Direct Stimulation of the Guanine Nucleotide Exchange Activity of p115 RhoGEF by $G\alpha_{13}$

Matthew J. Hart,* Xuejun Jiang,* Tohru Kozasa, William Roscoe, William D. Singer, Alfred G. Gilman, Paul C. Sternweis,† Gideon Bollag†

Signaling pathways that link extracellular factors to activation of the monomeric guanosine triphosphatase (GTPase) Rho control cytoskeletal rearrangements and cell growth. Heterotrimeric guanine nucleotide–binding proteins (G proteins) participate in several of these pathways, although their mechanisms are unclear. The GTPase activities of two G protein α subunits, $G\alpha_{12}$ and $G\alpha_{13}$, are stimulated by the Rho guanine nucleotide exchange factor p115 RhoGEF. Activated $G\alpha_{13}$ bound tightly to p115 RhoGEF and stimulated its capacity to catalyze nucleotide exchange on Rho. In contrast, activated $G\alpha_{12}$ inhibited stimulation by $G\alpha_{13}$. Thus, p115 RhoGEF can directly link heterotrimeric G protein α subunits to regulation of Rho.

Certain heterotrimeric G proteins, particularly G_{12} and G_{13}, regulate cell function through Rho-dependent pathways (*1*). One or both of these G proteins cause cytoskeletal rearrangements, Na^+/H^+ exchanger activation, cell transformation, stimulation of phospholipase D activity, and apoptosis in a Rho-dependent manner (*2*). Mouse fibroblasts deficient in $G\alpha_{13}$ fail to display thrombin-stimulated cell migration, a phenomenon thought to involve activation of Rho (*3*).

Activation of Rho can occur by stimulation of guanosine triphosphate (GTP) binding or by inhibition of its intrinsic GTPase activity (*4*). The S19N variant of Rho, in which Ser^{19} (S19) is changed to Asn (N), is thought to inhibit endogenous Rho function by forming nonproductive complexes with guanine nucleotide exchange factors (GEFs) for Rho (*5*). Thus, attenuation of heterotrimeric G protein–mediated pathways by expression of S19N Rho suggests that stimulation of GTP binding is a prominent mechanism for activation of Rho by relevant stimuli. Potential mediators of such stimulation are GEFs with specificity for Rho, including Dbl, Lbc, Lfc, Lsc, and p115 RhoGEF (*6, 7*). The NH_2-terminal region of p115 RhoGEF contains a domain characteristic of RGS (regulator of G protein signaling) proteins (*8, 9*) and specifically stimulates the intrinsic GTPase activity of the α subunits of G_{13} and G_{12} (*8*). We therefore tested whether these two G proteins might alter the function of the exchange factor, thereby providing a mechanism for regulation of Rho by heterotrimeric G proteins.

Physical interaction between $G\alpha_{13}$ and p115 RhoGEF was detected by immunoprecipitation (Fig. 1) (*10, 11*). Antibody to Myc (anti-Myc) immunoprecipitated $G\alpha_{13}$ from a lysate of transfected COS cells expressing Myc-tagged p115 RhoGEF. This interaction was observed in the presence of AlF_4^-, an activator of $G\alpha_{13}$. The conformation of guanosine diphosphate (GDP)–bound, AlF_4^--activated G protein α subunits resembles that of the transition state for GTP hydrolysis, and RGS proteins bind with highest affinity to Gα proteins in this conformation (*12*). $G\alpha_{13}$ did not coprecipitate with a truncated form of p115 RhoGEF that lacks the NH_2-terminal RGS domain [required for GTPase-activating protein (GAP) activity], but it did bind to p115 RhoGEF lacking the Dbl homology (DH) domain, which is required for GEF activity. Similar interactions were observed when antibodies to $G\alpha_{13}$ were used to immunoprecipitate proteins from cells expressing Myc-tagged p115 RhoGEF (Fig. 1C). Weaker interactions were detected between $G\alpha_{12}$ and Myc-tagged p115 RhoGEF, but other G protein α subunits ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_z$) were not detected in the anti-Myc immunoprecipitates, even though they were present in the lysates (*13*). These interactions are consistent with the specificity of p115 RhoGEF to stimulate the GTPase activity of only $G\alpha_{13}$ and $G\alpha_{12}$ (*8*).

Association of purified $G\alpha_{13}$ with recombinant p115 RhoGEF stimulated the capacity of the latter protein to facilitate dissociation of GDP from Rho (Fig. 2, A and B) (*14, 15*). In contrast, several other G protein α subunits, including $G\alpha_{12}$, did not influence the nucleotide exchange activity of p115 RhoGEF (Fig. 2, A and C). Stimulation of p115 RhoGEF activity required activation of $G\alpha_{13}$ (Fig. 2D). Thus, treatment of $G\alpha_{13}$ with AlF_4^- [AMF (*15*)] or binding of GTP-γ-S to $G\alpha_{13}$ supported

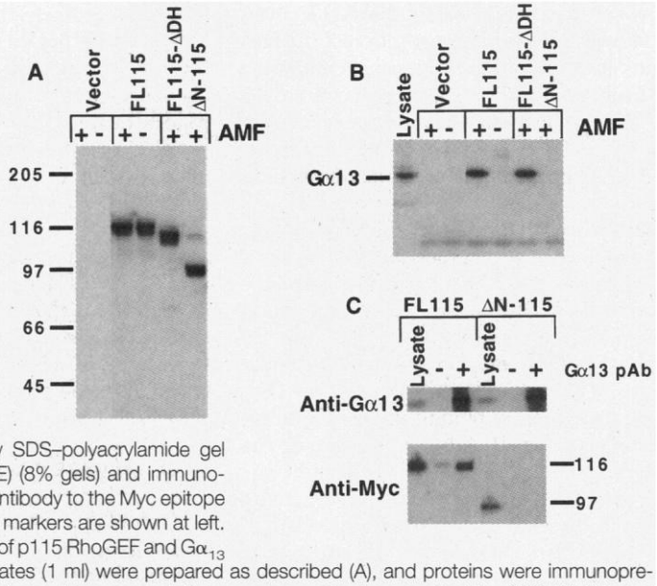

Fig. 1. Binding of $G\alpha_{13}$ to p115 RhoGEF. (**A**) Expression of p115 RhoGEF in COS cells. Myc-epitope–tagged versions (*10*) of full-length p115 RhoGEF (FL115), p115 RhoGEF with a deletion of residues 466 to 547 in the Dbl homology region (FL115-ΔDH), and p115 RhoGEF with a deletion of residues 1 to 249 (ΔN-115) were expressed in COS cells, and lysates were prepared in the presence or absence of AMF. Portions (20 μl) of lysates were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (8% gels) and immunoblotted with a monoclonal antibody to the Myc epitope (BAbCO). Molecular weight markers are shown at left. (**B**) Coimmunoprecipitation of p115 RhoGEF and $G\alpha_{13}$ with anti–Myc epitope. Lysates (1 ml) were prepared as described (A), and proteins were immunoprecipitated with anti–Myc epitope. Immunoprecipitates were washed, separated by SDS-PAGE, and immunoblotted with the $G\alpha_{13}$ antiserum, B860 (*11*). Left lane, lysate (20 μl) from control COS. (**C**) Coimmunoprecipitation with $G\alpha_{13}$ antiserum. Lysates (1 ml) of COS cells transfected with FL115 or ΔN-115 as in (A) were prepared in the presence of AMF, and proteins were immunoprecipitated with vehicle or antiserum B860 to $G\alpha_{13}$ as indicated. Proteins were separated by SDS-PAGE and immunoblotted with either B860 antiserum (anti-$G\alpha_{13}$) or anti–Myc epitope. Portions (20 μl) of lysates were included for reference; pAb, polyclonal antibody. Molecular weight markers are shown at right.

M. J. Hart, W. Roscoe, G. Bollag, Onyx Pharmaceuticals, 3031 Research Drive, Richmond, CA 94806, USA.
X. Jiang, T. Kozasa, W. D. Singer, A. G. Gilman, P. C. Sternweis, Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75235–9041, USA.

*These authors contributed equally to the work.
†To whom correspondence should be addressed. E-mail: sternwei@utsw.swmed.edu (P.C.S.) and bollag@onyx-pharm.com (B.G.).

p115-mediated dissociation of GDP from Rho, but binding of GDP-β-S to $G\alpha_{13}$ did not. Thus, stimulation of p115 RhoGEF exchange activity by $G\alpha_{13}$ presumably depends on receptor-mediated activation of the G protein.

The NH_2-terminal RGS domain of p115 RhoGEF is apparently required for binding $G\alpha_{13}$, and this domain might inhibit nucleotide exchange activity—a restraint that could be relieved by interaction with $G\alpha_{13}$. Consistent with this hypothesis, truncated p115 RhoGEF lacking the RGS domain was more active than the full-length protein (Fig. 3A). Furthermore, addition of the isolated RGS domain [as a glutathione S-transferase (GST) fusion protein] caused partial inhibition of $G\alpha_{13}$-stimulated p115 RhoGEF activity (Fig. 3B), presumably by sequestration of the α subunit. Although these data do not preclude additional sites of interaction of $G\alpha_{13}$ with p115 RhoGEF, they are consistent with a primary mode of action through the RGS domain.

The inability of $G\alpha_{12}$ to stimulate nucleotide exchange on Rho is puzzling, particularly because p115 RhoGEF stimulates the GTPase activity of both $G\alpha_{12}$ and $G\alpha_{13}$, and the AMF-stimulated forms of both α subunits are equipotent in attenuating this action (*8*). Conversely, $G\alpha_{12}$ blocked stimulation of p115 RhoGEF activity by $G\alpha_{13}$ (Fig. 3C). Thus, $G\alpha_{12}$ may compete with $G\alpha_{13}$ for binding to the RGS domain of p115 RhoGEF. However, this binding of $G\alpha_{12}$ to p115 RhoGEF is clearly not sufficient to stimulate Rho exchange activity. This ineffectiveness of $G\alpha_{12}$ may relate to the lower efficacy of p115 RhoGEF to activate the GTPase activity of $G\alpha_{12}$ compared with $G\alpha_{13}$ or may indicate an additional site of interaction between $G\alpha_{13}$ and p115 RhoGEF. Differential regulation of p115 RhoGEF by $G\alpha_{13}$ and $G\alpha_{12}$ indicates that signals transduced through $G\alpha_{13}$ might be modulated by $G\alpha_{12}$. The inability of $G\alpha_{12}$ to stimulate p115 RhoGEF raises the possibility that this G protein activates Rho through another RhoGEF.

The p115 RhoGEF protein appears to serve as a direct link between Rho GTPases and heterotrimeric G proteins. A correlate in *Drosophila* is DRhoGEF2, which transduces developmental signals mediated by the G protein Cta, a homolog of $G\alpha_{12}$ and $G\alpha_{13}$ (*8*, *16*). Furthermore, p115 RhoGEF is the first protein with an RGS domain to function as an effector of G protein action. Other effectors for G proteins can also serve as GAPs for their activators and thus stimulate their deactivation (*17*, *18*). This coupling of effector and deactivator may provide mechanisms for precise control by permitting rapid attenuation of signaling upon removal of the stimulus. Furthermore, Biddlecome *et al.* (*18*) have suggested that rapid deactivation of $G\alpha_q$ by phospholipase Cβ provides the opportunity for receptor-mediated reactivation of Gα while still present in a complex of G protein and effector. Thus, rapid GTPase cycles may permit the continued existence of receptor–G protein–effector complexes capable of generating higher amplitude signals because the signaling complex does not un-

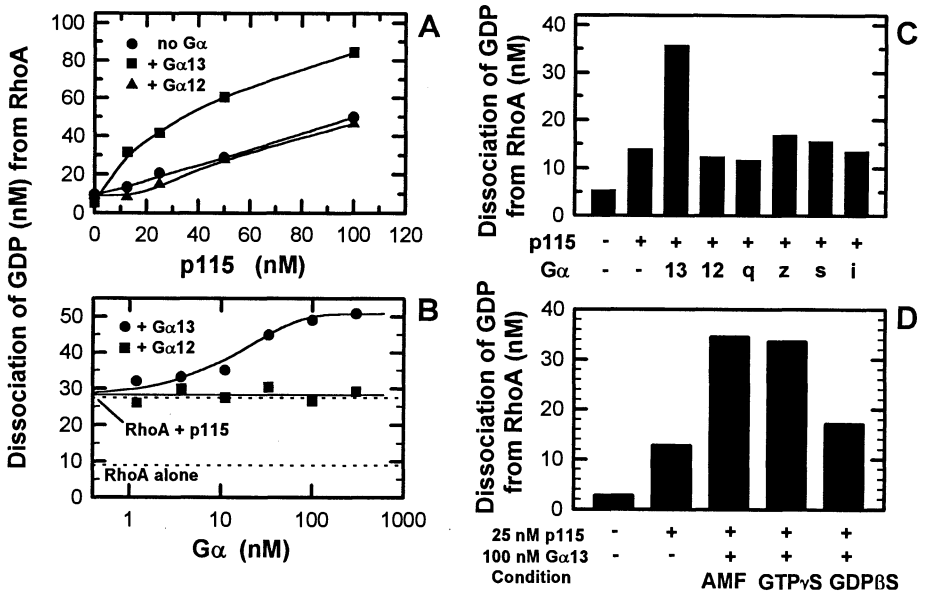

Fig. 2. Stimulation of Rho-dependent nucleotide exchange activity of p115 RhoGEF by $G\alpha_{13}$. (**A**) Dissociation of bound GDP from 100 nM RhoA (*14*, *15*) was examined after 10 min at 30°C in the absence or presence of 100 nM AMF-activated $G\alpha_{13}$ or $G\alpha_{12}$ and various concentrations of p115 RhoGEF, as indicated. (**B**) Dissociation of GDP from 100 nM RhoA after 10 min at 30°C in the presence of 25 nM p115 RhoGEF and the indicated concentrations of AMF-activated $G\alpha_{13}$ or $G\alpha_{12}$. The dashed lines indicate dissociation of GDP from RhoA alone or in the presence of p115 RhoGEF but no Gα. (**C**) Dissociation of GDP from 100 nM RhoA after 10 min of incubation at 30°C with p115 (25 nM) and various Gα subunits (100 nM), as indicated. (**D**) Dissociation of GDP from 100 nM RhoA after 10 min of incubation at 30°C with p115 and $G\alpha_{13}$ that had been treated as indicated with AMF, GTP-γ-S, or GDP-β-S.

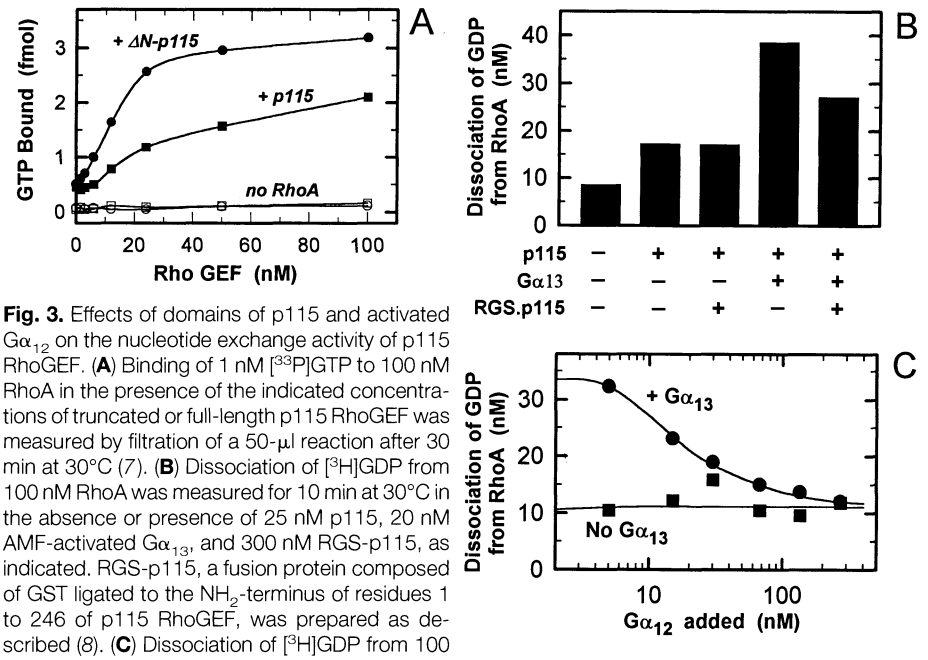

Fig. 3. Effects of domains of p115 and activated $G\alpha_{12}$ on the nucleotide exchange activity of p115 RhoGEF. (**A**) Binding of 1 nM [^{33}P]GTP to 100 nM RhoA in the presence of the indicated concentrations of truncated or full-length p115 RhoGEF was measured by filtration of a 50-μl reaction after 30 min at 30°C (*7*). (**B**) Dissociation of [^{3}H]GDP from 100 nM RhoA was measured for 10 min at 30°C in the absence or presence of 25 nM p115, 20 nM AMF-activated $G\alpha_{13}$, and 300 nM RGS-p115, as indicated. RGS-p115, a fusion protein composed of GST ligated to the NH_2-terminus of residues 1 to 246 of p115 RhoGEF, was prepared as described (*8*). (**C**) Dissociation of [^{3}H]GDP from 100 nM RhoA was measured for 10 min at 30°C with 25 nM p115 RhoGEF and in the absence or presence of 25 nM AMF-activated $G\alpha_{13}$ and the indicated concentrations of AMF-activated $G\alpha_{12}$. Assays for (B) and (C) were performed as described (*15*).

dergo dissociation and reformation.

The $G\alpha_{13}$-Rho pathway participates in phenomena such as cell migration, angiogenesis, and apoptosis (*2*, *3*). The identification of p115 RhoGEF as a critical link in this pathway will facilitate mechanistic understanding of these functions. Furthermore, RGS domains in other proteins may also impart sensitivity to regulation by G protein α subunits.

REFERENCES AND NOTES

1. N. Dhanasekaran and J. M. Dermott, *Cell Signal.* **8**, 235 (1996); A. Hall, *Science* **279**, 509 (1998).
2. A. M. Buhl, N. L. Johnson, N. Dhanasekaran, G. L. Johnson, *J. Biol. Chem.* **270**, 24631 (1995); R. Hooley, C.-Y. Yu, M. Symons, D. L. Barber, *ibid.*, **271**, 6152 (1996); C. Fromm, O. A. Coso, S. Montaner, N. Xu, J. S. Gutkind, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10098 (1997); H. Althoefer, P. Eversole-Cire, M. I. Simon, *J. Biol. Chem.* **272**, 24380 (1997); S. G. Plonk, S.-K. Park, J. H. Exton, *ibid.*, **273**, 4823 (1998).
3. S. Offermanns, V. Mancino, J.-P. Revel, M. I. Simon, *Science* **275**, 533 (1997).
4. M. S. Boguski and F. McCormick, *Nature* **366**, 643 (1993).
5. R.-G. Qiu, J. Chen, F. McCormick, M. Symons, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11781 (1995); S. Powers, K. O'Neill, M. Wigler, *Mol. Cell. Biol.* **9**, 390 (1989); S. A. Haney and J. R. Broach, *J. Biol. Chem.* **269**, 16541 (1994).
6. R. A. Cerione and Y. Zheng, *Curr. Opin. Cell Biol.* **8**, 216 (1996); I. P. Whitehead, S. Campbell, K. L. Rossman, C. J. Der, *Biochim. Biophys. Acta* **1332**, F1 (1997).
7. M. J. Hart *et al.*, *J. Biol. Chem.* **271**, 25452 (1996).
8. T. Kozasa *et al.*, *Science* **280**, 2109 (1998).
9. H. G. Dohlman and J. Thorner, *J. Biol. Chem.* **272**, 3871 (1997); M. R. Koelle, *Curr. Opin. Cell. Biol.* **9**, 143 (1997); D. M. Berman and A. G. Gilman, *J. Biol. Chem.* **273**, 1269 (1998).
10. EXV-Myc tagged (for COS cell transfections) and pAc-Glu–tagged (for baculovirus expression) proteins with deletions of the RGS or DH domains have been described previously (*7*). Full-length versions were constructed in the same vectors. Transfection, immunoprecipitation, and purification were performed as described previously (*7*). Cells were lysed in 20 mM tris (pH 7.5), 1 mM dithiothreitol (DTT), 100 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$, and 0.7% Triton X-100. Where indicated, AMF (50 μM $AlCl_3$, 10 mM $MgCl_2$, and 5 mM NaF) was included.
11. W. D. Singer, R. T. Miller, P. C. Sternweis, *J. Biol. Chem.* **269**, 19796 (1994).
12. D. M. Berman, T. Kozasa, A. G. Gilman, *ibid.*, **271**, 27209 (1996).
13. M. J. Hart, W. Roscoe, G. Bollag, unpublished data.
14. Gα subunits were prepared as described (*8*). RhoA was expressed as a fusion protein (GST-RhoA) in Sf9 cells, followed by affinity purification and removal of the GST tag as described previously [W. D. Singer, H. A. Brown, X. Jiang, P. C. Sternweis, *J. Biol. Chem.* **271**, 4504 (1996)].
15. Dissociation assay. RhoA (2.5 μM) was loaded with [^{3}H]GDP by incubation at 30°C for 1 hour with 25 μM GDP (10,000 cpm/pmol) in 50 mM Na-Hepes (pH 7.5), 50 mM NaCl, 4 mM EDTA, 1 mM DTT, and 0.1% Triton X-100. After addition of $MgCl_2$ to 9 mM and octylglucoside to 1%, Rho was incubated for 5 min at 30°C and separated from free GDP by rapid filtration through Sephadex-G50 that had been equilibrated with 50 mM Na-Hepes (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, and 1% octylglucoside. Dissociation of GDP from RhoA was measured at 30°C in 20 μl of 50 mM Na-Hepes (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 30 μM $AlCl_3$, 5 mM NaF, and 5 μM GTP-γ-S. Unless specified, G protein α subunits were first incubated with AMF (30 μM $AlCl_3$, 5 mM $MgCl_2$, and 5 mM NaF) before mixing with other proteins. Where indicated, α subunits were incubated for 60 min at 30°C with 25 μM GTP-γ-S or GDP-β-S (rather than AMF), 1 mM EDTA, and 0.5 mM $MgSO_4$, and dissociation assays included 5 μM nucleotide (and no AMF). Reactions were started by addition of [^{3}H]GDP-RhoA, and bound GDP was determined by filtration [J. K. Northup, M. D. Smigel, A. G. Gilman, *J. Biol. Chem.* **257**, 11416 (1982)] before and after incubation.
16. K. Barrett, M. Leptin, J. Settleman, *Cell* **91**, 905 (1997); U. Haecker and N. Perrimon, *Genes Dev.* **12**, 274 (1998).
17. G. Berstein *et al.*, *Cell* **70**, 411 (1992); V. Y. Arshavsky and M. D. Bownds, *Nature* **357**, 416 (1992); V. Z. Slepak *et al.*, *J. Biol. Chem.* **270**, 14319 (1995).
18. G. H. Biddlecome, G. Berstein, E. M. Ross, *J. Biol. Chem.* **271**, 7999 (1996).

19. We thank S. Gutowski and J. Laidlaw for technical assistance. Supported by American Heart Association grant 96G105 (T.K.); the Bayer Corporation (Onyx); NIH grant GM34497 and the Raymond and Ellen Willie Distinguished Chair in Molecular Neuropharmacology (A.G.G.); and NIH grant GM 31954, the Robert A. Welch Foundation, and the Alfred and Mabel Gilman Professorship in Molecular Pharmacology (P.C.S.).

20 March 1998; accepted 7 May 1998

Type IV Pili, Transient Bacterial Aggregates, and Virulence of Enteropathogenic *Escherichia coli*

David Bieber, Sandra W. Ramer, Cheng-Yen Wu, William J. Murray, Toru Tobe, Rosemary Fernandez, Gary K. Schoolnik*

Type IV bundle-forming pili of enteropathogenic *Escherichia coli* are required for the localized adherence and autoaggregation phenotypes. Whether these pili are also required for virulence was tested in volunteers by inactivating *bfpA* or *bfpT* (*perA*) encoding, respectively, the pilus subunit and the *bfp* operon transcriptional activator. Both mutants caused significantly less diarrhea. Mutation of the *bfpF* nucleotide-binding domain caused increased piliation, enhanced localized adherence, and abolished the twitching motility–dispersal phase of the autoaggregation phenotype. The *bfpF* mutant colonized the human intestine but was about 200-fold less virulent. Thus, BfpF is required for dispersal from the bacterial aggregate and for full virulence.

The type IV family of bacterial pili, produced by several human and animal pathogens, is thought to participate in the infectious process by promoting bacterial adherence to host cells (*1*). However, evidence for this putative pathogenic role comes largely from in vitro observations; for the only two species tested in humans, *Neisseria gonorrhoeae* and *Vibrio cholerae* 01, the type IV pili have been shown to be required for infectivity (*2*). Type IV pili are also associated in some bacterial species with twitching motility, a kind of nonflagellar movement thought to promote spread of the organism on body surfaces (*3*). However, the relevance of twitching motility for the infectious process has not been tested.

Enteropathogenic *Escherichia coli* (EPEC) is a type IV pilus–producing biotype and a common cause of childhood diarrhea in developing countries (*4*). Most EPEC serotypes isolated from patients and all experimentally infectious strains harbor an ~80-kb enteroadherence factor (EAF) plasmid (*5–7*) containing an operon that encodes a type IV pilus—designated the bundle-forming pilus (BFP)—because pilus filaments emanating from the bacterial surface appear to align along their longitudinal axes to form bundles of filaments (Fig. 1A) (*8*, *9*). BFP expression is induced during logarithmic-phase growth and regulated by physicochemical signals characteristic of the small intestine (*10*). BFP biogenesis is directed by an operon of 14 genes (*9*, *11*), including *bfp*A, which encodes the major repeating subunit of the pilus fiber (*12*). A transcriptional factor encoded by the *bfpT* (also called *per*A) operon is required for *bfp* operon expression and is located elsewhere on the EAF plasmid (*13*, *14*).

BFP expression is required for the development of EPEC microcolonies on tissue culture cell monolayers [the localized adherence (LA) phenotype] (*5*, *8*) and the formation of spherical bacterial aggregates in tissue culture media (the autoaggregation phenotype) (*15*). The latter is distinct from the "aggregative adherence" phenotype exhibited by some enteroaggregative *E. coli* in that the EPEC autoaggregation phenotype

D. Bieber, S. W. Ramer, C.-Y. Wu, R. Fernandez, G. K. Schoolnik, Department of Medicine, Division of Infectious Diseases and Geographical Medicine, and Department of Microbiology and Immunology, Stanford Program for Vaccine Research, Stanford University Medical Center, Stanford, CA 94305 USA.
W. J. Murray, Department of Biological Sciences, San Jose State University, San Jose, CA 95912 USA.
T. Tobe, Department of Bacteriology, Institute of Medicine, University of Tokyo, Tokyo, Japan.

*To whom correspondence should be addressed. E-mail: ml.gks@forsythe.stanford.edu