that at least one type of transmembrane ECM receptor, integrin β_1, can act as a mechanoreceptor in that it can transfer mechanical signals to the CSK by way of a specific molecular pathway. A cell's sensitivity to a mechanical stimulus therefore may be altered by changing ECM receptor number, location, or adhesion strength or by modulating focal adhesion formation. Other types of transmembrane molecules that interconnect with CSK filaments (for example, different integrin subunits, cadherins, or cell surface proteoglycans) may also transfer external mechanical signals to the CSK. The magnetic twisting device provides a simple method to directly address this possibility. In addition, these results suggest that transfer of force from integrins to the CSK may represent a proximal step in an intracellular mechanical signaling cascade that leads to global CSK rearrangements and simultaneous mechanotransduction events at multiple locations inside the cell (*21*, *28*). If cells use a tensegrity-based transduction system, then mechanical signal transfer throughout the entire cell would be essentially instantaneous and thus more rapid than any diffusion-based signaling system.

REFERENCES AND NOTES

1. Y. C. Fung, *Biomechanics: Mechanical Properties of Living Tissues* (Springer-Verlag, New York, 1988); D. W. Thompson, *On Growth and Form* (Cambridge Univ. Press, New York, 1977); E. S. Russell, *Form and Function* (Univ. of Chicago Press, Chicago, 1982); J. Koch, *Am. J. Anat.* **21**, 177 (1917).
2. F. Sachs, in *Cell Shape: Determinants, Regulation, and Regulatory Role*, W. D. Stein and F. Bronner, Eds. (Academic Press, San Diego, CA, 1989), pp. 63–92.
3. I. Komuro *et al.*, *J. Biol. Chem.* **266**, 1265 (1991); R. P. De Groot *et al.*, *J. Cell Sci.* **97**, 33 (1990); S.-P. Olesen *et al.*, *Nature* **331**, 168 (1988); J. B. Lansman *et al.*, *ibid.* **325**, 811 (1987).
4. P. Watson, *J. Biol. Chem.* **265**, 6569 (1990); T. J. Ryan, *J. Am. Acad. Dermatol.* **21**, 115 (1989).
5. K. G. Murti, K. Kaur, R. M. Goorha, *Exp. Cell Res.* **202**, 36 (1992).
6. B. Kacahr, P. Parakkal, J. Fex, *Hear. Res.* **45**, 179 (1990); L. J. Wilson and D. H. Paul, *J. Comp. Neurol.* **296**, 343 (1990).
7. L. F. Cipriano, *Physiologist* **34**, 72 (1991); T. J. Dennerll *et al.*, *J. Cell Biol.* **107**, 665 (1988); J. Kolega, *ibid.* **102**, 1400 (1986); R.-P. Franke *et al.*, *Nature* **307**, 648 (1984).
8. V. Wagner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3644 (1992); R. Wayne, M. P. Staves, A. C. Leopold, *J. Cell Sci.* **101**, 611 (1992); D. E. Ingber, *Curr. Opin. Cell Biol.* **3**, 841 (1991).
9. S. M. Albelda and C. A. Buck, *FASEB J.* **4**, 2868 (1990); K. Burridge *et al.*, *Annu. Rev. Cell Biol.* **4**, 487 (1988).
10. H. R. W. Wirtz and L. G. Dobbs, *Science* **250**, 1266 (1990); J. L. Samuel and H. H. Vandenburgh, *In Vitro Cell. Dev. Biol.* **26**, 905 (1990); R. C. Harris, M. A. Haralson, K. L. Badr, *Lab. Invest.* **66**, 548 (1992); B. E. Sumpio *et al.*, *Arch. Surg. (Chicago)* **123**, 1233 (1988); L. Terracio *et al.*, *In Vitro Cell Dev. Biol.* **24**, 53 (1988).
11. C. L. Bizal, J. P. Butler, P. A. Valberg, *J. Leukocyte Biol.* **50**, 240 (1991); P. A. Valberg and J. P. Butler, *Biophys. J.* **52**, 537 (1987); P. A. Valberg and D. F. Albertini, *J. Cell Biol.* **101**, 130 (1985); P. A. Valberg, *Science* **224**, 513 (1984).
12. Capillary endothelial cells were plated (3×10^4 cells per well) on fibronectin-coated bacteriological plastic (96-well Removawells, Immunolon II) and cultured for 6 to 10 hours in chemically defined medium (*27*) before bead addition. Spherical ferromagnetic beads (5.5 μm in diameter, 1 mg/ml) [W. Moller, C. Roth, W. Stahlhofen, *J. Aerosol Sci.* **21**, S657 (1990)] were coated with RGD-containing peptide (Peptite 2000), AcLDL, BSA, or anti-integrin β_1 antibodies as previously described (all at 50 μg/ml) (*13*).
13. M. A. Schwartz, C. Lechene, D. E. Ingber, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7849 (1991).
14. E. G. Hayman, M. D. Pierschbacher, E. Ruoslahti, *J. Cell Biol.* **100**, 1948 (1985).
15. AcLDL binds to specific transmembrane receptors on the endothelial cell surface; however, it does not normally play a role in cell adhesion. The small but statistically significant resistance to deformation that these beads did exhibit (deviation from 90°) may be due to generalized distortion of elements of the submembranous CSK that are known to be highly deformable [N. O. Petersen, W. B. McConnaughey, E. L. Elson, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5327 (1982)]. Local nonspecific CSK deformation was also observed when this cell magnetometry system was used with ingested ferromagnetic particles that were uncoated, irregularly shaped, and contained within intracellular lysosomes (*11*). Thus, establishment of a specific molecular path for force transmission appears to be required for efficient signal transfer as well as an effective CSK response.
16. G. H. Nuckolls, L. H. Romer, K. Burridge, *J. Cell Sci.* **102**, 753 (1992).
17. M. Hay and U. De Boni, *Cell Motility Cytoskeleton* **18**, 63 (1991).
18. P. A. Janmey, U. Euteneur, P. Traub, M. Schliwa, *J. Cell Biol.* **113**, 155 (1991).
19. E. G. Fey *et al.*, *ibid.* **98**, 1973 (1984); J. J. Wolosewick and K. R. Porter, *ibid.* **82**, 114 (1979).
20. D. Stamenovic, *Physiol. Rev.* **70**, 1117 (1990); T. A. McMahon, *Muscles, Reflexes, and Locomotion* (Princeton Univ. Press, Princeton, NJ, 1984); I. M. Ward, *Mechanical Properties of Solid Polymers* (Wiley, New York, 1983); J. G. Pinto and Y. C. Fung, *J. Biomech.* **6**, 597 (1973); Y. C. Fung, *Am. J. Physiol.* **213**, 1532 (1967); I. S. Sokolnikoff, *Mathematical Theory of Elasticity* (McGraw-Hill, New York, 1956).
21. D. E. Ingber, *J. Cell Sci.* **104**, 613 (1993); D. E. Ingber *et al.*, in *Physical Forces and the Mammalian Cell*, J. A. Frangos and C. L. Ives, Eds. (Academic Press, San Diego, CA, 1993), pp. 61–78; D. E. Ingber and J. Folkman, in *Cell Shape: Determinants, Regulation, and Regulatory Role*, W. Stein and F. Bronner, Eds. (Academic Press, San Diego, CA, 1989), pp. 3–31; D. E. Ingber and J. D. Jamieson, in *Gene Expression During Normal and Malignant Differentiation*, L. C. Andersson, C. G. Gahmberg, P. Ekblom, Eds. (Academic Press, Orlando, FL, 1985), pp. 13–32; B. Fuller, *Portfolio Art News Annu.* **4**, 112 (1961).
22. K. S. Zaner and P. A. Valberg, *J. Cell Biol.* **109**, 2233 (1989).
23. J. Sims, S. Karp, D. E. Ingber, *J. Cell Sci.* **103**, 1215 (1992).
24. R. E. Buxbaum and S. R. Heidemann, *J. Theor. Biol.* **134**, 379 (1988); H. C. Joshi *et al.*, *J. Cell Biol.* **101**, 697 (1985).
25. G. W. Brodland and R. Gordon, *J. Biomech. Eng.* **112**, 319 (1991).
26. I. Yahara and G. M. Edelman, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 608 (1972).
27. D. E. Ingber, *ibid.*, p. 3579; _____ and J. Folkman, *J. Cell Biol.* **109**, 317 (1989); *Cell* **58**, 803 (1989).
28. K. J. Pienta and D. S. Coffey, *Med. Hypotheses* **34**, 88 (1991).

29. We thank W. Moller and W. Stahlhofen for providing ferromagnetic microbeads, G. Plopper for assistance in the immunostaining studies, J. Fredberg and P. Valberg for helpful discussions, and J. Folkman for reviewing the manuscript. This work was supported by grants from the National Institutes of Health (HL-33009 and CA45548) and the Space Biology Program at the National Aeronautics and Space Administration (NAG-9-430) and by a Faculty Research Award from the American Cancer Society (D.E.I.).

11 December 1992; accepted 2 April 1993

Expression of *Pseudomonas aeruginosa* Virulence Genes Requires Cell-to-Cell Communication

Luciano Passador, James M. Cook, Michael J. Gambello, Lynn Rust, Barbara H. Iglewski*

Pseudomonas aeruginosa **is an opportunistic human pathogen that causes a variety of infections in immunocompromised hosts and individuals with cystic fibrosis. Expression of elastase, one of the virulence factors produced by this organism, requires the transcriptional activator LasR. Experiments with gene fusions show that gene *lasI* is essential for high expression of elastase. The *lasI* gene is involved in the synthesis of a diffusible molecule termed *Pseudomonas* autoinducer (PAI). PAI provides *P. aeruginosa* with a means of cell-to-cell communication that is required for the expression of virulence genes and may provide a target for therapeutic approaches.**

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen capable of secreting many extracellular virulence factors. Among these are the products of the *aprA* gene, which encodes alkaline protease (*1*), the *toxA* gene encoding toxin A (*2*, *3*), and the products of the *lasA* and *lasB* genes, each of which encodes a protease with elastolytic activity (*4*, *5*). The role of elastase as a virulence factor is supported by the list of substrates that it uses, including elastin (*1*), human immunoglobulins G and A (*6*, *7*), some collagens (*8*), serum α_1-proteinase inhibitor (*9*), and components of the complement system (*10*).

The product of the *lasR* gene (LasR) has been shown to be required for the transcrip-

Department of Microbiology and Immunology, University of Rochester, School of Medicine and Dentistry, Rochester, NY 14620.

*To whom correspondence should be addressed.

tion of *aprA*, *lasA*, and *lasB* (*11–14*) and to enhance *tox*A expression (*12, 14*). Thus, it appears that LasR may be a global regulator of genes involved in the virulence of *P. aeruginosa*.

An incomplete open reading frame of approximately 209 bp has been identified distal to *lasR* (*14*) and named ORF2. To study this gene, we used a 703-bp probe encompassing an Eco RV–Eco RI fragment from pMJG1.7 (*11*) to screen a subgenomic library of PAO1. A 5.4-kb Eco RV fragment was identified, isolated, and subcloned into pBluescriptII KS$^+$ (Stratagene) to construct pJMC30. The cloned insert was further subcloned into a 1.8-kb Eco RI fragment, and a 4.0-kb Cla I fragment. These were inserted into pBluescriptII KS$^+$ to construct pJMC31 and pJMC32, respectively.

The DNA sequence upstream of the Eco RI site within ORF2 has been reported (*11*). We determined the nucleotide sequence of the remainder of the open reading frame by the dideoxy chain termination method (*15*), using pJMC30 through pJMC32 as templates. A putative Shine-Dalgarno region centered at ten nucleotides upstream of the suggested start of translation has been identified. The high frequency of *P. aeruginosa* codon usage (*16*) and the high GC content (59%) throughout the open reading frame are good indicators that ORF2 represents an actual *P. aeruginosa* gene. The translated product of ORF2 has a deduced molecular mass of 22,847 daltons. No likely transmembrane regions were detected with the University of Wisconsin GCG DNA–Protein analysis software (*17*). The GenBank–European Molecular Biology Laboratory sequence database was searched by means of the GCG DNA–Protein analysis software package (*17*), and we found that the highest degree of similarity was to the *Vibrio fischeri luxI* gene product (*18*). Regulation of the genes required for luminescence in *V. fischeri* involves both LuxR and a small effector molecule that is freely diffusible and is termed autoinducer (AI), the synthesis of which is catalyzed by the product of the *luxI* gene, AI synthetase (*19–21*). *Vibrio fischeri* AI exhibits its similarity to a group of autoregulatory molecules seen in *Streptomyces* spp. (*22*), and both have been postulated to be involved in cell-cell communication (*22, 23*).

The LuxR protein is a member of a group of transcriptional activators defined by sequence similarity in a COOH-terminal, helix-turn-helix–containing region proposed to be involved in binding DNA (*24*). Included in this group is LasR, the overall amino acid sequence of which exhibits 27% identity to that of LuxR (*14*). The COOH-terminal domains of LasR and LuxR are ~53% identical, suggesting a similar biological function in binding DNA (*14*). Furthermore, the regions spanning

```
LasI   1 MIVQIGRRE..EFDKKLLGEMHKLRAQVFKERKGWDVSVIDEMEIDGYDA 48
         |.: | :.:  .:... . :: .|| ||||:| :||: | :::| |:||.
LuxI   1 MTIMIKKSDFLAIPSEEYKGILSLRYQVFKQRLEWDLVVENNLESDEYDN 50

      49 LSPYYMLIQEDTPEAQVFGCWRILDTTGPYMLKNTFPELLHGKEAPCSPH 98
         .: |:.  :||  ..| ||||:|.|||-||||..||||| ...|| .|:
      51 SNAEYIYACDDT..ENVSGCWRLLPTTGDYMLKSVFPELLGQQSAPKDPN 98

      99 IWELSRFAI.NSGQKGSLGFSDCTLEAMRALARYSLQNDIQTLVTVTTVG 147
         | ||||||: ..: | . : |:.|:. : |: :..: .:| ..||||..:
      99 IVELSRFAVGKNSSKINNSASEITMKLFEAIYKHAVSQGITEYVTVTSTA 148

     148 VEKMMIRAGLDVSRPGPH..LKIGIERAVALRIELNAKTQIALYGGVLVEQRLAVS 201
         :|::: |  :.. |:|..    :| .:.|.|.:.:|.. . |:..
     149 IERFLKRIKVPCHRIGDKEIHVLGDTKSVVLSMPINEQFKKAVLN 193
```

Fig. 1. Comparison of the amino acid sequence of LasI to that of LuxI. Optimal alignment was found with the local homology algorithm of Smith and Waterman (*33*). Dots in the sequence denote a gap inserted to allow correct alignment of the downstream sequence. The numbers preceding and following each line indicate the position of the respective amino acid at the beginning and end of each line. Comparison symbols between the two sequences are defined as follows: identical amino acids are denoted by a line, two dots denote conservative amino acid replacements, and one dot denotes evolutionary related substitutions. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

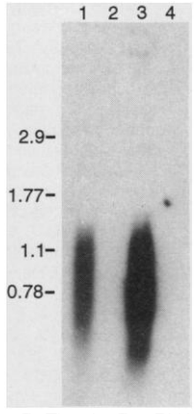

Fig. 2. Northern analysis of *lasR* mRNA. Total RNA from strain PAO1 (lane 1), PAO-RI (lane 2), PAO-RI(pMJG1.7) (lane 3), or PAO-RI-(pSW200) (lane 4) was separated by electrophoresis on a 1.2% agarose gel containing 6.2% formaldehyde and transferred to nitrocellulose. The RNA was hybridized to a ^{32}P-labeled 649-bp Eco RV fragment obtained from pMJG1.7 that is specific to an internal region of *lasR*. The *lasR* mRNA consistently appeared as a smear, perhaps indicating a short half-life. RNA molecular size standards are indicated at left (in kilobases). RNA was obtained, separated by electrophoresis, and probed as described (*11*).

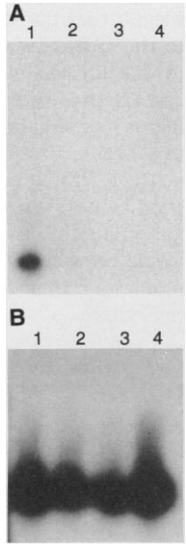

Fig. 3. Northern analysis of *lasI* mRNA. We used a 700-bp fragment obtained by Sal I digestion of pJMC31 and corresponding to the region downstream of the Eco RI site 214 bp internal to *lasI* to probe total RNA from *P. aeruginosa* PAO1 (lane 1), PAO-RI (lane 2), PAO-RI(pMJG1.7) (lane 3), and PAO-RI(pSW200) (lane 4) (**A**). RNA was isolated, separated by electrophoresis, and probed as described in Fig. 2. We monitored the integrity of the RNA by hybridizing it with a pilin gene probe (**B**) as described (*11*).

the AI recognition site of LuxR also exhibit 36% identity to a region in the NH_2-terminus of LasR (*14*). Five of the seven LuxR point mutations that decrease sensitivity to AI are conserved between LasR and LuxR (*25*). On the basis of the similarities between LuxR and LasR and between ORF2 and *luxI*, and given the location of *luxI* and ORF2 relative to *luxR* and *lasR* (the gene for LasR), respectively, ORF2 was renamed *lasI*.

The amino acid sequence of the putative LasI (the *lasI* gene product) is 34.6% identical and 55.9% similar to that of LuxI (the *luxI* gene product or AI synthetase) (Fig. 1). Although LuxI is 193 amino acid residues in length, LasI contains 201 amino acids. Given the similarity of LasI to LuxI, we suspected that LasI might have a similar function. Thus, we designed an assay system to investigate the function of LasI with respect to LasR and LasB, the *lasB* gene product.

Pseudomonas aeruginosa strain PAO-RI, in which the *lasR* gene is inactivated, exhibits no detectable elastase activity or antigen (*11*). However, the elastolytic phenotype can be partially restored if *lasR* is supplied in trans (*11*). To quantify the specific effects of *lasR* on expression of the elastase structural gene *lasB*, we used PAO-RI carrying a plasmid-encoded *lasB::lacZ* translational fusion [pTS400 (*26*)] as a reporter system. In this system, the expression of *lasB* is monitored by measuring the β-galactosidase production (*27*) from PAO-RI carrying the fusion in the presence [PAO-RI(pTS400-1.7) (*26*)] or absence [PAO-RI(pTS400)] of *lasR* supplied in trans.

The addition of *lasR* in trans exhibited only a small effect on *lasB* expression (Table 1). Therefore, the presence of only *lasR*, even on a multicopy plasmid, does not restore elastolytic activity to the amounts seen in wild-type strain PAO1. Northern (RNA) analysis indicated that *lasR* was transcribed in the wild-type PAO1 and in PAO-RI when *lasR* was supplied in trans on a multicopy plasmid (Fig. 2, lanes 1 and 3) but not in PAO-RI or PAO-RI carrying the vector control (Fig. 2, lanes 2 and 4). Thus, the *lasR* gene product alone was not sufficient for maximal expression of elastase.

Northern analysis of total RNA with a 700-bp *lasI*-specific probe shows that the

Table 1. Expression of *lasB* in the presence of *lasR* and *lasI*.

Strain*	Phenotype†	β-gal‡	ECR (%)§
PAO1(pTS400)	R^+I^+	2664 ± 218	100
PAO-RI(pTS400)	R^-I^-	7 ± 1	5
PAO-RI(pTS400-1.7)	R^+I^-	28 ± 8	5
PAO-RI(pLPL106)	R^+I^+	6807 ± 812	323
PAO-RI(pLPL108)	R^-I^+	5 ± 1	2.5

*Strains were grown at 32°C with shaking in LB medium containing carbenicillin (200 μg/ml) for 18 hours. †Phenotype of the strain with respect to *lasR* (R) and *lasI* (I) is shown. A plus sign indicates that a functional gene is present either on the chromosome or as a plasmid-encoded copy. A minus sign indicates that the gene is not expressed. ‡Samples were taken after 18 hours of growth, and β-galactosidase (β-gal) assays were performed as described (*27*). β-Galactosidase values are expressed as Miller units (*27*). Values shown indicate the mean ± SD of at least three determinations. §The elastolytic activity of each strain, obtained by the ECR assay (*13*), is expressed as a percentage of the value obtained for PAO1(pTS400).

Table 2. Expression of the *lasB::lacZ* fusion in strains grown in cell-free supernatants of *E. coli* TB1 or *P. aeruginosa* PAO-RI carrying *lasI* or a control plasmid.

Spent medium*	β-gal (Miller units)†	
	MG4(pTS400)	MG4(pTS400-1.7)
TB1(pSW200)/LB	31 ± 3	28 ± 1
TB1(pLASI-1)/LB	46 ± 2	1151 ± 34
PAO-RI(pSW200)/A‡	192 ± 35	68 ± 7
PAO-RI(pLASI-2)/A‡§	237 ± 40	522 ± 14

*Supernatants from 18- to 24-hour cultures of the indicated strain grown in the medium shown were used as the source of spent medium. Cultures contained either ampicillin (100 μg/ml) (in the case of *E. coli* TB1) or carbenicillin (200 μg/ml) (in the case of *P. aeruginosa* PAO-RI) to ensure maintenance of the plasmid. The supernatants were filter-sterilized by passage through a 0.45-μm filter, and we tested portions for cellular contamination by incubating them at 37°C for 24 hours. Glycerol (0.05%) and the appropriate antibiotic were added to portions of supernatants shown to be cell-free and were used to grow the *E. coli* MG4 strains. †β-Galactosidase assays were carried out as described (*27*). Cultures were grown in the supernatant at 32°C with shaking. Samples for the assay were taken at 4 hours after inoculation into the supernatant. Values shown indicate the mean ± SD of at least three determinations. ‡A medium is essentially as described (*27*) except that glycerol (0.4%), yeast extract (0.05%), and 1 mM $MgSO_4$ were used. §Plasmid pLASI-2 is described in (*31*).

lasI message was present in PAO1 (Fig. 3A). However, *lasI* message was not detected in samples of RNA obtained from strain PAO-RI either alone or with other plasmids. This confirms that the *lasI* gene is not transcribed in PAO-RI and that the incomplete *lasI* gene found in pMJG1.7 is not sufficient to produce a stable *lasI* transcript. All lanes contained the same amount of RNA, and there was no evidence of RNA degradation (Fig. 3B).

Given the similarities of *lasI* to *luxI*, *lasI* may be responsible for the production of a factor that activates LasR in a manner similar to the LuxR-AI system. In order to address this question, we subcloned *lasR* and *lasI* onto pTS400, which carries the *lasB::lacZ* translational fusion, to construct pLPL106 (*28*). In this construct, the normal chromosomal position and spacing of *lasR* and *lasI* was maintained to ensure that all regulatory features would be preserved. We used pLPL106 to determine whether the presence of both the *lasR* and *lasI* genes would affect expression of *lasB* relative to the presence of the *lasR* gene or the *lasI* gene alone. The presence of both *lasR* and *lasI* genes resulted in an increase in *lasB* expression over *lasR* alone and even over that seen in parental strain PAO1 (Table 1). The presence of *lasI* alone exhibited no significant effect on *lasB* expression. Presumably, *lasB* expression is higher in PAO-RI(pLPL106) than in PAO1(pTS400) because multiple copies of *lasR* and *lasI* are provided.

The elastolytic activity of strains carrying only *lasR* [PAO-RI(pTS400-1.7)] or *lasI* [PAO-RI(pLPL108)] exhibited no significant difference when compared with the $lasR^-$ control strain [PAO-RI(pTS400)] (Table 1). The activity of these strains was far below that seen in the wild-type control strain PAO1(pTS400). The presence of *lasR* alone only resulted in a small increase in activity. These results suggest that neither *lasR* nor *lasI* alone can account for wild-type amounts of elastase activity. Furthermore, the results from the elastin Congo Red (ECR) assays (*13*) correlated well with the respective results from the β-galactosidase assays (Table 1). These data indicate that both *lasR* and *lasI* are required for maximal expression of *lasB* and that *lasI* may function in a manner similar to the *luxI* gene of *V. fischeri*.

To determine whether *lasI* is involved in the production of a diffusible molecule like the AI molecule in *V. fischeri*, we grew the lac^- *Escherichia coli* MG4 strain (*29*) containing both *lasR* and the *lasB::lacZ* fusion [MG4(pTS400-1.7)] or containing the fusion alone [MG4(pTS400)] in cell-free supernatants of *E. coli* TB1 (*30*) carrying *lasI* [TB1(pLASI-1) (*31*)] or the control vector [TB1(pSW200)]. Supernatant from cultures of the strain that carries *lasI* increased expression of the fusion more than 40-fold when *lasR* was present (Table 2). No significant effect was seen in the absence of *lasR*. Furthermore, supernatants from cultures of the control strain do not exhibit any effect, regardless of the presence of *lasR*. The same experiments were carried out with *P. aeruginosa* PAO-RI carrying either a control plasmid (pSW200) or *lasI* (pLASI-2) (*31*) as the source of supernatant. Experiments suggest that the diffusible factor is either unstable in *P. aeruginosa* LB supernatants or that the LB medium contains inhibitors that are not broken down by *P. aeruginosa* (*32*). Furthermore, when grown in LB medium, *P. aeruginosa* often produces large amounts of pigment that might interfere with the β-galactosidase assay. For these reasons, we used modified A medium (Table 2). Medium from *P. aeruginosa* PAO-RI(pLASI-2) cultures increased expression of *lasB* approximately eightfold (Table 2). No significant effect was observed when the control strain was used as a source of spent medium.

The data presented in this study indicate that LasI is involved in the production of a diffusible AI-like molecule that we term the *P. aeruginosa* autoinducer (PAI) and that both LasR and PAI are required for maximum *lasB* expression. The experiments carried out with the *E. coli* strains also indicate that LasR protein and PAI are sufficient for the expression of *lasB* and that no other *P. aeruginosa*–derived factors are required. Thus, LasR, a regulator of virulence, requires a diffusible inducer molecule produced by the pathogen.

To be successful, a pathogen must be able to sense its environment and modulate the expression of those genes necessary to establish itself in a new niche. This is important in the case of *P. aeruginosa* because it is found in many different environments, such as burn tissue or the lungs of individuals affected with cystic fibrosis or neutropenia. The latter two environments result in different (chronic-localized versus acute-disseminated) lung infections, highlighting the importance of understanding how virulence factor genes are regulated and of identifying the signals involved.

The possibility also exists that the LasR-PAI system could serve as a mechanism for cell-cell communication between *P. aeruginosa* cells or between *P. aeruginosa* and other bacterial cells to allow coordinate expression of virulence-associated genes. When carried out in response to cell density or other environmental and nutritional stimuli, this communication could result in a concentrated attack on the host.

REFERENCES AND NOTES

1. K. Morihara and J. Y. Homma, in *Bacterial Enzymes and Virulence*, I. A. Holder, Ed. (CRC Press, Boca Raton, FL, 1985), pp. 49–79.
2. B. H. Iglewski and D. Kabat, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2284 (1975).
3. B. H. Iglewski, J. C. Sadoff, M. J. Bjorn, E. S. Maxwell, *ibid.* **75**, 3211 (1978).
4. R. A. Bever and B. H. Iglewski, *J. Bacteriol.* **170**, 4309 (1988).
5. E. Kessler and M. Saffrin, *ibid.*, p. 5241.
6. G. Doring, J. Obernesser, K. Botzenhart, *Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. A* **249**, 89 (1981).
7. L. W. Heck *et al.*, *J. Immunol.* **6**, 2253 (1990).
8. L. W. Heck, K. Morihara, W. B. McRae, E. J. Miller, *Infect. Immun.* **51**, 115 (1986).
9. K. Morihara, H. Tsuzuki, K. Oda, *ibid.* **24**, 188 (1979).
10. D. R. Schultz and K. D. Miller, *ibid.* **10**, 128 (1974).
11. M. J. Gambello and B. H. Iglewski, *J. Bacteriol.* **173**, 3000 (1991). After publication of this paper, we were informed that the pMG designation was in conflict with other constructs listed in a plasmid data bank. To avoid confusion, we have renamed pMG1.7 [as originally referenced in the Gambello and Iglewski paper] as pMJG1.7.
12. M. J. Gambello, S. A. Kaye, B. H. Iglewski, *Infect. Immun.* **61**, 1180 (1983).
13. D. S. Toder, M. J. Gambello, B. H. Iglewski, *Mol. Microbiol.* **5**, 2003 (1991).
14. M. J. Gambello, thesis, University of Rochester (1991).
15. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977); the GenBank–European Molecular Biology Laboratory accession number for the sequence is L04681.
16. S. E. West and B. H. Iglewski, *Nucleic Acids Res.* **16**, 9323 (1988).
17. J. Devereaux, P. Haeberli, O. Smithies, *ibid.* **12**, 387 (1984).
18. J. M. Cook and B. H. Iglewski, unpublished results.
19. E. A. Meighen, *Microbiol. Rev.* **55**, 123 (1991).
20. A. Eberhard, *J. Bacteriol.* **109**, 1101 (1972).
21. A. Eberhard *et al.*, *Biochemistry* **20**, 2444 (1981).
22. T. Beppu, *Gene* **115**, 159 (1992).
23. H. B. Kaplan and E. P. Greenberg, *J. Bacteriol.* **163**, 1210 (1985).
24. S. Henikoff, J. C. Wallace, J. P. Brown, *Methods Enzymol.* **183**, 111 (1990).
25. G. S. Shadel, R. Yound, T. O. Baldwin, *J. Bacteriol.* **172**, 3980 (1990); J. Schlock, D. vanRiet, D. Kolibachuk, E. P. Greenberg, *ibid.*, p. 3974.
26. A. Sinai and B. H. Iglewski, unpublished results. To construct pTS400-1.7, we subcloned the 1.7-kb fragment of pMJG1.7 carrying the *lasR* gene into the unique Tth 111I site downstream of *lacZ*.
27. J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972).
28. We subcloned an Eco RV–Sal I fragment from pJMC30 into pBluescriptII SK$^+$ to produce pLPL100. We then subcloned a 1.1-kb Bam HI–Sfi I fragment from pMJG1.7 that extends from the Bam HI site in the multiple cloning site of pBluescriptII SK$^+$ up to the Sfi I site just before the end of the *lasR* coding sequence into pLPL100 to construct pLPL104. This plasmid carries the entire reconstructed *lasR* gene and the entire *lasI* coding sequence. A 2.4-kb Bam HI–Xho I fragment from pLPL104 that carries both *lasR* and *lasI* was then subcloned into Bam HI– and Sal I–digested pSW200 (*11*) or into the Tth 111I site downstream of *lacZ* on pTS400 to construct pLPL106, which contains the *lasB::lacZ* translational fusion in addition to *lasR* and *lasI*.
29. G. Ralling, S. Bodrug, T. Linn, *Mol. Gen. Genet.* **201**, 379 (1985).
30. Gibco–Bethesda Research Laboratories Life Technologies, *Focus* **6**, 4 (1984).
31. The plasmid pLASI-1 carries the *lasI* gene as a 989-bp fragment that extends from an Nla IV site 90 bp upstream of the start of *lasI* up to a Sal I site distal to the gene. We constructed it by subcloning a 300-bp Nla IV–Eco RI fragment from pMJG1.7 (*11*) and a 689-bp Eco RI–Sal I fragment from pJMC31 into Sma I– and Sal I–digested pBluescriptII SK$^+$. Plasmid pLASI-2 consists of the same fragment containing the *lasI* gene used in pLASI-1 cloned onto pSW200, which carries the pRO1600 replicon, which allows replication in *P. aeruginosa* (*11*).
32. L. Passador and B. H. Iglewski, unpublished results.
33. T. F. Smith and M. S. Waterman, *Adv. Appl. Math.* **2**, 482 (1981).

34. We thank P. Greenberg and K. Tucker for discussions, T. Linn for the gift of *E. coli* MG4, and J. Nezezon for technical assistance. Supported by PHS grant AI33713 and by NIH grants T32GM07356 (to M.J.G.) and T32AI07362 (to L.R.).

16 October 1992; accepted 12 February 1993

GDNF: A Glial Cell Line–Derived Neurotrophic Factor for Midbrain Dopaminergic Neurons

Leu-Fen H. Lin, Daniel H. Doherty, Jack D. Lile, Susan Bektesh, Frank Collins*

A potent neurotrophic factor that enhances survival of midbrain dopaminergic neurons was purified and cloned. Glial cell line–derived neurotrophic factor (GDNF) is a glycosylated, disulfide-bonded homodimer that is a distantly related member of the transforming growth factor–β superfamily. In embryonic midbrain cultures, recombinant human GDNF promoted the survival and morphological differentiation of dopaminergic neurons and increased their high-affinity dopamine uptake. These effects were relatively specific; GDNF did not increase total neuron or astrocyte numbers nor did it increase transmitter uptake by γ-aminobutyric–containing and serotonergic neurons. GDNF may have utility in the treatment of Parkinson's disease, which is marked by progressive degeneration of midbrain dopaminergic neurons.

Parkinson's disease is characterized by degeneration of dopaminergic neurons in the midbrain that innervate the striatum (*1*). Current treatments are aimed at pharmacologically augmenting striatal dopamine but do not prevent continued neuron degeneration. Neurotrophic factors that specifically prevent this degeneration and increase the functional activity of the remaining dopaminergic neurons are therefore of substantial clinical interest.

The search for such neurotrophic factors has focused on dissociated cultures of embryonic midbrain, where high-affinity dopamine uptake and expression of tyrosine hydroxylase (TH) can be used as markers for dopaminergic neuron survival and differentiation (*2*). These bioassays have established the existence of dopaminergic neurotrophic activity in conditioned media derived from primary glial cells (*3*) and from several cell lines with the properties of glia (*4*). However, these factors have not been characterized.

Here we report the characterization of a specific dopaminergic neurotrophic factor secreted by one of these glial cell lines, rat B49 (*5*). This factor, termed GDNF (for glial cell line–derived neurotrophic factor), was purified to apparent homogeneity (*6*) on the basis of its ability to promote dopamine uptake in midbrain cultures (*7*). Purified GDNF produced a single peak on reversed-phase high-performance liquid chromatography (RP-HPLC) but a broad smear on SDS–polyacrylamide gel electrophoresis (SDS-PAGE), a property suggestive of glycosylation (Fig. 1). The presence of *N*-linked glycosylation was confirmed by treatment with *N*-glycanase, which decreased the apparent molecular mass of GDNF from ~20 kD to ~15 kD (Fig. 1). GDNF behaved like a disulfide-bonded dimer; its apparent molecular mass on nonreducing SDS gels was 32 to 42 kD compared with 18 to 22 kD on reducing gels (Fig. 1).

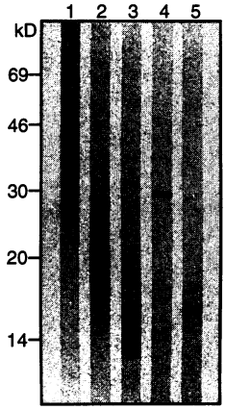

Fig. 1. SDS-PAGE of GDNF. Samples were heated to 100°C for 10 min with or without reducing agent (200 mM dithiothreitol) or after treatment with *N*-glycanase to remove Asn-linked sugars. The positions of molecular weight markers are indicated on the left. Lane 1, purified B49 cell GDNF (25 ng), detected by silver staining; lane 2, purified B49 cell GDNF (25 ng) after reduction of disulfide bonds, detected in an immunoblot with antibodies to rhGDNF; lane 3, purified B49 cell GDNF (25 ng) after treatment with *N*-glycanase, detected in an immunoblot with antibodies to rhGDNF; lane 4, purified rhGDNF (2 μg), detected by Coomassie brilliant blue staining; lane 5, purified rhGDNF (2 μg) after reduction of disulfide bonds, detected by Coomassie brilliant blue staining.

Synergen, Inc., 1885 33rd Street, Boulder, CO 80301.

*To whom correspondence should be addressed.