Specific Involvement of G Proteins in Regulation of Serum Response Factor-mediated Gene Transcription by Different Receptors*

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Regulation of serum response factor (SRF)-mediated gene transcription by G protein subunits and G protein-coupled receptors was investigated in transfected NIH3T3 cells and in a cell line that was derived from mice lacking Goq and Gα11. We found that the constitutively active forms of the α subunits of the Goq and Gα12 class of G proteins, including Goq, Gαq11, Goq14, Goq16, and Gα13, can activate SRF in NIH3T3 cells. We also found that the type 1 muscarinic receptor (m1R) and α1-adrenergic receptor (AR)-mediated SRF activation is exclusively dependent on Goq11, while the receptors for thrombin, lysophosphatidic acid (LPA), thromboxane A2, and endothelin can activate SRF in the absence of Goq11. Moreover, RGS12 but not RGS2, RGS4, or Axin was able to inhibit Gαq and Gα13-mediated SRF activation. And RGS12, but not other RGS proteins, blocked thrombin- and LPA-mediated SRF activation in the Goq11-deficient cells. Therefore, the thrombin, LPA, thromboxane A2, and endothelin receptors may be able to couple to Gα12/13. On the contrary, receptors including β2- and α2-ARs, m2R, the dopamine receptors type 1 and 2, angiotensin receptors type 1 and 2, and interleukin-8 receptor could not activate SRF in the presence or absence of Goq11, suggesting that these receptors cannot couple to endogenous G proteins of the Gq12 or Gq classes.

Hormones, neurotransmitters, and many other biologically active molecules, such as lysophosphatidic acid (LPA),¹ thrombin, catecholamines, endothelin, etc., transduce their signals through heterotrimeric G proteins (1, 2). Molecular cloning has revealed at least four classes of G protein α subunits: Goq, Gαq, Gα12, and Gα13 (3). The Goq subunits and Gαq subunits regulate adenyl cyclase activities, while the Gα12 subunits regulate phospholipase C activities. However, the function of the Gα12 class of G proteins, which includes Gα12 and Gα13, remains to be elucidated. Activated forms of Gα12 and Gα13, when transfected into fibroblast cells, were shown to induce transformation phenotypes (4–6), suggesting that this class of G proteins may be involved in cell growth regulation. Moreover, Gα12 and Gα13 were shown to induce formation of stress fibers in fibroblast cells through small G protein RhoA (7). This observation was supported by the report that Gα13 activated serum response factor (SRF) through RhoA (8). The in vivo function of Gα13 was also investigated using the gene-targeting technique in mice. Mice lacking Gα13 are embryonic lethal apparently due to the failure to develop vasculature structures, indicating that Gα13 may be involved in the function of endothelial cells (9). In the same study, thrombin-mediated chemotaxis of fibroblasts lacking Gα13 was blocked, indicating that the thrombin receptor couples to Gα13. This is consistent with the observation that thrombin as well as a thromboxane A2 receptor agonist could stimulate the binding of a photo-affinity GTP analog to Gα13 (10). However, there were contradictory reports with regard to the involvement of the Gq class of G proteins in RhoA and SRF activation (7, 8, 16).

RGS (regulator of G protein signaling) proteins belong to a growing family of proteins that contains homologous RGS domains (11, 12). Some of these proteins such as GIAP and RGS4 were shown to inhibit G protein-mediated signaling by interacting with the Goq and Gαq subunits and stimulating their GTPase activities (13), which are also referred to as GTPase-activating protein activities. RGS2 and RGS4 were found to inhibit Goq-mediated activation of phospholipase Cβ (14–16), implying that they may function as GTPase-activating protein for Goq. Interestingly, most of these RGS proteins were unable to inhibit Gq-mediated signaling, and they could not stimulate the GTPase activity of the Goq proteins (13, 14). Regulation of the G12/13 proteins by RGS proteins has not been investigated.

In this report, we characterized the abilities of the Gq protein subunits and of a number of GPCRs to stimulate SRF-mediated gene transcription using a cotransfection system. We found that the α subunits of the Goq and Gα12 class of G proteins can induce SRF activation in a C3-dependent manner. We also found that the activation of SRF by some of GPCRs depends exclusively on Goq11, while others do not. Those that activate SRF independently of Goq11 may act through Goq12/13. In addition, we, for the first time, identified a RGS protein, RGS12, that can inhibit the Gα12/13 function.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Luciferase Assay—NIH3T3 and the Goq-deficient cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum at 37°C under 5% CO2. The Goq-deficient cell line was established from mice lacking both Goq and Gα11 (17). For transfection, cells (5 × 10⁴ cells/well) were seeded into 24-well plates the day before transfection. Cells were transfected with 0.5 µg of DNA/well using LipofectAMINE Plus (Life Technologies, Inc.), as suggested by the manufacturer. The transfection was stopped after 3 h by switching to culture medium containing 0.5% fetal bovine serum. Cell extracts were collected 24 h later for luciferase assays.
Luciferase assays were performed using Boehringer Mannheim Constant Light Luciferase Assay Kit as instructed. Cell lysates were first taken for determining in a Wallac multichannel the fluorescence intensity emitted by GFP proteins, which are cotransfected with the luciferase reporter gene plasmid and used to normalize the transfection efficiency. The Wallac counter (Wallac AG&G, Finland) is capable of counting both fluorescence and luminescence. Then, the luciferase substrate was added to the cell lysates, and luciferase activities were determined by measuring luminescence intensity using the same counter. Luminescence intensities were normalized against fluorescence intensities. DNA concentrations were adjusted if transfection of any of the cDNAs resulted in significant differences between normalized and non-normalized data.

**Construction of Expression Plasmids—**All of the G protein subunits and GPCRs were in pCMV expression vectors as described previously (18–20). The SRE.L-luciferase reporter plasmid was constructed as described in Ref. 7, except the luciferase gene was used as the reporter instead of the chimeraphenolic acetyltransferase gene. The CRE-luciferase reporter gene plasmid was purchased from Stratagene, La Jolla, CA. RGS2, RGS4, and RGS12 (kindly provided by Sheng-Cai Lin) were also in the pCMV vector. Axin (kindly provided by F. Costantini) was in the pcDNA3 vector (Invitrogen). C3 was kindly provided by Alan Hall.

**RESULTS**

A cotransfection system was used to characterize signal transduction pathways mediated by G proteins that lead to the regulation of SRF-dependent gene transcription. SRF-mediated gene transcription was evaluated by determining the activity of luciferase, the production of which is regulated by a transcription regulatory sequence element, called SRE.L. SRE.L is a derivative of c-Fos serum response element (SRE), to which SRF but not tertiary complex factor binds (21). Thus, SRE.L-mediated production of luciferase mainly depends on the activity of SRF. The abilities of various G protein subunits to regulate SRE.L-mediated gene transcription were determined by cotransfecting NIH3T3 cells with the reporter gene plasmid and cDNA encoding one of constitutively active Gα subunits. We found that cells expressing activated α subunit of Gαi, Gαq, Gα14, Gα16, Gα22, or Gα13, produced markedly higher levels of luciferase than those expressing the control β-galactosidase (LacZ), whereas expression of activated Gαo or Gαo, did not (Fig. 1). This indicates that the α subunits of the Gαi and Gα12 classes of G proteins can lead to SRF activation. The finding that C3 blocked SRF activation by the G protein α subunits suggests that the small GTP-binding protein RhoA (Fig. 1) may mediate the SRF activation. C3 (Clostridium butulinum C3 transferase) is a specific RhoA inactivator, which ADP-ribosylates RhoA (21). In our transfection system, C3 inhibited only RhoA-induced but not Cdc42- (Fig. 1) or Rac1- (data not shown) induced SRF activation, indicating that C3 acted specifically.

Activation of SRF by the Gαq and Gα12 family of G proteins allows us to determine which receptors can couple to these G proteins to activate SRF. Many cells, including fibroblasts, contain endogenous receptors for thrombin and LPA, which belong to a superfamily of GPCR. In addition, the thrombin receptor was shown previously to couple to the G12/13 proteins (10) so that it may function as a positive control. We found that both thrombin and LPA were able to stimulate SRE.L-mediated gene transcription in NIH3T3 cells transfected with the reporter gene plasmids (Fig. 2A). This result suggests that NIH3T3 cells contain endogenous receptors for thrombin and LPA. A number of other GPCRs were also tested for their abilities to stimulate SRE.L-mediated gene transcription in transfected 3T3 cells. Cells expressing m1R and α1-AR showed marked increases in luciferase activities in response to carbachol and norepinephrine, respectively (Fig. 1B). Neither carbachol nor norepinephrine elicited any change in the luciferase activity in cells transfected with the reporter gene plasmid alone (Fig. 1A), indicating that there are no endogenous receptors for carbachol and norepinephrine in NIH3T3 cells. A mutant of α1-AR, α1-ARΔ2, was also tested in the same transfection system. α1-ARΔ2, which was unable to couple to Goαq/11 to activate phospholipase C (22), lost the ability to activate SRF in the presence of ligand norepinephrine. This suggests that α1-AR-mediated SRF activation appears to depend on Goαq/11. Furthermore, we tested Gq-coupling m2R and IL-8 receptor and Gq-coupling β2-AR. None of these receptors was able to stimulate SRF activity in response to their ligands (Fig. 2B). These results are consistent with the observation that the Gq and G11 are not involved in regulation of SRF.
It has been demonstrated by various approaches that m1R and α1-AR couple to the Gq proteins (18, 23). Although the receptors for thrombin and LPA have not been rigorously tested for their abilities to couple to the G proteins of the Gq family, they were shown previously to stimulate inositol phosphate accumulation (24), suggesting that they may couple to the Gq proteins. To test the roles of Gq/11 in SRF activation by these receptors, a fibroblast cell line derived from mice lacking Gq/11 was used. Thrombin, LPA, carbachol, and norepinephrine were added to cells expressing the muscarinic receptors, α1R and α2-ARs, IL-8 receptor, and β2-AR, respectively (B). Data are processed and presented as described in the legend to Fig. 1.

Thus, if any receptor can induce SRF activation in this cell line, the cDNA encoding the thrombin receptor and the reporter gene transcription (data not shown) may be due to the lack of endogenous receptors. Therefore, these two ligands were tested in the Gq/11-deficient cells transiently expressing α1R and m1R, respectively. The ligands were still unable to elicit responses, even in the presence of the recombinant receptors (Fig. 3B). However, the responses of cells to norepinephrine were restored when Goq was reintroduced back into the Gq/11-deficient cells by cotransfection with α1R and the reporter gene (Fig. 3B). The same result was also observed for carbachol when m1R and Goq were coexpressed in the Gq/11-deficient cells (Fig. 3B). Therefore, we conclude that α1-AR and m1R are dependent exclusively on Gq/11 in SRF activation. This conclusion further strengthens the idea that the Gq proteins are capable of activating RhoA and SRF. Since α1-AR is able to couple to all the members of the Gq class of G proteins, including Gq14 and Gq16 (19), the inability of α1R to activate SRF in the Gq/11-deficient cell line suggests that there are not sufficient levels of endogenous Gq14 and Gq16 in this cell line. Thus, if any receptor can induce SRF activation in this cell line, it would suggest that this receptor is able to couple to G proteins other than the Gq class, which would be the G12/13 proteins. Thus, the Gq/11-deficient cell line may be used for testing the coupling of receptors to the G proteins of the G12 class. We tested a number of receptors in this Gq/11-deficient cell line, including the endothelin receptors 1a and 1b, thromboxane A2 receptor, angiotensin (AT) receptors type 1a and type 2, α2-AR and β2-AR, and dopamine receptors type 1 and 2. Endothelin was able to activate the SRF-L-mediated gene transcription
in cells expressing endothelin receptor type 1a (C), and thromboxane A2 receptor (D). The next day, cells were lysed 6 h after the addition of thrombin (A), LPA (B), endothelin (C), and U46619 (D) with concentrations indicated in the figure. Data are processed and presented as described in the legend to Fig. 1.

The Gbgα1-deficient cells were cotransfected with 0.15 μg of SRE.L-luciferase reporter plasmid, 0.15 μg of GFP expression construct, and 0.2 μg of LacZ (A, B), the endothelin receptor type 1a (C), and thromboxane A2 receptor (D). The next day, cells were lysed 6 h after the addition of thrombin (A), LPA (B), endothelin (C), and U46619 (D) with concentrations indicated in the figure. Data are processed and presented as described in the legend to Fig. 1.

As expected, cells expressing m2R showed marginal luciferase activities than those expressing the control LacZ (Fig. 5A), since Gq and G12/13-mediated effects shown by cells expressed Gqα1 or Gα12 alone. This suggests that Gα12 or Gα13 may work synergistically with Gbgα12 or Gbgα13 in regulation of SRF.

The wild-type Gbgq12 or Gbgα13, when expressed in NIH3T3 cells, also showed significant stimulation of SRF-mediated transcription (Fig. 6A), although the activity is usually one-fifth of that of the QL mutant when the same amount of DNA is used in transfection (data not shown). The relative high activities of the wild-type Gbgq12 or Gbgα13 may be due to the slow intrinsic GTPase activities of these Gbg subunits (10, 25, 26). The SRF activation by the wild-type Gbgq12 and Gbgα13 allowed us to test if the RGS proteins could inhibit Gbgq12/13-mediated effects. We tested RGS2, RGS4, RGS12, and Axin. RGS2 and RGS4 are among the well characterized RGS proteins, which show GTPase-activating protein activities for the Gq and/or Gα bg families of α subunits (13, 14, 27, 28). RGS12 (29) and Axin (30) are two recently cloned proteins that contain the RGS domains but have not been tested for their abilities to regulate G protein-mediated signaling. Coexpression of RGS12 significantly inhibited Gbgq12- and Gbgα13-induced SRF activation (Fig. 6A), whereas RGS2, Axin (Fig. 6A), and RGS4 (data not shown) showed little effects. Moreover, expression of RGS12 did not inhibit activated RhoA-mediated SRF activation (data not shown), indicating that inhibition of Gbgq12/13-mediated effects by RGS12 is not due to nonspecific inhibition of downstream proteins. The inhibition by RGS12 is also unlikely to be the result of the changes in the expression levels of cotransfected G proteins, since coexpression of RGS12, Axin, or RGS2 did not alter the expression levels of Gbgq12 or Gbgα13 (Fig. 6E). Therefore, RGS12 is likely to affect the function of Gbgq12/13 directly. The effects of the RGS proteins on LPA and thrombin-mediated SRF activation were also tested in the Gαq11-deficient fibroblast cell line, where LPA and thrombin-mediated SRF activation is presumably mediated by the G12 family of G proteins. The Gαq11-deficient cells were cotransfected with SRE.L re-
induced CRE-mediated gene transcription (Fig. 6C), suggesting that none of the RGS proteins could inhibit the G \(_a\) function. However, all the RGS proteins except Axin were also able to inhibit norepinephrine-induced SRF activation in 3T3 cells expressing \(\alpha_1\)-AR (Fig. 6D). As demonstrated earlier, \(\alpha_1\)-AR-mediated SRF activation is dependent on G\(_{q/11}\) proteins. Thus, inhibition of \(\alpha_1\)-AR-mediated SRF by the RGS proteins suggests that these RGS proteins may inhibit G\(_{q/11}\). Both RGS2 and RGS4 were also able to inhibit norepinephrine-induced phospholipase C activation in COS-7 cells coexpressing \(\alpha_1\)-AR.\(^2\) Thus, the action of RGS12 may not be specific to the G\(_{12}\) class of G proteins.

**DISCUSSION**

In this report, we have characterized the involvement of G protein subunits in activation of SRF by a number of GPCRs. Our findings that G\(_{12}\) and G\(_{13}\) can activate SRF are consistent with previous reports that G\(_{12}\) activates SRF through RhoA (8) and that G\(_{12/13}\) induces formation of stress fibers via RhoA (7). The literature, however, appears to be inconsistent with regard to the involvement of G\(_a\) in SRF activation (8, 31). Our findings described in this report demonstrate that not only G\(_a\), but also all other members of this class of G proteins can activate SRF probably through RhoA. The fact that m1R and \(\alpha_1\)-AR use G\(_{q/11}\) exclusively in activation of SRF strongly supports the involvement of G\(_q\) in SRF activation. Not all G\(_q\)-coupled receptors, however, activate SRF in G\(_{q/11}\)-dependent pathways. Receptors, including the endothelin receptors and thromboxane receptor A2, both of which are known to couple to G\(_i\) to activate phospholipase C, can activate SRF in a G\(_{q/11}\)-independent way (32). Moreover, receptors for thrombin and LPA can also lead to RhoA and SRF activation independently of G\(_{q/11}\), although these two receptors may also be able to couple to the G\(_i\) proteins in addition to the G\(_q\) proteins. Interestingly, among those we tested none of the receptors that are previously known to predominantly couple to G\(_i\) or G\(_\alpha\) can induce activation of SRF in the presence or absence of G\(_{q/11}\).

Unlike the \(\alpha\) subunits of the G\(_i\) and G\(_\alpha\) classes, the expression of activated G\(_{12}\) or G\(_{13}\) does not activate SRF. In addition, G\(_i\) does not appear to activate SRF because the \(\beta\)-AR agonist isoprenaline did not stimulate SRF-mediated transcription in cells expressing \(\beta_2\)-AR, while isoprenaline was able to stimulate CRE-mediated transcription. The inability of \(\beta\)-AR to activate SRF also suggests that \(\beta_2\)-AR cannot couple to endogenous G\(_{12/13}\) in the fibroblasts. It is also apparent that m1R and \(\alpha_1\)-AR as well as \(\alpha_2\)-AR, \(\beta_2\)-AR, the IL-8 receptor, and D1 and D2 receptors are unable to couple to G\(_{12}\) or G\(_{13}\), because these receptors could not activate SRF in the G\(_{q/11}\)-deficient cells. The thromboxane A2 and thrombin receptors were shown previously to couple to G\(_{12}\) and G\(_{13}\) using different methods (9, 10, 33). Thus, SRF activation by these receptors should at least in part be mediated by G\(_{12/13}\). The LPA receptor and endothelin receptors may also be able to couple to G\(_{12/13}\) unless there exist yet-to-be identified G protein \(\alpha\) subunits that can also activate SRF in the G\(_{q/11}\)-deficient mouse fibroblast cells. Under the same premise, the inhibition of LPA and thrombin-induced SRF activation by RGS12 may be attributed to the inhibition of G\(_{12/13}\). In fact, RGS12 can inhibit recombinant G\(_{13}\) and G\(_{13}\)-mediated SRF activation (Fig. 6A). The inability of RGS2 and RGS4 to inhibit G\(_{12/13}\); LPA-, or thrombin-mediated SRF activation indicates that these two RGS proteins do not act on G\(_{12/13}\). Therefore, there is apparent specificity in interactions between RGS proteins and G proteins. Although Axin contains a RGS domain, it is unable to regulate any of the known G proteins.

\(^2\) D. Wu, unpublished data.
Gβγ subunits are also involved in regulation of SRF, especially together with Gαq and Gα13. Since SRF is downstream of the signal cascade, we do not know where the Gα-mediated pathways interact with the Gβγ-mediated ones. It appears that the interactions are upstream of RhoA because C3 was able to completely abolish carbachol-mediated effects in cells coexpressing m1R and m2R. It is also not clear how Gαq and Gα13 regulate RhoA and SRF. Recent studies suggested that the Tec family of nonreceptor tyrosine kinases may be regulated by Gαq and Gβγ. In addition, Gβγ was shown to regulate Btk, a member of the Tec family, via phosphatidylinositide 3-kinase (35). Thus, the synergistic activation of SRF by Gα and Gβγ may lie in the kinases. Further studies are needed to better understand these questions.

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