Analysis of the Gs/Mitogen-activated Protein Kinase Pathway in Mutant S49 Cells*

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Heterotrimeric G protein-coupled receptors can activate the mitogen-activated protein kinase (MAPK) cascade. Recent studies using pharmacological inhibitors or dominant-negative mutants of signaling molecules have advanced our understanding of the pathways from G protein-coupled receptors to MAPK. However, molecular genetic analysis of these pathways is inadequate in mammalian cells. Here, using the well characterized Gαo- and protein kinase A-deficient S49 mouse lymphoma cells, we provide the molecular genetic evidence that Gαo is responsible for transducing the β-adrenergic receptor signal to MAPK in a protein kinase A-dependent pathway involving Rap1 and Raf (but not Ras) molecules.

G proteins serve their physiological roles by transducing signals from a broad class of cell-surface receptors to specific effector proteins (1–4). A variety of intracellular signal transduction pathways are regulated by G proteins, including the mitogen-activated protein kinase (MAPK) pathway (5). Although the activation mechanism of the MAPK cascade by receptors with intrinsic tyrosine kinase activity has been well studied, the route from G proteins to the MAPK cascade in mammalian cells is less understood (6, 7).

Recent studies in cultured cell lines with pharmacological inhibitors and dominant-negative mutants of certain signaling molecules have revealed the participation of some molecular components in the regulation of MAPK by G protein-coupled receptors (for review, see Ref. 5). Although the detailed biochemical steps are far from clear, these studies have shown that G protein-coupled receptors use pathways very similar to those utilized by receptor tyrosine kinases to activate the prototype Raf/MEK/MAPK cascade. Gαs and Gαi-coupled receptors transmit the signals to MAPK through a pathway involving tyrosine kinase, adapter proteins Shc and Grb2, guanine nucleotide exchange factor Sos, Ras, and Raf in most cases (for review, see Ref. 5). Phosphatidylinositol 3-kinase has been implicated to act upstream of tyrosine kinases in the Gαi/MAPK pathway in some cells (8–12).

For receptors coupled to Gαs, overexpressing Gβγ or Gαi subunits in COS-7 cells has shown that whereas Gβγ subunits have the capacity to stimulate MAPK, the ability of Gαo to stimulate MAPK is controversial (13, 14). It is also unclear if cAMP and protein kinase A (PKA) participate in the Gαi-coupled receptor/MAPK pathway. Whereas one group reported that cAMP, forskolin, and Gαi-coupled receptors can stimulate MAPK in COS-7 cells (13), another reported that cAMP and PKA do not mediate activation of MAPK by Gαi-coupled receptors in COS-7 cells (14). It was proposed that the Gαi-coupled β-adrenergic receptor used the Gβγ subunit to activate the MAPK pathway through Ras and used the Gαi subunit to inhibit MAPK activation through cAMP and PKA (14). These contradictory results regarding whether the α-subunit or the βγ-subunits of Gα protein mediate the receptor stimulation of MAPK and whether PKA is involved in the Gαi/MAPK pathway in mammalian cells prompted us to address this question genetically. For the most part, signaling by heterotrimeric G proteins has not been studied genetically in mammalian cells.

S49 mouse lymphoma cells have played an important historical role in G protein research (15). A variant of S49 cells lacking Gαo was instrumental in defining the function of and characterizing Gαo (15). Elevation of intracellular cAMP levels results in growth arrest in the G1 phase of the cell cycle and later (after several days) in cell death (16, 17). Mutants have been selected that are resistant to cytosis. These mutants include cyc− (which lacks Gαo) (18), UNC (which has a mutation of arginine at position 372 of Gαo and thus uncouples the interaction of Gαo with the receptors) (19), and kin− (which lacks protein kinase A activity) (20).

These Gαi- and PKA mutant S49 cells should be very useful in a molecular genetic study to understand the role of Gαo and PKA in the β-adrenergic receptor/MAPK signaling system. In this study, using these mutant S49 cells, we demonstrate that Gαo transduces the β-adrenergic receptor signal to MAPK in a protein kinase A-dependent pathway involving Rap1 and Raf (but not Ras) molecules.

EXPERIMENTAL PROCEDURES

S49 Mouse Lymphoma Cells—S49 mouse lymphoma T cells were obtained from the Cell Culture Facility at the University of California at San Francisco and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum as described previously (22–25). Transfection efficiency was ~20%.

Immunoprecipitation and Immunoblot Analysis—S49 whole cell extracts were prepared as follows. Cells were harvested from 10-cm plates and washed twice with cold phosphate-buffered saline, and pellets were resuspended in 0.8 mL of extraction buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1% deoxycholate, 1% Triton X-100, 0.5% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.02 mg/mL leupeptin). Resuspended pellets were passed five times through a 26-gauge needle and centrifuged at 5000 rpm for 5 min at 4 °C to remove insoluble material, and the supernatant was saved as the whole cell extract. For immunoprecipitation, 10 μL of protein G-agarose was added to the whole cell lysate to
RESULTS

\( G_{\alpha} \) Is Required for Transmitting the \( \beta \)-Adrenergic Receptor Signal to MAPK—In wild-type S49 mouse lymphoma cells, the agonist isoproterenol activates the endogenous \( G_{\alpha} \)-coupled \( \beta \)-adrenergic receptor, leading to the stimulation of MAPK activity (Fig. 1A). Isoproterenol-induced increase in MAPK activity was not sensitive to pertussis toxin and could be blocked by the \( \beta \)-adrenergic receptor-specific antagonist propranolol (data not shown). To genetically determine whether \( G_{\alpha} \) by the \( \beta \)-adrenergic receptor failed to increase the activity of MAPK, although the stimulation of MAPK by the endogenous \( G_{\alpha} \)-coupled somatostatin receptor was normal. The isoproterenol effect could be blocked by the \( \beta \)-receptor antagonist propranolol (data not shown). In unc cells, stimulation of the \( \beta \)-adrenergic receptor failed to increase the activity of MAPK, although the stimulation of MAPK by the endogenous \( G_{\alpha} \)-coupled somatostatin receptor was normal. As expected, the \( \beta \)-adrenergic receptor signal to MAPK—

To further confirm the necessity of \( G_{\alpha} \) for this signal transduction pathway, we tested another allele of the \( G_{\alpha} \) mutant, the UNC S49 cells. The UNC mutant of \( G_{\alpha} \) has a single amino acid change at position 372 (from arginine to proline), six residues from the carboxyl terminus (19, 26). This mutant \( G_{\alpha} \) fails to couple to the \( \beta \)-adrenergic receptor. Thus, the \( \beta \)-adrenergic receptor signal is unable to be delivered to downstream targets in vivo. As shown in Fig. 1 (B and D), in UNC mutant S49 cells, stimulation of the \( \beta \)-adrenergic receptor failed to stimulate MAPK activity. Again, stimulation of MAPK by the endogenous \( G_{\alpha} \)-coupled somatostatin receptor was normal. These data reaffirm that \( G_{\alpha} \) is necessary for \( \beta \)-adrenergic receptor/MAPK signaling. The inability of unc- and UNC mutant S49 cells to respond to isoproterenol in stimulating MAPK was not due to gross abnormality of G protein expression in these mutants since we found that expression of \( G_{\alpha} \), \( G_{\beta} \), and \( G_{\alpha} \) proteins in all cell lines was similar, except that \( G_{\alpha} \) was missing in unc- cells (Fig. 1C).

\( G_{\alpha} \) Is the Signal Transducer in the \( \beta \)-Adrenergic Receptor/MAPK Pathway—The requirement for \( G_{\alpha} \) may be due to its signaling role or to its requirement in maintaining the structural integrity of trimeric G proteins or both. To distinguish between the signaling versus structural role, we introduced into unc- cells (a null \( G_{\alpha} \) mutant background) a \( G_{\alpha} \) mutant that still complexes with the \( G_{\beta} \) subunit and is still able to couple to the \( \beta \)-adrenergic receptor (that is, the structural role is still fulfilled), but is unable to stimulate its downstream target adenyl cyclase (that is, the signaling role is impaired). If such a mutant is unable to rescue the unc- mutant response to \( \beta \)-adrenergic receptor stimulation of MAPK, then \( G_{\alpha} \) is very likely the signal transducer. If such a mutant is able to rescue the unc- response to \( \beta \)-adrenergic receptor stimulation of MAPK, \( G_{\alpha} \) is probably needed for structural integrity. Therefore, we tested two \( G_{\alpha} \) mutants with amino acid changes in the effector contact region of \( G_{\alpha} \), previously described to be defec-
active in stimulating adenyl cyclase, but still interacting with Gβγ and the β-adrenergic receptor (27, 28). As shown in Fig. 2, wild-type Gα rescues the cdc5 cell response to β-adrenergic stimulation of MAPK, whereas neither of the two mutants could rescue the response. Furthermore, expression of a constitutively activated Gα mutant (αQ227L, with Gln227 changed to Leu) (29) leads to stimulation of MAPK, indicating that Gα is not only required but also sufficient to activate the MAPK pathway. Thus, we conclude that Gα is the signal transducer in the β-adrenergic receptor/MAPK pathway.

PKA Is Required for Transmitting the β-Adrenergic Receptor Signal to MAPK—Binding of isoproterenol to the β-adrenergic receptor results in the activation of Gα, leading to stimulation of adenyl cyclase and elevation of the intracellular levels of cAMP. Most cAMP-mediated intracellular responses are mediated through protein kinase A in mammalian cells (30). As shown in Fig. 3, stimulation of MAPK by the β-adrenergic receptor is blocked in kin− mutant S49 cells that lack protein kinase A activity, whereas stimulation of MAPK by the Gα-coupled somatostatin receptor is normal in kin− cells. This result further demonstrates that the Gα/adenyl cyclase/cAMP/protein kinase A cascade links the β-adrenergic receptor to MAPK in S49 mouse lymphoma cells.

Downstream Components of the PKA/MAPK Pathway in S49 Cells—Activation of MAPK by PKA in some cells has been proposed to act through a Ras-independent but B-Raf- and Rap1-dependent signaling pathway (31). To examine the role of Ras, Raf, and Rap1 in the PKA/MAPK pathway in S49 cells, we tested the effects of dominant-negative mutants of Ras, Raf, and Rap1 (Fig. 4). Expression of a dominant-negative Ras mutant (RasN17) (32) in S49 cells had no effect on the stimulation of MAPK by Gα-coupled β-receptors (Fig. 4A), whereas it inhibited the MAPK stimulation by Gα-coupled somatostatin receptors (Fig. 4B). Transfection of a dominant-negative Raf mutant (a truncated Raf mutant with the conserved region 1, which interferes with the activation of endogenous Raf including B-Raf) (33–36) into S49 cells blocked the MAPK stimulation by both Gα- and Gβγ-coupled receptors (Fig. 4). While there was no effect on the stimulation of MAPK by the Gα-coupled somatostatin receptor, transfection of a dominant-negative Rap1 mutant (RapIN17) (31) blocked the MAPK stimulation by the Gα-coupled β-receptor (Fig. 4C). These data suggest that in S49 cells, as in some other mammalian cells, the PKA/MAPK pathway requires Rap1 and Raf, but not Ras (31, 37).

DISCUSSION

In summary, using various mutant S49 mouse lymphoma cells, we have provided the first genetic evidence for Gα and protein kinase A transducing the β-adrenergic receptor signal to MAPK. In cdc5 mutant S49 cells that lack Gα proteins, stimulation of the β-adrenergic receptor failed to activate MAPK. In UNC mutant S49 cells that Gα is unable to couple to the β-adrenergic receptor, MAPK could not be stimulated by the β-receptor. Two Gα mutants that can complex with the Gβγ subunit and β-receptor, but are unable to stimulate adenyl cyclase, failed to rescue the cdc5 mutant response to β-receptor stimulation of MAPK. Wild-type Gα rescued the cdc5 cell response. Furthermore, in kin− mutant S49 cells that lack protein kinase A activity, stimulation of MAPK by the β-receptor was blocked. Moreover, dominant-negative mutants of Rap1 or Raf, but not Ras, suppressed the β-receptor-induced MAPK stimulation. These data collectively demonstrate that the Gα-coupled adrenergic receptor uses Gα, transducing the signal to a PKA-, Rap1-, and Raf-dependent, pathway leading to MAPK activation in S49 mouse lymphoma cells.

Previously, Faure et al. (13) have shown that overexpressing Gβγ or constitutively activated Gα could lead to activation of MAPK and that cAMP, forskolin, and Gα-coupled receptors could stimulate MAPK in COS-7 cells. On the other hand, Crespo et al. (14) reported that only overexpression of Gβγ subunits, but not the activated Gα subunit, could increase MAPK activity in COS-7 cells. It was proposed that in COS-7 cells, whereas Gβγ transduces a positive signal to increase MAPK activity, Gα, through protein kinase A, inhibits the MAPK stimulation (14). The reason for this discrepancy is not...
clear. The suggestion that Gβγ mediates the β-adrenergic receptor signal to MAPK is based on two types of experiments (14). One is that, as mentioned above, overexpression of Gβγ could lead to increased MAPK activity. Another is that overexpression of a Gβγ-binding fragment from the β-adrenergic receptor kinase protein or Gα could attenuate the stimulation of MAPK by β-adrenergic receptors.

We were unable to perform a genetic analysis of the role of Gβγ subunits due to the lack of null mutants of Gβ or Gγ subunits in S49 cells. Gβγ is likely required in a structural role for the integrity of G protein function, but is unlikely to play a major signaling role for the following reasons. First, in c-raf mutant cells, the basal activity of MAPK is similar to that in wild-type cells. If Gβγ is the major signal transducer, as in Saccharomyces cerevisiae, then in c-raf cells, MAPK could be constitutively active, as in Gα null mutant cells in S. cerevisiae (38, 39). Second, two mutant Gα (αa89 and αa389) subunits did not rescue the c-raf cell response despite being able to release Gβγ upon receptor stimulation. Third, in S49 cells, the isoforms of adenylyl cyclases can be stimulated by Gα upon receptor stimulation. These data suggest that if Gβγ is needed, it is for structural reasons only, not for activating downstream targets. Therefore, in S49 mouse lymphoma cells, Gα, not Gβγ, transduces the receptor signal to MAPK.

In the fission yeast Schizosaccharomyces pombe, the α-subunit of G protein carries the signal to the MAPK pathway (41). In the budding yeast S. cerevisiae, the βγ-subunit of G protein couples the receptor to the MAPK cascade (38, 39). Given that the α-subunit of Gα transduces Gα-coupled receptor signal to MAPK in S49 cells and that the βγ-subunit of Gα likely conveys the message to Gα-coupled receptors to MAPK (15, 42, 43), these different usages of the α and βγ subunits might be reminiscent of S. pombe versus S. cerevisiae MAPK signaling pathways. Thus, mammalian cells have both yeast pathways that are utilized by different families of G proteins.

The cAMP and protein kinase A effect on the MAPK pathway depends on cell type: in some cells, they are stimulatory to the MAPK pathway, whereas in other cells, they are inhibitory (44, 45). A recent study has determined that these stimulatory or inhibitory effects are dictated by the expression of B-Raf (31). Protein kinase A directly activates the small G protein Rap1, which in turn, selectively and directly activates B-Raf, leading to the activation of MAPK. We found that B-Raf is expressed in S49 cells and that isoproterenol could stimulate B-Raf activity in S49 cells. Also, we have examined the activation of MEK, an upstream activator of MAPK, and obtained results similar to those for MAPK activation. Thus, we propose the activation sequence as β-adrenergic receptor/Gα/adenylyl cyclase/cAMP/ PKA/Rap1/B-Raf/MEK/MAPK. This Gα/MAPK pathway represents the first complete biochemical pathway for G protein/MAPK signaling.

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