinase family, such as Lyn and Fyn, also have been shown to interact with PI 3-kinase via their SH3 domains present in p85 [52].

The activation of platelets has been used as a model to explore the regulation of PI 3-kinase in relation to G-protein-coupled receptors. The thrombin receptor seems to be coupled to at least one $G_q$-like and one $G_i$-like G-protein. Ptdlns(3)P is found at low but detectable levels in resting platelets, while Ptdlns(3,4)$P_2$ and Ptdlns(3,4,5)$P_3$ are produced only in response to thrombin. Activators of G-proteins, such as GTPγS, have been shown to activate PI 3-kinase in a dose–response manner in permeabilized platelets, thus implicating G-proteins in the regulation of PI 3-kinase [53].

Stimulation of PI 3-kinase activity in platelets seems to be dependent on the activation of PKC. Both calcium ionophores and PMA stimulate changes in 3-phosphoinositides in platelets, but neither one alone, or in combination, is as potent as thrombin. This suggests that factors other than products of PLC-derived second messengers are required for full activation [54]. Upon thrombin stimulation of platelets, increased PI 3-kinase activity can be detected in anti-phosphotyrosine, anti-Src, and anti-Fyn immunoprecipitates, implicating tyrosine kinases and p85/p110 PI 3-kinase in the thrombin-stimulated synthesis of 3-phosphorylated inositol lipids [55], thus suggesting that a fraction of the PI 3-kinase is probably binding directly to either a protein-tyrosine kinase or to a phosphorylated substrate molecule. Tyrosine kinases are further implicated in this process, since the tyrosine kinase inhibitor tyrphostin AG-213 inhibits thrombin-stimulated Ptdlns(3,4)$P_2$ production in platelets [56].

Some candidate targets of phosphoinositides have been suggested in recent reports. Ptdlns(3,4)$P_2$ and Ptdlns(3,4,5)$P_3$ are able to activate calcium-independent PKC isoforms *in vitro*, and these lipids are able to induce an increase in the phosphorylation of pleckstrin, the major PKC substrate in platelets. PI 3-kinase also seems to be upstream of the protein kinases AKT and $p70^{S6}$ kinase, and there is evidence for direct binding of Ptdlns(3,4)$P_2$ and Ptdlns(3,4,5)$P_3$ to the PH domains of AKT and the kinase that phosphorylates AKT, called PDK [44]. SH2 domains have been shown to bind Ptdlns(3,4,5)$P_3$ in competition with tyrosine phosphorylated proteins, raising the possibility that this lipid can recruit SH2-containing proteins to the cellular membrane [57].

The initial suggestion for the involvement of some PI 3-kinase activity mediating the pathway between G-protein-coupled receptors and MAP kinase came from preliminary observations that wortmannin was able to block MAP kinase activity after stimulation of muscarinic receptors or Gβγ proteins. Several papers report similar effects in different systems. Ferby *et al.* [58] have shown that the phospholipid mediator platelet-activating factor (PAF), a very well known activator of MAP kinase and PI 3-kinase activities by G-protein-coupled receptor in blood cells, acts in a wortmannin-sensitive manner. PAF-induced MAP kinase activation in guinea pig neutrophils and in CHO cells carrying the PAF receptor was partially inhibited by wortmannin, while on the other hand wortmannin completely blocked somatostatin-induced MAP kinase activation in CHO cells expressing a cloned somatostatin receptor (SSTR4) [59]. SSTR4 does not cause any $Ca^{2+}$ signaling in the cells, but does potently activate MAP kinase. These findings raised a reasonable possibility that PI 3-kinase might be involved in G-protein-dependent MAP kinase activation in a $Ca^{2+}$-independent pathway. In rat 3Y1 fibroblasts stimulated with vasopressin, a similar additive effect was observed with wortmannin treatment and PKC down-regulation [60].

Hawes *et al.* [61] were able to show that wortmannin and LY294002 block the Ras and MAP kinase activation induced by LPA, $\alpha_2$-adrenergic receptor, or Gβγ proteins. In contrast, wortmannin and LY294002 have a lesser effect on MAP kinase activation stimulated by PMA or EGF. This inhibition is limited to the MAP kinase signaling pathway, in that PI 3-kinase inhibitors do not affect $\alpha_2$-adrenergic receptor-mediated phosphoinositide hydrolysis.

The identity of the PI 3-kinase involved in this wortmannin-sensitive pathway has been explored recently. Ferby *et al.* [58] have shown that the stable transfection of a murine macrophage cell line (P388D1), expressing an inducible dominant-negative p85 (Δp85) that lacks the p110 binding site, does not affect the MAP kinase stimu-

lated by PAF, whereas Hawes *et al.* [61] demonstrated that transient transfection in COS-7 cells of one analogous dominant-negative p85 leads to blockage of the pathway after stimulation with the $\alpha_2$-adrenergic receptor of $G\beta\gamma$. The discrepancy in these reports can be explained by the different cell lines and the type of transfection used. $\Delta$p85 may inhibit the $G\beta\gamma$ signal by binding directly to $PIP_3$. Binding of $\Delta$p85 to $PIP_3$ may disrupt PI 3-kinase-dependent signaling by preventing $PIP_3$ from competing with phosphoproteins for binding to an SH2 domain. Furthermore, using purified fractions from rat liver, the presence of one activity synergistically stimulated by both phosphotyrosyl peptides and $G\beta\gamma$ proteins has been shown; this fraction was identified as the dimer p85$\alpha$/p110$\beta$ [62]. The biological relevance and the molecular details of this phenomenon remain to be determined.

On the other hand, p110$\gamma$ was speculated to be the PI 3-kinase activity downstream of heterotrimeric GTPase-linked receptors on the basis of its sensitivity to $G\alpha$-GTP and $G\beta\gamma$-subunits *in vitro* and its expression in myeloid-derived cells. The transfection of a dominant negative form of PI 3-kinase $\gamma$ was able to block the MAP kinase activation by muscarinic receptors and $G\beta\gamma$, and the transfection of PI 3-kinase $\gamma$ alone was able to induce a dose-dependent activation of MAP kinase, suggesting that PI 3-kinase $\gamma$ was both necessary and sufficient for MAP kinase activation, in a pathway involving a Src tyrosine kinase, Shc, Sos, and Ras [21].

The exact role of the Src and Shc proteins is far from clear. One of the products of PI 3-kinase, $PIP_3$, is capable of binding with high affinity to the SH2 domains of proteins such as Src and the p85 subunit of PI 3-kinase $\alpha$ [57]. In addition, $PIP_3$ can compete with tyrosine-phosphorylated proteins for binding to these sites. $PIP_3$ may block phosphoprotein binding to SH2 domain-containing proteins or even supplant phosphoproteins bound to an SH2 domain. It is therefore possible that $PIP_3$ may serve as an intermediate in the $G\beta\gamma$-mediated MAP kinase kinase signaling pathway.

The identity and physiological relevance of some specific Src kinases acting in this pathway and the molecules intervening need to be determined. In fact, recent data suggest the involvement of an additional 100-kDa tyrosine-phosphorylated protein for the $G_i$-mediated MAP kinase activation. This protein preferentially binds to the C-terminal SH3 domain of Grb2 following LPA or thrombin stimulation, suggesting a model in which the p100–Grb2 complex, together with an upstream non-Src tyrosine kinase and a wortmannin-sensitive PI 3-kinase, links $G_i$ to Ras/MAP kinase activation, with Src and Shc functioning in a separate pathway [63].

In summary, G-protein-coupled receptor stimulation of MAP kinases is dependent upon heterotrimeric G-proteins, lipid kinases, tyrosine kinases, and low-molecular-mass G-proteins. However, the precise relationship between these elements remains poorly defined. It is probable that a number of elements remain to be characterized, and there is no doubt that to dissect the components of these signal transduction pathways in detail will keep researchers busy for some time.

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## References


1. Schlessinger J and Ullrich A, Growth factor signaling by receptor tyrosine kinases. *Neuron* **9:** 383–391, 1992.
2. Fantl WJ, Johnson DE and Williams LT, Signaling by receptor tyrosine kinases. *Annu Rev Biochem* **62:** 453–481, 1993.
3. Post GR and Brown JH, G protein-coupled receptors and signaling pathways regulating growth responses. *FASEB J* **10:** 741–749, 1996.
4. Cano E and Mahadevan LC, Parallel signal processing among mammalian MAPKs. *Trends Biochem Sci* **20:** 117–122, 1995.
5. Cohen P, The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Biochem Sci* **7:** 353–361, 1997.
6. Denhardt DT, Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: The potential for multiplex signaling. *Biochem J* **318:** 729–747, 1996.
7. Salcini AE, McGlade J, Pelicci G, Nicoletti I, Pawson T and Pelicci PG, Formation of Shc–Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. *Oncogene* **9:** 2827–2836, 1994.
8. Erpel T and Courtneidge SA, Src family protein tyrosine kinases and cellular signal transduction pathways. *Curr Opin Cell Biol* **7:** 176–182, 1995.
9. Watson S and Arkinstall S, *The G-Protein Linked Receptor Facts Book*. Academic Press, London, 1994.
10. Berman DM, Wilkie TM and Gilman AG, GAIP and RGS4 are GTPase-activating proteins for the $G_i$ subfamily of G protein $\alpha$ subunits. *Cell* **86:** 445–452, 1996.
11. Neer EJ, Intracellular signaling: Turning down G-protein signals. *Curr Biol* **7:** 31–33, 1997.
12. Baffy G, Yang L, Raj S, Manning DR and Williamson JR, G protein coupling to the thrombin receptor in Chinese hamster lung fibroblasts. *J Biol Chem* **269:** 8483–8487, 1994.
13. Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marmé D and Rapp UR, Protein kinase C$\alpha$ activates RAF-1 by direct phosphorylation. *Nature* **364:** 249–252, 1993.
14. Cook SJ and McCormick F, Kinetic and biochemical correlation between sustained p44ERK1 activation and lysophosphatidic acid-stimulated DNA synthesis in Rat-1 cells. *Biochem J* **320:** 237–245, 1996.
15. Faure M, Voyno-Yasenetskaya TA and Bourne HR, cAMP and $\beta\gamma$ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J Biol Chem* **269:** 7851–7854, 1994.
16. Crespo P, Xu N, Simonds WF and Gutkind JS, Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits. *Nature* **369:** 418–420, 1994.
17. Lemmon MA, Ferguson KM and Schlessinger J, PH domains: Diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* **85:** 621–624, 1996.
18. Gibson TJ, Hyvonen M, Musacchio A, Saraste M and Birney E, PH domains. *Trends Biochem Sci* **19:** 349–353, 1994.
19. van Biesen T, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, Sakaue M, Luttrell LM and Lefkowitz RJ, Receptor-tyrosine-kinase- and $G\beta\gamma$-mediated MAP kinase

activation by a common signalling pathway. *Nature* **376:** 781–784, 1995.
20. Daub H, Weiss FU, Wallasch C and Ullrich A, Role of transactivation of the EGF receptor in signaling by G-protein-coupled receptors. *Nature* **379:** 557–560, 1996.
21. Lopez-Ilasaca M, Crespo P, Pellici PG, Gutkind JS and Wetzker R, Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase γ. *Science* **275:** 394–397, 1997.
22. Hordijk PL, Verlaan I, van Corven EJ and Moolenaar WH, Protein tyrosine phosphorylation induced by lysophosphatidic acid in Rat-1 fibroblasts. Evidence that phosphorylation of MAP kinase is mediated by the $G_i$-$p21^{ras}$ pathway. *J Biol Chem* **269:** 645–651, 1994.
23. Cazaubon SM, Ramos-Morales F, Fischer S, Schweighoffer F, Strosberg AD and Couraud P-O, Endothelin induces tyrosine phosphorylation and GRB2 association of Shc in astrocytes. *J Biol Chem* **269:** 24805–24809, 1994.
24. Ohmichi M, Sawada T, Kanda Y, Koike K, Hirota K, Miyake A and Saltiel AR, Thyrotropin-releasing hormone stimulates MAP kinase activity in GH3 cells by divergent pathways. Evidence of a role for early tyrosine phosphorylation. *J Biol Chem* **269:** 3783–3788, 1994.
25. Chen Y, Grall D, Salcini AE, Pelicci PG, Pouysségur J and Van Obberghen-Schilling E, Shc adaptor proteins are key transducers of mitogenic signaling mediated by the G protein-coupled thrombin receptor. *EMBO J* **15:** 1037–1044, 1996.
26. Touhara K, Hawes BE, van Biesen T and Lefkowitz RJ, G protein βγ subunits stimulate phosphorylation of Shc adapter protein. *Proc Natl Acad Sci USA* **92:** 9284–9287, 1995.
27. Ptasznik A, Traynor-Kaplan A and Bokoch GM, G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase · Shc adapter protein signaling complexes. *J Biol Chem* **270:** 19969–19973, 1995.
28. Ishida M, Marrero MB, Schieffer B, Ishida T, Bernstein KE and Berk BC, Angiotensin II activates $pp60^{c\text{-}src}$ in vascular smooth muscle cells. *Circ Res* **77:** 1053–1059, 1995.
29. Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ and Lefkowitz RJ, Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gβγ subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* **271:** 19443–19450, 1996.
30. Langhans-Rajasekaran SA, Wan Y and Huang X-Y, Activation of Tsk and Btk tyrosine kinases by G protein βγ subunits. *Proc Natl Acad Sci USA* **92:** 8601–8605, 1995.
31. Rivard N, McKenzie FR, Brondello J-M and Pouysségur J, The phosphotyrosine phosphatase PTP1D, but not PTP1C, is an essential mediator of fibroblast proliferation induced by tyrosine kinase and G protein-coupled receptors. *J Biol Chem* **270:** 11017–11024, 1995.
32. Li RY, Gaits F, Ragab A, Ragab-Thomas JMF and Chap H, Tyrosine phosphorylation of an SH2-containing protein tyrosine phosphatase is coupled to platelet thrombin receptor via a pertussis toxin-sensitive heterotrimeric G-protein. *EMBO J* **14:** 2519–2526, 1995.
33. Zwick E, Daub H, Aoki N, Yamaguchi-Aoki Y, Tinhofer I, Maly K and Ullrich A, Critical role of calcium-dependent epidermal growth factor receptor transactivation in PC12 cell membrane depolarization and bradykinin signaling. *J Biol Chem* **272:** 24767–24770, 1997.
34. Clark EA and Brugge JS, Integrins and signal transduction pathways: The road taken. *Science* **268:** 233–239, 1995.
35. Dikic I, Tokiwa G, Lev S, Courtneidge SA and Schlessinger J, A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383:** 547–550, 1996.
36. Tokiwa G, Dikic I, Lev S and Schlessinger J, Activation of Pyk2 by stress signals and coupling with JNK signaling pathway. *Science* **273:** 792–794, 1996.
37. Della Rocca GJ, van Biesen T, Daaka Y, Luttrell DK, Luttrell LM and Lefkowitz RJ, Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of $G_i$- and $G_q$-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J Biol Chem* **272:** 19125–19132, 1997.
38. Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T and Gutkind JS, The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81:** 1137–1146, 1995.
39. Minden A, Lin A, Claret FX, Abo A and Karin M, Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81:** 1147–1157, 1995.
40. Coso OA, Teramoto H, Simonds WF and Gutkind JS, Signaling from G protein-coupled receptors to c-Jun kinase involves βγ subunits of heterotrimeric G proteins acting on a Ras and Rac1-dependent pathway. *J Biol Chem* **271:** 3963–3966, 1996.
41. Yamauchi J, Nagao M, Kaziro Y and Itoh H, Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors. Involvement of Gβγ and $G\alpha_{q/11}$ subunits. *J Biol Chem* **272:** 27771–27777, 1997.
42. Fromm C, Coso OA, Montaner S, Xu N and Gutkind JS, The small GTP-binding protein Rho links G protein-coupled receptors and $G\alpha_{12}$ to the serum response element and to cellular transformation. *Proc Natl Acad Sci USA* **94:** 10098–10103, 1997.
43. Ridley AJ, Rho: Theme and variations. *Curr Biol* **6:** 1256–1264, 1996.
44. Toker A and Cantley LC, Signaling through the lipid products of phosphoinositide-3-OH kinase. *Nature* **387:** 673–676, 1997.
45. Vanhaesebroeck B, Leevers SJ, Panayotou G and Waterfield MD, Phosphoinositide 3-kinases: A conserved family of signal transducers. *Trends Biochem Sci* **22:** 267–272, 1997.
46. Volinia S, Dhand R, Vanhaesebroeck B, MacDougall LK, Stein R, Zvelebil MJ, Domin J, Panaretou C and Waterfield MD, A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. *EMBO J* **14:** 3339–3348, 1995.
47. Stoyanov B, Volinia S, Hanck T, Rubio I, Loubtchenkov M, Malek D, Stoyanova S, Vanhaesebroeck B, Dhand R, Nürnberg B, Gierschik P, Seedorf K, Hsuan JJ, Waterfield MD and Wetzker R, Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science* **269:** 690–693, 1995.
48. Stephens LR, Eguinoa A, Erdjument-Bromage H, Lui M, Cooke F, Coadwell J, Smrcka AS, Thelen M, Cadwallader K, Tempst P and Hawkins PT, The Gβγ sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell* **89:** 105–114, 1997.
49. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD and Downward J, Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370:** 527–532, 1994.
50. Rubio I, Rodriguez-Viciana P, Downward J and Wetzker R, Interaction of Ras with phosphoinositide 3-kinase γ. *Biochem J* **326:** 891–895, 1997.
51. Roche S, Koegl M and Courtneidge SA, The phosphatidylinositol 3-kinase α is required for DNA synthesis induced by some, but not all, growth factors. *Proc Natl Acad Sci USA* **91:** 9185–9189, 1994.
52. Pleiman CM, Clark MR, Gauen LKT, Winitz S, Coggeshall KM, Johnson GL, Shaw AS and Cambier JC, Mapping of sites on the Src family protein tyrosine kinases $p55^{blk}$, $p59^{fyn}$, and $p56^{lyn}$ which interact with the effector molecules phospholipase C-γ2, microtubule-associated protein kinase, GTPase-

activating protein, and phosphatidylinositol 3-kinase. *Mol Cell Biol* **13:** 5877–5887, 1993.
53. Grand RJ, Turnell AS and Grabham PW, Cellular consequences of thrombin-receptor activation. *Biochem J* **313:** 353–368, 1996.
54. Kucera GL and Rittenhouse SE, Human platelets form 3-phosphorylated phosphoinositides in response to α-thrombin, U46619, or GTPγS. *J Biol Chem* **265:** 5345–5348, 1990.
55. Gutkind JS, Lacal PM and Robbins KC, Thrombin-dependent association of phosphatidylinositol-3 kinase with $p60^{c\text{-}src}$ and $p59^{fyn}$ in human platelets. *Mol Cell Biol* **10:** 3806–3809, 1990.
56. Guinebault C, Payrastre B, Sultan C, Mauco G, Breton M, Levy-Toledano S, Plantavid M and Chap H, Tyrosine kinases and phosphoinositide metabolism in thrombin-stimulated human platelets. *Biochem J* **292:** 851–856, 1993.
57. Rameh LE, Chen C-S and Cantley LC, Phosphatidylinositol $(3,4,5)P_3$ interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. *Cell* **83:** 821–830, 1995.
58. Ferby IM, Waga I, Hoshino M, Kume K and Shimizu T, Wortmannin inhibits mitogen-activated protein kinase activation by platelet-activating factor through a mechanism independent of p85/p110-type phosphatidylinositol 3-kinase. *J Biol Chem* **271:** 11684–11688, 1996.
59. Sakanaka C, Ferby I, Waga I, Bito H and Shimizu T, On the mechanism of cytosolic phospholipase $A_2$ activation in CHO cells carrying somatostatin receptor: Wortmannin-sensitive pathway to activate mitogen-activated protein kinase. *Biochem Biophys Res Commun* **205:** 18–23, 1994.
60. Nishioka N, Hirai S, Mizuno K, Osada S, Suzuki A, Kosaka K and Ohno S, Wortmannin inhibits the activation of MAP kinase following vasopressin V1 receptor stimulation. *FEBS Lett* **377:** 393–398, 1995.
61. Hawes BE, Luttrell LM, van Biesen T and Lefkowitz RJ, Phosphatidylinositol 3-kinase is an early intermediate in the Gβγ-mediated mitogen-activated protein kinase signaling pathway. *J Biol Chem* **271:** 12133–12136, 1996.
62. Kurosu H, Maehama T, Okada T, Yamamoto T, Hoshino S, Fukui Y, Ui M, Hazeki O and Katada T, Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110β is synergistically activated by the βγ subunits of G proteins and phosphotyrosyl peptide. *J Biol Chem* **272:** 24252–24256, 1997.
63. Kranenburg O, Verlaan I, Hordijk PL and Moolenaar WH, $G_i$-mediated activation of the Ras/MAP kinase pathway involves a 100 kDa tyrosine-phosphorylated Grb2 SH3 binding protein, but not Src nor Shc. *EMBO J* **16:** 3097–3105, 1997.