In fibroblasts, the G protein α subunits Ga12 and Ga13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly, whereas G protein βγ subunits instead exert a disruptive influence. We show here that the latter can, however, stimulate the formation of stress fibers and focal adhesions in epithelial-like HeLa cells. Transient expression of βγ with γ2, γ3, and γ13 in quiescent HeLa cells induced stress fiber formation and focal adhesion assembly as did expression of the constitutively active Ga12. Co-expression of βγ with Ga12 and the C-terminal fragment of the β-adrenergic receptor kinase, both of which are known to bind and sequester free βγ, blocked βγ-induced stress fiber and focal adhesion formation. Inhibition was also noted with co-expression of a dominant negative mutant of Rho. Botulinum C3 exoenzyme, which ADP-ribosylates and inactivates Rho, and a Rho-associated protein kinase inhibitor, Y-27632, similarly inhibited βγ-induced stress fiber and focal adhesion assembly. These results indicate that G protein βγ subunits regulate Rho-dependent actin polymerization in HeLa cells.

Rho family small GTP-binding proteins play a major role in regulating the actin polymerization necessary for cytoskeleton formation, determination of cell shape, and regulatory responses including chemotaxis and mitogenesis (1). In fibroblasts, the formation of stress fibers generally parallels the assembly of focal adhesions. For instance, some stimuli involving lysophosphatidic acid (LPA)1, thrombin, and bombesin induce both. G proteins of the G12 subfamily have been shown to defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: LPA, lysophosphatidic acid; G protein, guanine nucleotide binding regulatory protein; GEF, guanine nucleotide exchange factor.
mM phorbol 12-myristate 13-acetate for 24 h. Fixed cells were immuno-

stained using antibodies against \( \alpha \), \( \beta \), and vinculin followed by second-

ary antibodies, fluorescein isothiocyanate-conjugated goat anti-rabbit

IgG, and tetramethylrhodamine isothiocyanate-conjugated goat anti-
mouse IgG, as described earlier (18). The cells were also stained for

F-actin with tetramethylrhodamine phalloidin (Sigma).

After applying coverslips, slides were examined under a laser scanning

microscope (FLUOVIEW Olympus) equipped for fluorescence. All ex-

periments were performed at least three times with similar results.

RESULTS AND DISCUSSION

Fig. 1 and 2 illustrate the effects of transient co-expression of

\( \beta \) with various \( \gamma \) subunits, including \( \gamma_2 \), \( \gamma_5 \), \( \gamma_7 \), and \( \gamma_{12} \) in quiescent HeLa cells. Combinations of \( \beta \) with all individual \( \gamma \) subunits stimulated the formation of thick stress fibers (Fig. 1) as well as focal adhesion assembly, as assessed by localization of vinculin at the leading edges and middle body in cells (Fig. 2). Such stress fibers and focal adhesions were not observed in expression-negative cells, which were seen among surrounding transfected cells (Figs. 1 and 2). Expression of the GTPase-deficient Ga12 subunit (Ga12Q229L) also induced actin stress fiber and focal adhesion assembly (Fig. 3, A, B, I, and J) as previously observed in Swiss 3T3 cells (2). However, the other GTPase-deficient \( \alpha \) subunits, Ga12 (Ga12Q205L), Ga11, (Ga11Q209L), and Ga5 (Ga5Q227L), were without such effects (Fig. 3, C–F), although Ga11Q209L appeared to induce the formation of thin actin fibers because of bright staining of transfected cells with rhodamine phalloidin (Fig. 3, E and F). Activated Ga12 and Ga11 were also ineffective in Swiss 3T3 cells (2, 3).

In contrast with Ga12Q229L subunits, it was reported that microinjection of \( \beta \gamma \) into Swiss 3T3 cells (2) and transfection of \( \beta \gamma \) into NIH 3T3 cells (10) did not stimulate stress fiber forma-
Expression of $\beta_1$, or $\gamma_2$ alone failed to stimulate the formation of actin stress fibers (data not shown), suggesting that $\beta\gamma$ complexes are necessary for this purpose. To test whether the $\beta\gamma$ complex is indeed involved in induction of stress fiber formation and focal adhesion assembly, we co-expressed Go$_{12}$ and the C-terminal fragment of the $\beta$-adrenergic receptor kinase, both of which are expected to bind and sequester free $\beta\gamma$, and showed this to prevent the $\beta_1\gamma_2$-induced actin stress fiber formation (Fig. 4, C and E) and focal adhesion assembly (Fig. 4, D and F). In contrast, Go$_{12}$ and the C-terminal fragment of the $\beta$-adrenergic receptor kinase did not prevent Go$_{12}$Q229L-induced stress fiber formation and focal adhesion assembly (data not shown).

It is well known that actin stress fiber formation and focal adhesion assembly are induced by activation of Rho in several cells and tissues (1). To determine whether the effects of $\beta\gamma$ in HeLa cells were Rho-dependent, cells were co-transfected with $\beta_1\gamma_2$ and dominant negative mutants of Rho family GTP-binding proteins. As shown in Fig. 5 (A and B), co-transfection of RhoT19N completely inhibited $\beta_1\gamma_2$-induced stress fiber formation and focal adhesion assembly. In contrast, co-transfection of dominant negative mutants of other Rho family GTP-binding proteins, Rac (RacT17N) and Cdc42 (Cdc42T17N), was without effect (Fig. 5, C–F). These dominant negative mutants seemed to be functional, because RacT17N and Cdc42T17N diminished the phosphorylation of c-Jun N-terminal kinase induced by LPA in HeLa cells (19) when the phosphorylation was determined by immunoblotting with phospho-specific c-Jun N-terminal kinase antibody (data not shown). Go$_{12}$Q229L-induced stress fiber formation and assembly of focal adhesion was also inhibited by co-transfection of RhoT19N (data not shown). To confirm the Rho dependence, cells were co-transfected with $\beta_1\gamma_2$ and the botulinum C3 exoenzyme, which ADP-ribosylates and inactivates Rho, or $\beta_1\gamma_2$-transfected cells were treated with Y-27632, a Rho-associated protein kinase (p160ROCK) inhibitor (17). In both cases, actin stress fiber formation and focal adhesion assembly were prevented (Fig. 5, G–J). These results clearly demonstrated that $\beta\gamma$ subunits regulate stress fiber formation and assembly of focal adhesion in a Rho and p160ROCK-dependent manner. The stimulation by expression of Go$_{12}$Q229L was also prevented by C3 exoenzyme co-transfection and Y-27632 treatment (data not shown).

A previous study showed the tyrosine kinase inhibitor tyrophostin A25 to inhibit the formation of stress fibers stimulated by LPA but not by constitutively active Rho in quiescent Swiss 3T3 cells, indicating the existence of a protein-tyrosine kinase acting in the LPA pathway upstream of Rho (20). Another study showed that tyrophostin A25 and tyrophostin AG 1478 inhibit the formation of stress fibers stimulated by constitutively active Go$_{13}$Q226L but not by Go$_{13}$Q229L (3). Therefore, we examined the effects of several kinds of tyrosine kinase inhibitors on $\beta_1\gamma_2$-transfected cells. Tyrophostin A25 (Fig. 6, C and D) and tyrophostin AG 1478 (data not shown) did not significantly influence the stress fiber formation and focal adhesion assembly induced by $\beta\gamma$ or Go$_{12}$Q229L. Similarly, a selective inhibitor of the Src family of protein tyrosine kinases PP2 did not inhibit $\beta\gamma$-induced stress fiber formation (data not shown). Although these tyrosine kinase inhibitors did not influence stress fiber formation, they effectively inhibited tyrosine kinase-induced stress fiber formation and focal adhesion assembly induced by LPA.
stress kinase C in these cells; tyrophostin A25 and PP2 decreased tyrosine phosphorylation of focal adhesion kinase-like protein (about 125 kDa) stimulated by G_{12},Q229L when phosphorylation was determined by antibody against phosphotyrosine (data not shown). Unlike these tyrosine kinase inhibitors, genistein blocked the formation of actin stress fibers stimulated by both βγ and G_{12},Q229L (Fig. 6, E and F). These results suggested that genistein-sensitive but not tyrophostin-sensitive or Src-like tyrosine kinases are involved in the signaling with βγ and G_{12},Q229L-induced Rho-dependent stress fiber formation.

Because βγ subunits can stimulate phospholipase C-β, which results in up-regulation of protein kinase C (21, 22), we examined whether protein kinase C activation was required for the formation of actin stress fibers by βγ. However, down-regulation of endogenous protein kinase C by a 24-h exposure of cells to 1 μM phorbol 12-myristate 13-acetate or treatment of cells with protein kinase C inhibitor Ro31–8220 for 3 h before fixation did not significantly inhibit βγ-induced stress fiber formation (Fig. 6, G and H).

There have been several reports that G protein-coupled receptors, including LPA and muscarinic receptors, are linked with phosphatidylinositol 3-kinase in a βγ-dependent fashion in cells (23, 24). To examine whether activation of phosphatidylinositol 3-kinase is required for βγ-induced stress fiber formation, the cells were treated with the phosphatidylinositol 3-kinase inhibitors wortmannin and LY-294002 for 3 h before fixation. No inhibition was observed (Fig. 6, I and J). To verify that these inhibitors for protein kinase C and phosphatidylinositol 3-kinase and protein kinase C down-regulation actually inhibited the respective pathways, we determined effects of these treatments on the phosphorylation of c-Jun N-terminal kinase induced by LPA (25, 26). All inhibitors and protein kinase C depletion diminished the phosphorylation of c-Jun N-terminal kinase (data not shown), indicating that these treatments effectively inhibited these signaling pathways.

It is generally accepted that the βγ-mediated signal pathway mainly acts through G_{i/o} coupled receptors, and when cells were transiently transfected with cDNA encoding the δ-opioid receptor and stimulated by (D-Ala{sub 2},D-Leu{sub 5})enkephalin, stress fiber formation and focal adhesion assembly were induced (data not shown). However, co-transfection with the C-terminal fragment of the β-adrenergic receptor kinase or treatment with pertussis toxin did not significantly block these effects. Thus δ-opioid receptors may couple not only with G_{i/o} but also with G_{12} and G_{13} for whose α subunits more effectively stimulate Rho than βγ subunits in HeLa cells.

The present study clearly demonstrated that βγ subunits, like G_{12}, regulate Rho-dependent actin polymerization, resulting in stress fiber formation and focal adhesion assembly in HeLa cells. Some recent reports have indicated that G_{12} and G_{13} are able to bind directly to p115-RhoGEF (4) or PDZ-RhoGEF (5) and that G_{12} but not G_{13} stimulates the GDP-GTP exchange reaction of p115-RhoGEF (4). Therefore, it is possible that βγ subunits also directly interact with RhoGEF. In the budding yeast Saccharomyces cerevisiae, the βγ complex has been shown to associate with Cdc24, a GEF for Cdc42, suggesting a cascade from βγ to actin organization via Cdc42 (27, 28). The large number of RhoGEFs so far found share Dbl and pleckstrin homology domains (29). Some members of the family also have Src homology 2, Src homology 3, GTPase-activating protein, RasGEF, and/or serine/threonine kinase domains, suggesting that they may interact with various molecules. In addition, analyses of the expression of RhoGEF family members have revealed that most are subject to varying degrees of tissue restriction (29). The different cell-specific responses to βγ subunits observed in HeLa and NIH 3T3 cells may thus result from differential expression of the RhoGEF regulated by βγ in these cells.

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REFERENCES
1. Hall, A. (1998) Science 279, 509–514
2. Buhl, A. M., Johnson, N. L., Dhanasekaran, N., and Johnson, G. L. (1995) J. Biol. Chem. 270, 4653–4659
3. Gohla, A., Harhammer, R., and Schultz, G. (1998) J. Biol. Chem. 273, 145–151
4. Hart, M. J., Jiang, X., Kozasa, T., Roseoe, W., Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Science 280, 2112–2114
5. Fukuhara, S., Murga, C., Zehar, M., Iigishi, T., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 5868–5879
6. Ridley, A. J., and Hall, A. (1994) EMBO J. 13, 2600–2610
7. Coso, O. A., Teramoto, H., Simonds, W. F., and Gutkind, J. S. (1996) J. Biol. Chem. 271, 3963–3966
8. Yamauchi, J., Kazirio, Y., and Itoh, H. (1999) J. Biol. Chem. 274, 1957–1965
9. Lin, H. C., Duncan, J. A., Kazirio, T., and Gilman, A. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5057–5060
10. Ueda, H., Yamauchi, J., Itoh, H., Morishita, R., Kazirio, Y., Kato, K., and Asano, T. (1999) J. Biol. Chem. 274, 12124–12128
11. Morishita, R., Nakayama, H., Isebe, T., Matsuda, T., Hashimoto, Y., Okano, T., Fukuda, Y., Mizuno, K., Ohno, S., Kazirio, O., Kato, K., and Asano, T. (1999) J. Biol. Chem. 275, 29469–29475
12. Yamauchi, J., Kazirio, Y., and Itoh, H. (1985) Biochem. Biophys. Res. Commun. 124, 694–700
13. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Leffkowitz, R. J. (1994) J. Biol. Chem. 269, 6193–6197
14. Nemoto, Y., Nambu, T., Kazirio, S., and Narumiya, S. (1991) J. Biol. Chem. 266, 19312–19319
15. Evans, C. J., Keith, D. E., Jr., Morrison, H., Magendzo, K., and Edwards, R. H. (1995) Science 258, 1952–1955
16. Morishita, R., Kato, K., and Asano, T. (1998) Eur. J. Biochem. 174, 87–94
17. Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997) Nature 389, 990–994
18. Ueda, H., Saga, S., Shinozawa, H., Morishita, R., Kato, K., and Asano, T. (1997) J. Cell Biol. 131, 1503–1511
19. Collins, L. R., Minden, A., Karin, M., and Brown, J. H. (1996) J. Biol. Chem.
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20. Nobes, C. D., Hawkins, P., Stephens, L., and Hall, A. (1995) *J. Cell Sci.* **108**, 225–233
21. Camps, M., Hou, C., Sidiropoulos, D., Stock, J. B., Jakobs, K. H., and Gierschik, P. (1992) *Eur. J. Biochem.* **206**, 821–831
22. Clapham, D. E., and Neer, E. J. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37**, 167–203
23. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271**, 12133–12136
24. Lopez-Ilasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S., and Wetzker, R. (1997) *Science* **275**, 394–397
25. Nagao, M., Yamauchi, J., Kaziro, Y., and Itoh, H. (1998) *J. Biol. Chem.* **273**, 22292–22294
26. Lopez-Ilasaca, M., Gutkind, J. S., and Wetzker, R. (1998) *J. Biol. Chem.* **273**, 2505–2508
27. Zhao, Z. S., Leung, T., Manser, E., and Lim, L. (1995) *Mol. Cell. Biol.* **15**, 5246–5257
28. Nern, A., and Arkowitz, R. A. (1998) *Nature* **391**, 195–198
29. Whitehead, I. P., Campbell, S., Rossman, K. L., and Der, C. J. (1997) *Biochim. Biophys. Acta* **1332**, 1–23