Communication

Gα12 and Gα13 Stimulate Rho-dependent Stress Fiber Formation and Focal Adhesion Assembly*

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Rho, a member of the Ras superfamily of GTP-binding proteins, regulates actin polymerization resulting in the formation of stress fibers and the assembly of focal adhesions. In Swiss 3T3 cells, heterotrimeric G protein-coupled receptors for lysophosphatidic acid and gastrin releasing peptide stimulate Rho-dependent stress fiber and focal adhesion formation. The specific heterotrimeric G protein subunits mediating Rho-dependent stress fiber and focal adhesion formation have not been defined previously. We have expressed GTPase-deficient, constitutively activated G protein α subunits and mixtures of β and γ subunits in Swiss 3T3 cells. Measurement of actin polymerization and focal adhesion formation indicated that GTPase-deficient α12 and α13 but not the activated forms of α2 or αq stimulated stress fiber and focal adhesion assembly. Combinations of β and γ subunits were unable to stimulate stress fiber or focal adhesion formation. Gα12 and α13-mediated stress fiber and focal adhesion assembly was inhibited by botulinum C3 exoenzyme, which ADP-ribosylates and inactivates Rho, indicating that α2 or α13 but not other G protein α subunits or βγ complexes, regulate Rho-dependent responses. The results define the integration of G12 and G13 with the regulation of the actin cytoskeleton.

The heterotrimeric G protein-coupled receptors for lysophosphatidic acid (LPA),1 gastrin releasing peptide (GRP) or bombesin, and thrombin are capable of stimulating the growth of specific cell types (1–3). LPA and thrombin receptors have been shown to stimulate Ras GTP loading which is required for the mitogenic response to these ligands (4, 5). Ras GTP loading in response to LPA and thrombin activation of their receptors requires the heterotrimeric G proteins Gi or Gq (5–7). GRP receptors are mitogenic in Swiss 3T3 cells, lung epithelia, and small cell lung carcinoma cells (2, 9); GRP receptors activate Gq and phospholipase C activity but stimulate little or no GTP loading of Ras (10). In addition to the stimulation of mitogenic responses, the LPA, GRP, and thrombin receptors regulate the polymerization of actin to produce stress fibers and the assembly of focal adhesions (11). The stimulation of stress fiber and focal adhesion assembly has been demonstrated to be regulated by Rho (12, 13).

The polymerization of actin and the actin cytoskeleton is important for cell shape and regulatory responses including chemotaxis and mitogenesis. In quiescent Swiss 3T3 cells, the formation of stress fibers generally parallels the assembly of focal adhesions. Focal adhesions are oligomeric protein complexes that include p125bands, talin, α-actinin, vinculin, and other proteins (14, 15). Focal adhesions link actin stress fibers to integrins at the inner surface of the plasma membrane. How Rho regulates the assembly of focal adhesions and the formation of stress fibers is currently ill-defined. Similarly, the heterotrimeric G proteins that couple LPA, GRP, and thrombin receptors to Rho activation have not been defined. Pertussis toxin does not inhibit receptor-stimulated actin polymerization or focal adhesion assembly, indicating that neither Gi nor Gq mediates these responses (16). Similarly, calcium ionophores and phorbol esters do not stimulate Rho-dependent responses suggesting that Gq activation of phospholipase C is not involved (16).

To define which heterotrimeric G protein subunits were involved in stimulating Rho-dependent actin polymerization to produce stress fibers and focal adhesion assembly, we microinjected expression plasmids encoding GTPase-deficient mutant heterotrimeric G protein α subunits into the nuclei of serum-starved, quiescent Swiss 3T3 cells. For each G protein α subunit, the conserved glutamine (Q) adjacent to the G3 sequence of the GDP/GTP binding domain of the polypeptide was mutated to a leucine (L). The Gln → Leu mutation functionally inhibits the GTPase activity of the polypeptide resulting in a constitutively activated G protein α subunit (17, 18). This mutation corresponds to residue 229 in α12 and 226 in α13. In addition, different combinations of β and γ subunits were microinjected. Each expression plasmid used in the experiments was characterized previously for functional expression (19–21). Following microinjection, cells were stained with rhodamine-phalloidin to identify stress fibers or anti-vinculin antibody for the identification of focal adhesions. Microinjected cells were marked by coinjection of G protein subunit expression plasmids with a plasmid encoding β-galactosidase.

**EXPERIMENTAL PROCEDURES**

For microinjection, Swiss 3T3 cells were plated at approximately 10% confluency on acid-washed glass coverslips in Dulbecco’s modified Eagle’s medium (DMEM) with 5% bovine calf serum (BCS) and 5% newborn calf serum (NCS). The next day, cells were rinsed three times and placed in 0.1% BCS/DMEM. Twenty-four h later, cells were rinsed three times in DMEM in the absence of serum and incubated for an additional 18 h before microinjection. Injections were performed with

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1 The abbreviations used are: LPA, lysophosphatidic acid; GRP, gastrin releasing peptide; DMEM, Dulbecco’s modified Eagle’s medium; BCS, bovine calf serum; NCS, newborn calf serum; PDGF, platelet-derived growth factor.
an Eppendorf automated microinjection system with needles pulled from glass capillaries on a vertical pipette puller (Kapfig, Tujunga, CA). The cDNAs used for expression were inserted in either pCMV5 or pCDNA3 and have been characterized for functional expression. All plasmids were prepared by cesium chloride gradient centrifugation and used at 100 ng/μl for microinjection into the nuclei of Swiss 3T3 cells. The botulinum C3 exoenzyme pGEX2T vector was a gift from Drs. S. Dillon and L. Feig, Tufts Medical School, Boston, MA. The C3 fusion protein was induced, cleaved with thrombin, and purified as described (22). The pCMV5RhoAQL plasmid was a gift from Dr. Sim Winitz, Scripps Research Foundation, LaJolla, CA. Two- to three-h postnuclear injection cells were fixed in 3% paraformaldehyde for 10 min. Cells were rinsed in phosphate-buffered saline and permeabilized using 0.2% Triton X-100 for 5 min. The fixed and permeabilized cells were then incubated with DMEM/8% BCS/5% NCS for 15 min. β-Galactosidase was stained using a rabbit anti-β-galactosidase antibody (Cappel) and a secondary FITC-conjugated sheep anti-mouse antibody (Sigma) and a secondary FITC-conjugated donkey anti-rabbit antibody (Pierce). Rho-adhesions were stained using a mouse monoclonal anti-vinculin antibody (Sigma) and a secondary FITC-conjugated goat anti-mouse (Cappel) antibody. When cells were stained for vinculin, a rhodamine-conjugated goat anti-rabbit antibody (Cappel) was used for detection of β-galactosidase. Coverslips were mounted on slides and examined with a Nikon Diaphot-TMD microscope with epi fluorescence. Images of cells were captured using the IPLAB Spectrum digital image analysis program (Signal Analytics Co., Vienna, VA). All experiments were done at least 3 times with similar results.

RESULTS AND DISCUSSION

Fig. 1 shows that constitutively activated RhoA having glutamine 63 mutated to leucine (RhoQL), which corresponds to the G61L mutation in p21ras, induces stress fiber formation in Swiss 3T3 cells. Strikingly, the Gln → Leu mutant α subunits for G12 (α12QL) and G13 (α13QL), when expressed in Swiss 3T3 cells, mimicked activated RhoQL in stimulating the formation of stress fibers. The activated forms of α12 and α13 were capable of stimulating stress fiber formation in quiescent Swiss 3T3 cells. Activated, GTPase-deficient forms of α12 (α12QL) or α13 (α13QL) were unable to induce stress fiber formation (Fig. 2).

Expression of α2QL appears to disorder stress fibers and cause a loss of cortical actin along the cytoplasmic surface of the plasma membrane. Expression of β12 or β13 also did not induce stress fiber formation in Swiss 3T3 cells. Similarly, neither β21 nor β23 had any effect on the actin network when injected into Swiss 3T3 cells (not shown). In addition, treatment of quiescent Swiss 3T3 cells with forskolin to stimulate cAMP synthesis, phorbol esters to activate protein kinase C, and ionomycin to elevate intracellular calcium does not induce stress fiber formation (16). Our results demonstrate that α12 and α13, but not other G protein α subunits or second messengers, stimulate stress fiber formation.

Expression of RhoQL in quiescent Swiss 3T3 cells also stimulates focal adhesion assembly (Fig. 3). Microinjection and expression of α12QL and α13QL mimicked RhoQL in stimulating focal adhesion assembly, as measured by the localization of vinculin, at the leading edge of cells. Thus, activated forms of α12 and α13 regulate the polymerization of actin and the assembly of focal adhesions similar to that observed with RhoQL. To demonstrate that the activity of α12QL and α13QL were Rho-dependent, the cells were injected with recombinant, purified botulinum C3 exoenzyme. Botulinum C3 exoenzyme has been shown to catalyze the ADP-ribosylation of Asn-41 in the Rho polypeptide resulting in the inhibition of Rho activity (23, 24). Fig. 4 shows that microinjection of botulinum C3 exoenzyme inhibits LPA-stimulated stress fiber formation. To demonstrate that the botulinum C3 exoenzyme was selectively inhibiting Rho-dependent effects on the actin cytoskeleton, cells were also stimulated with platelet-derived growth factor (PDGF). PDGF has been shown to stimulate Rac1-dependent actin polymerization that is associated with membrane ruffling.
PDGF-stimulated membrane ruffling is unaffected by microinjection of botulinum C3 exoenzyme (Fig. 4, middle panel). Cells were then fixed and stained with an anti-vinculin antibody to detect focal adhesions. Injected cells were detected by staining for β-galactosidase.

These studies clearly demonstrate that α12 and α13 regulate Rho-dependent actin polymerization resulting in stress fiber formation and the assembly of focal adhesions. G12 and G13 have been shown previously to interact with the thrombin receptor (27), and G13 was shown to couple to the bradykinin receptor (28), two receptors that stimulate actin polymerization responses in Swiss 3T3 cells. Both G12 and G13 subunits are expressed in Swiss 3T3 cells as determined by immunoblotting (not shown). Our results suggest that α12 and α13 behave similarly in their ability to stimulate Rho-dependent stress fiber formation and focal adhesion assembly. This finding indicates...
that $\alpha_{12}$ and $\alpha_{13}$ probably interact with a common effector regulating Rho activation. Whether this effector is a dbl- or lbc-like Rho exchange factor (29, 30) or a Rho GDI or GDS protein characterized by Takai and co-workers (31, 32) is presently unclear. Nonetheless, the results dearly demonstrate that $\alpha_{12}$ and $\alpha_{13}$ integrate heterotrimeric G protein-coupled receptors with the regulation of Rho. Thus, it is becoming increasingly apparent that specific G protein subunits differentially regulate the activation of low molecular weight GTP-binding proteins of the Ras and Rho families (33, 34). The Ras and Rho superfamily of low molecular weight GTP-binding proteins are key regulators of major phenotypic responses of cells including growth, apoptosis, chemotaxis, and cell shape. The ability of seven transmembrane receptors to couple to specific G proteins determines the ability of receptor agonists to regulate the responses controlled by different low molecular weight GTP-binding proteins. $G_{12}^\alpha$ and $G_{13}^\alpha$-coupled receptors will function to regulate specific actin cytoskeleton responses. Activated Rho exchange factors have been shown to alter the growth and to transform NIH3T3 cells (29). It is probable that the ability of $\alpha_{12}$ and $\alpha_{13}$ to alter the growth and to transform specific cell types is related in part to the regulation of Rho and the downstream functions controlled by Rho. It will be interesting to determine if regulatory events such as activation of $Na^+/H^+$ antiporter activity by $\alpha_{12}$ and $\alpha_{13}$ (35, 36) involves Rho-dependent pathways as well.

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