Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*

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A proteinaceous elicitor of the plant defense reaction known as the hypersensitive response was isolated from *Erwinia amylovora*, the bacterium that causes fire blight of pear, apple, and other rosaceous plants. The elicitor, named harpin, is an acidic, heat-stable, cell-envelope–associated protein with an apparent molecular weight of 44 kilodaltons. Harpin caused tobacco leaf lamina to collapse and caused an increase in the pH of bathing solutions of suspension-cultured tobacco cells. The gene encoding harpin (*hrpN*) was located in the 40-kilobase *hrp* gene cluster of *E. amylovora*, sequenced, and mutated with Tn*5*tac1. The *hrpN* mutants were not pathogenic to pear, did not elicit the hypersensitive response, and did not produce harpin.

The hypersensitive response (HR) of higher plants is characterized by the rapid, localized death of tissues containing an incompatible pathogen (a microorganism that is pathogenic only on other plants). It is associated with the defense of plants against many bacteria, fungi, nematodes, and viruses (*1*). The molecular basis for HR is unknown, but physiological and genetic observations with bacteria suggest that the same factor that elicits HR in nonhosts also is required for pathogenicity in hosts (*1, 2*). Production of this factor is controlled by *hrp* genes, which are highly conserved among many species of plant pathogenic bacteria (*2, 3*). Functional clusters of *hrp* genes have been cloned from *Erwinia amylovora* and *Pseudomonas syringae* and have been shown to confer on nonpathogenic bacteria the ability to elicit HR after infiltration of bacterial suspensions into the intercellular spaces of leaves of tobacco and other plants (*4, 5*).

The *hrp* gene cluster from *E. amylovora* (Fig. 1), contained in the cosmid pCPP430, is expressed particularly well in *Escherichia coli* (*4*). We report here the isolation of a proteinaceous elicitor of the HR from *Escherichia coli* DH5α(pCPP430) and from *E. amylovora*, the bacterium that causes the devastating disease of apple, pear, and other rosaceous plants known as fire blight (*6*). We propose the name harpin for the HR-elicitor from *E. amylovora* and *hrpN* for the gene that encodes it.

Tobacco leaves infiltrated (*7*) with cell-free culture supernatants of *E. amylovora* Ea321, Ea321(pCPP430) or *E. coli* DH5α (pCPP430) showed no HR, whereas leaves infiltrated with a centrifuged and filter-sterilized preparation from sonicated cells of *E. coli* DH5α(pCPP430) showed a strong HR within 12 hours (Fig. 2). The HR-eliciting activity of this cell-free elicitor preparation (CFEP) (*8*) was heat-stable but highly sensitive to protease (*9*).

Further purification was achieved by holding the CFEP in a boiling-water bath for 10 min and removing the insoluble material by centrifugation. The remaining soluble material was electrophoresed on an SDS-polyacrylamide gel (Fig. 3). A protein band corresponding to 44 kD was uniquely present in all preparations with HR-eliciting activity (Fig. 3). Similarly, after resolution of the CFEP on an isoelectric-focusing (IEF) granulated gel bed (*10*) or by ion-exchange chromatography (*11*), the fractions with HR-eliciting activity always contained a 44-kD protein.

The 44-kD protein, named harpin, was electroeluted from a preparative SDS-polyacrylamide gel (*12*). At concentrations ≥500 nM (≥25 μg/ml), harpin elicited HR in leaves of tobacco (Fig. 2, sectors 6 and 13). Harpin also elicited HR in tomato and *Arabidopsis thaliana*. Purified harpin was protease-sensitive, heat-stable, and had a *p*I of 4.3 on thin-layer IEF gels (*13*).

Because supernatants from *E. amylovora* Ea321(pCPP430) or *E. coli* DH5α (pCPP430) did not elicit HR, we postulated that harpin was not secreted but rather was present in or on the bacteria. Whole bacteria treated with protease failed to elicit HR, whereas bacteria incubated with protease together with 0.5 mM phenylmethylsulfonyl fluoride (PMSF, a protease inhibitor) did (Table 1). Treatment of bacteria with increasing amounts of protease resulted in a decreased ability to elicit HR that correlated with the disappearance of harpin detectable in SDS-polyacrylamide gels (Table 1). After centrifugation of CFEP at 105,000*g* for 1 hour, most HR-eliciting activity was found in the supernatant. However, when the cell suspension was brought to 30 mM $MgCl_2$, before sonication, most activity was associated with the sedimented membrane fraction. Gel-permeation chromatography of unheated CFEP also indicated association of the elicitor with a high molecular weight ($>10^6$ daltons) fraction, probably membrane vesicles (*14*). Only the membrane fraction of *E. amylovora* Ea321(pCPP430) reacted with an antiserum raised in response to harpin (*15*), further supporting the cell-envelope location of harpin (Fig. 4).

Cell suspensions of Ea321(pCPP430) or DH5α(pCPP430) in log-phase maintained their HR-eliciting activity for less than 0.5 hour and 1 hour, respectively, in the presence of tetracycline (40 μg/ml), an inhibitor of protein synthesis. Once cells lost

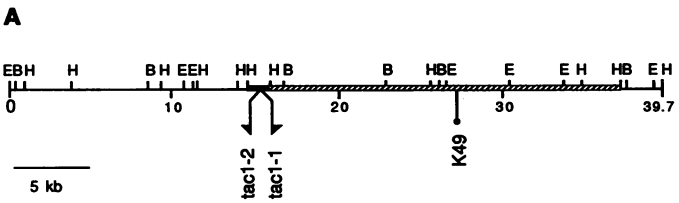

Fig. 1. (**A**) Physical map of the *hrp* gene cluster of *E. amylovora* (*4, 18, 29*), showing restriction sites: B, Bam HI; E, Eco RI; H, Hind III. Gene *hrpN*, encoding harpin, is contained in the 1.3 kb Hind III fragment indicated by the solid bar. The shaded region (including *hrpN*) contains that part of the *hrp* gene cluster in which most transposon insertions, exemplified by K49, a Tn*10* mini-*kan* (*30*) insertion, abolish the HR and pathogenicity phenotypes. Most insertions to the left of *hrpN* abolish pathogenicity to pear and eliminate or greatly disrupt HR-elicitation. Flags indicate the orientation of the outward-facing P_{tac} promoter of Tn*5*tac1 (*28*) in the tac1-1 and tac1-2 insertions. The two tac1 and K49 mutations were marker-exchanged into the *E. amylovora* 321 genome. The mutations abolished the ability of *E. amylovora* to cause disease in immature pear fruit, to elicit HR in tobacco leaves, and to produce harpin; similarly, they abolished the ability of *E. coli* DH5α(pCPP430) to elicit HR and to produce harpin. The effect of the K49 mutation on harpin production probably is regulatory. (**B**) Deduced amino acid sequence of harpin. The DNA sequence is accessioned in GenBank (#M92994). Amino acids confirmed by NH_2-terminal sequence analysis of the purified protein are underlined.

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HR-eliciting activity, harpin was not detected. However, when the protease inhibitor PMSF (0.5 mM) was included, the bacteria retained HR-eliciting activity and possessed detectable harpin for more than 2 hours. More protease was required per cell to destroy harpin produced by *E. coli* DH5α(pCPP430) than by Ea321(pCPP430), suggesting that *E. coli* DH5α(pCPP430) produces more harpin or degrades it more slowly, or both.

The ability of bacterial strains to elicit the HR in intact tobacco leaves is related genetically to their ability to elicit a K^+/H^+ exchange reaction (XR) in tobacco cell suspension cultures (TCSCs) (*16*); both reactions require the *hrp* gene cluster (*17*).

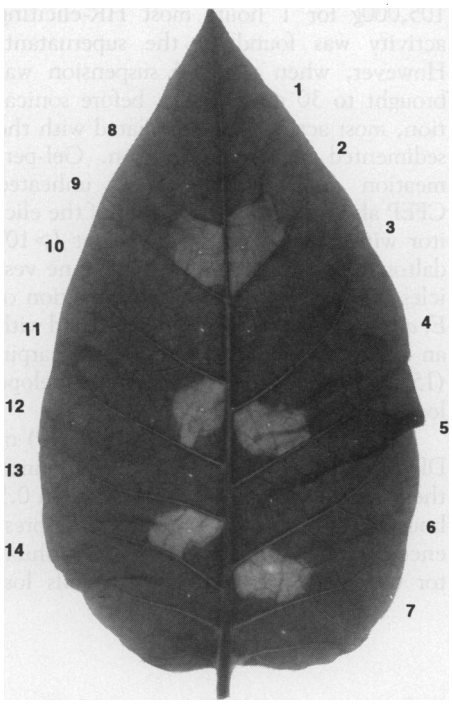

Fig. 2. Tobacco leaf showing responses 24 hours after infiltration of sectors (*7*) with the following preparations: 1, living *E. coli* DH5α (pCPP9) (1 × 10^8/ml); 2, *E. coli* DH5α (pCPP430) (1 × 10^8/ml); 3, *E. coli* DH5α (pCPP430K49) (1 × 10^8/ml); 4, *E. amylovora* Ea321 (1 × 10^8/ml); 5, Ea321K49, an *hrp* mutant (1 × 10^8/ml), 8, heat-treated CFEP from *E. coli* DH5α(pCPP9); 9, heat-treated CFEP from *E. coli* DH5α(pCPP430); 10, heat-treated CFEP from *E. coli* DH5α(pCPP430K49); 11, heat-treated CFEP from *E. amylovora* Ea321; 12, heat-treated CFEP from Ea321K49; 6, harpin (1.1 μM) from *E. coli* DH5α(pCPP430) eluted from SDS-polyacrylamide gel; 7, same preparation as 6, but protease treated for 2 hours then heated for 10 min to inactivate protease; 13, harpin (1 μM) from *E. amylovora* Ea321 eluted from SDS-polyacrylamide gel; 14, same preparation as 13 but with protease treatment as sample 7. Harpin solutions <~0.3 μm do not cause collapse of infiltrated tissue; spotty and incomplete collapse is caused by harpin between 0.3 and 0.5 μm.

We tested the ability of harpin to raise the pH of TCSC bathing solution, an indicator of the XR (Fig. 5). Cells of *E. amylovora*, grown in rich medium and added to TCSCs caused an increase in pH of the bathing solution after 2 to 3 hours. Addition of purified harpin caused an increase in pH within 1 hour. *Erwinia amylovora* mutant Ea321K49, which did not produce harpin in culture, and strains of *E. coli* containing mutated *hrp* gene clusters failed to elicit the XR.

Harpin also was isolated from *E. amylovora* Ea321, which had been preincubated for 5 hours in an HR-inducing medium (*18*). Harpin from *E. amylovora* Ea321 was identical in physical and biological properties to that isolated from *E. coli* DH5α (pCPP430). No protease-sensitive, heat-stable, HR-eliciting activity, associated with a 44-kD protein, was seen in cell-free extracts from *E. coli* DH5α(pCPP9), which harbors the vector of pCPP430 (Fig. 3, lane 5). On the basis of the visual intensities of the 44-kD bands on SDS-polyacrylamide gels (Fig. 3, lanes 7 and 8), we estimate that Ea321 produces about 10% as much harpin as does *E. coli* DH5α(pCPP430).

The structural gene encoding harpin, designated *hrpN*, was located within the *hrp* gene cluster (Fig. 1) by hybridization with an oligonucleotide probe corresponding to the ninth to the fifteenth amino acid residues from the NH_2-terminus of harpin (*19*). The 1.3-kb Hind III fragment that hybridized was cloned into pBluescript M13+ (Stratagene, La Jolla, California) and designated pCPP1084. A unique 44-kD protein, which was immunoprecipitated by antiserum raised against harpin (*15*), was expressed from pCPP1084 in the T7 RNA polymerase/promoter expression system (*20*). Insertions of Tn5tac1 in *hrpN* (*21*) (Fig. 1) abolished the ability of *E. coli* DH5α(pCPP430) to elicit HR on tobacco or produce harpin detectable on Western blots. Ea321T5, a derivative of *E. amylovora* Ea321 containing the marker-exchanged tac1-1 mutation, failed to elicit

Table 1. Protease sensitivity of the HR-eliciting activity of whole cells of *E. amylovora* Ea321(pCPP430). Cells were grown in LB medium, harvested by centrifugation, and resuspended in 0.1 volume of 5 mM potassium phosphate (pH 6.5) containing tetracycline (40 μg/ml). After incubation with protease (Sigma P5147), as indicated, at 37°C for 5 min, 100 μl of each cell suspension was infiltrated into tobacco leaves. Leaf sector collapse was assayed at 24 hours. At the time of infiltration, portions of protease-treated cell mixtures were lysed, held in boiling water for 10 min, centrifuged for 10 min at 12,000*g*, and electrophoresed on a 10% SDS-polyacrylamide gel to detect harpin. Electrophoresis was done for 2 hours at 15 mA, followed by staining with Coomassie blue R-250. Cell-free supernatant, produced from the LB culture, was filter-sterilized and then concentrated with the Centriprep-10 (Amicon, Danvers, Massachusetts).

Protease per milliliter	Tissue collapse	Harpin detected
0	+	+
5 μg	+	+
10 μg	+	+
20 μg	Weak	+
40 μg	−	−
80 μg	−	−
80 μg + 0.5 mM PMSF	+	+
Cell-free supernatant	−	−

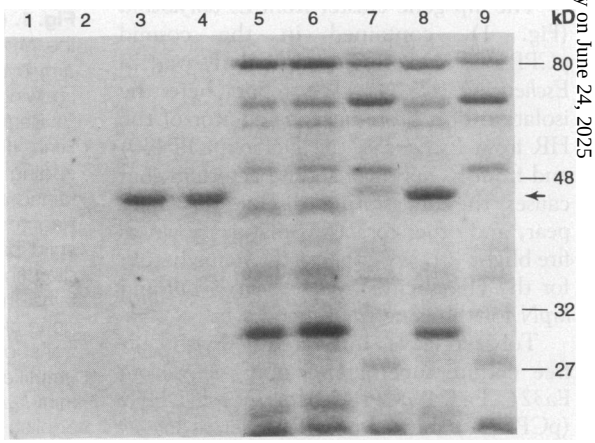

Fig. 3. SDS-polyacrylamide gel electrophoresis of CFEPs and purified harpin. Lanes: 1, purified harpin (1.5 μg) from *E. coli* DH5α(pCPP430) incubated with protease (*9*) for 1 hour; 2, purified harpin (1.5 μg) from *E. amylovora* Ea321 incubated with protease for 1 hour; 3, same as 1, but without treatment with protease; 4, same as 2, but without treatment with protease; 5, CFEP (5 μg) from *E. coli* DH5α(pCPP9) treated at 100°C for 10 min; 6, CFEP (5 μg) from *E. coli* DH5α(pCPP430K49) treated at 100°C for 10 min; 7, CFEP (5 μg) from *E. amylovora* Ea321 treated at 100°C for 10 min; 8, CFEP (5 μg) from *E. coli* DH5α(pCPP430) treated at 100°C for 10 min; 9, CFEP (5 μg) from *E. amylovora* Ea321K49 treated at 100°C for 10 min. Samples from the preparations in lanes 3, 4, 7, and 8 elicited HR in tobacco leaves. Samples were prepared as described (*8*) and brought to 125 mM tris-HCl (pH 6.8) 4% SDS, 20% glycerol, boiled for 3 min, then electrophoresed through a 10% (w/v) polyacrylamide gel with 0.1% SDS at 15 mA for 2 hours in a Mighty Small apparatus according to instructions (Hoefer Scientific Instruments, San Francisco, California). The gel was stained with 0.025% Coomassie Blue R-250. Low-range prestained molecular weight standards (Bio-Rad 161-0305) were used and calibrated with an unstained protein marker (Bio-Rad 161-0304). Arrow indicates region corresponding to 44 kD.

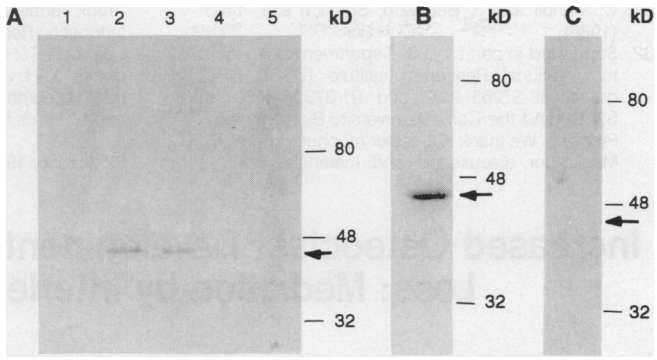

Fig. 4. Subcellular location of elicitor protein. Log-phase cells (1.5 ml) of strain Ea321(pCPP430) were fractionated (*31*). Proteins from each fraction were electrophoresed and transferred to Immobilon-P membrane (Millipore, Bedford, Massachusetts). The Amplified Alkaline Phosphatase Immuno-Blot Assay Kit (170-6412, Bio-Rad Richmond, California) was used in a Western blot to detect the elicitor protein with an antiserum raised in rabbit in response to harpin (*15*). (**A**) Fractions in lanes: 1, periplasm; 2, membrane; 3, whole cells; 4, supernatant; 5, cytoplasm. (**B**) Harpin purified by high-performance liquid chromatography (*19*) hybridized with antiserum. Arrows indicates 44 kD, based on the molecular weight markers used in Fig. 3. (**C**) Normal serum control. CFEP from *E. coli* DH5α(pCPP430) hybridized with pre-immune serum.

HR in tobacco leaves or to cause fire blight in highly susceptible immature pear fruits (*22*). Both pathogenic and HR-eliciting abilities were restored to Ea321T5 by pCPP1084, in trans, and the resulting strains produced harpin.

DNA sequence data from the 1.3-kb Hind III fragment revealed that *hrpN* is 1155 base pairs long, and it encodes a 385–amino acid protein (Fig. 1). The 15 NH_2-terminal residues revealed by amino acid sequencing corresponded to those deduced from the DNA sequence (Fig. 1). The deduced amino acid sequence of harpin (Fig. 1), which corresponded closely with the analyzed amino acid composition, reveals a glycine-rich protein with a high degree of hydrophilicity. It appears to have an open structure, which may explain its heat stability and sensitivity to proteases. A FASTA search (*23*) of GenBank for similar proteins revealed similarity only with other glycine-rich proteins, such as several plant cell wall proteins and keratins.

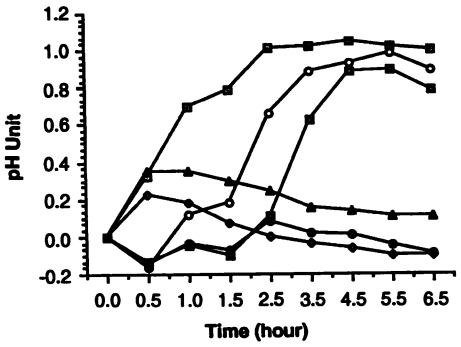

Fig. 5. Changes in pH of bathing solution of tobacco cell-suspension cultures (TCSC). Control values (no additive) were subtracted. Open squares, harpin (60 nM); open circles, cells of *E. coli* DH5α(pCPP430) (5 × 10^7 cells per milliliter); filled squares, cells of *E. amylovora* Ea321 (5 × 10^7 cells per milliliter); triangles, cells of *E. coli* DH5α(pCPP430K49) (5 × 10^7 cells per milliliter); diamonds, cells of *E. amylovora* Ea321K49 (5 × 10^7 cells per milliliter); filled circles, cells of *E. coli* DH5α(pCPP9) (5 × 10^6 cells per milliliter). TCSCs were shaken at room temperature with the indicated preparations. The pH was measured at the intervals indicated. All preparations that elicited HR in tobacco leaves (Fig. 2) also caused a pH increase in the TCSC medium.

The properties of the *E. amylovora* harpin protein are consistent with numerous physiological observations that were made after the discovery in 1963 that bacteria can elicit HR (*24*). These have indicated that elicitation of HR requires de novo protein synthesis and production of a labile factor by bacteria in close proximity to plant cells, and that each bacterial cell typically kills only one plant cell, thus explaining the requirement for high numbers of bacteria to cause death of enough plant cells to produce macroscopically visible collapse of the tissue (*1*, *25*).

The nonpathogenic phenotype of the *hrpN* mutation in Ea321T5 also is characteristic of *hrp* mutations in other phytopathogenic bacteria (*2*) and indicates that harpin is a primary determinant of pathogenicity in *E. amylovora*. That harpin has an essential role in both disease and plant defense reactions is puzzling but may be based on differential proteolysis or differential expression of *hrp* genes in host and nonhost plants (*18*).

Toxins, plant cell wall–degrading enzymes, and phytohormones contribute to the virulence (degree of pathogenicity) of certain members of the important group of phytopathogenic bacteria that possess limited host ranges and produce necrotic lesions after multiplication in compatible hosts (*26*). The *hrp* genes, in contrast, are absolutely required for pathogenicity by these bacteria, which include species of *Erwinia*, *Pseudomonas*, and *Xanthomonas*. The conservation of the *hrp* genes suggests that the *E. amylovora* harpin may be the archetypical disease determinant for these pathogens.

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7. D. W. Bauer and S. V. Beer, *Mol. Plant-Microbe Interact.* **4**, 493 (1991). Tobacco plants (*Nicotina tabacum* L. "Xanthi") were grown in artificial soil mix to a height of 90 to 100 cm. Plants were moved from the greenhouse to the laboratory <24 hours before infiltration with bacterial suspensions. For strains of *E. amylovora*, ~2 × 10^8 colony-forming units (cfu) per milliliter was used, and for *E. coli* about 5 × 10^7 cfu/ml was used in 5 mM potassium phosphate buffer (pH 6.5). Infiltration of the leaf lamina (~50 μl/cm^2 required) was done with a needle-less syringe through a small hole made with a dissecting needle. During infiltration, air in the intercellular spaces of mesophyll tissue is displaced, and the treated areas appear water-soaked for 30 to 90 min, until the liquid disappears. Initial indication of the developing HR is flaccidity of the previously water-soaked areas, which develops after 4 to 8 hours. HR was scored 24 hours after infiltration, when collapse of the infiltrated area was complete. Adjacent tissues are not visibly affected. Infiltration with suspensions containing less than a threshold concentration of bacteria (~5 × 10^6 cfu/ml for *E. coli* DH5α(pCPP430) and ~10^7 cfu/ml for *E. amylovora*) has no effect. Suspensions containing concentrations near the threshold needed for collapse cause blotchy or spotty collapse, which occurs on a somewhat extended schedule.
8. CFEPs were produced from *E. coli* DH5α (pCPP430) grown in LB medium (*27*) to an OD_{620} of 0.8. Cells were collected by centrifugation and resuspended in 0.1 the original volume of 5 mM potassium phosphate (pH 6.5) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor. Cells in a 10-ml bacterial suspension in an ice bath were disrupted by sonication with a Model W-225R Sonicator Ultrasonic Cell Disrupter (Heat System–Ultrasonics) for 10 min with a power output of 4 and a 40% duty cycle. Debris was removed by centrifugation at 12,000*g* for 1 hour, and the supernatant liquid was passed through a 0.2-μm, pore-size membrane filter. At dilutions up to ~1:10, the resulting CFEP elicited HR in tobacco leaves.
9. In the absence of PMSF, CFEP lost all HR-eliciting activity after 3 hours at 37°C or 6 to 8 hours at 4°C. Incubation of CFEPs with Protease type XIV (Sigma, P5147) at 100 μg/ml, for 1 hour at 37°C destroyed elicitor activity and eliminated the 44-kD protein band detectable in SDS-polyacrylamide gels. When harpin was incubated with protease that had been held at 100°C for 10 min to inactivate the enzyme, the preparation retained HR-eliciting activity.
10. An isoelectric focusing (IEF) gel bed was prepared with Bio-lyte gel (Bio-Rad, Richmond, CA) containing 2% (w/v) wide-range ampholytes (pH = 3 to 10; Sigma, P-1522). IEF was performed on a Bio-Phoresis Horizontal Electrophoresis Cell (Bio-Rad) with electrode solutions of 1 M H_3PO_4 (anode) and 1 M NaOH (cathode), according to instructions (Bio-Rad).
11. Ion-exchange chromatography was performed with a 1.5 cm in diameter open column of DE52 anion-exchange resin (Whatman, supplied by W&R Balston, Kent, UK) at 1 ml/min. Bound

material was eluted stepwise from the resin with 10 mM potassium phosphate (pH 6.5) containing 0.1 mM PMSF and 50 mM to 100 mM KCl. Fractions that were eluted with 90 mM KCl elicited HR.

12. The elicitor protein was eluted from SDS-polyacrylamide with an Elutrap apparatus (Schleicher & Schuell, Nashua, NH) at 150 V for 15 hours. The eluted protein (200 μg/ml) was dialyzed overnight against 2 liters of 5 mM potassium phosphate (pH 6.5) containing 0.1 mM PMSF.
13. Thin-layer IEF gels (Servalyte Precotes, pH 3 to 10, #42965 (Serva, Westbury, NY) and wide-range molecular weight markers (pI 4.6 to 9.6) (Bio-Rad) were used as directed.
14. Gel-permeation was carried out on a Sephadex G-100 (Pharmacia) column in 33 mM NaCl, 1 mM EDTA, 10 mM KPO_4 (pH 6.8), 1 mM β-mercaptoethanol, and 0.1 mM PMSF, at 4°C. HR-eliciting activity eluted in the void volume, as determined by elution of Blue Dextran (Pharmacia).
15. Antibodies were raised in rabbits in response to injection with harpin. Three injections of purified harpin (*19*) (100, 100, and 50 μg, respectively) were made at 2- to 3-week intervals. The antiserum was harvested after 8 weeks; immunoglobulin G was precipitated with ammonium sulfate and preabsorbed with sonicated *E. coli* DH5α(pCPP9) lysate. The specificity of the antiserum was confirmed by reaction in Western blots of harpin purified by high-performance liquid chromatography (*19*). No reaction was seen with pre-immune serum when Western blots containing resolved CFEP from DH5α(pCPP430) were hybridized.
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19. Harpin was purified to homogeneity from CFEPs by ion-exchange chromatography and then elution from a reverse-phase column (Yamamura model AQ-303) with a gradient of 20 to 80% acetonitrile in water containing 0.25% trifluoroacetic acid. Pure harpin (100 ng) was absorbed to a nylon membrane and the NH_2-terminal sequence determined by the Cornell University Biotechnology Program Protein Analysis Facility.
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21. pCPP1012, an 8.4-kb Bam HI subclone of pCPP430 in pBluescript M13+ containing *hrpN*, was mutagenized with Tn5tac1 (*28*). Two insertions in opposite orientations that mapped in the 1.3-kb Hind III fragment were marker-exchanged into pCPP430 in *E. coli* MC4100, and into *E. amylovora* Ea321. Mutant phenotypes were tested in the presence of 0.5 mM isopropylthiogalactoside. The location of the insertion in each of the resulting mutants was confirmed by probing Southern (DNA) blots with the 1.3-kb Hind III fragment.
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32. Supported in part by U.S. Department of Agriculture National Research Institute (USDA NRI) grants 89-37263-4692 and 91-37303-6430 to S.V.B., and the Cornell University Biotechnology Program. We thank K. Loeffler for photography, V. Macko for discussions and materials, Springbrook Farms (Alton, NY) and M. J. Hattingh (Stellenbosch, Republic of South Africa) for gifts of pear fruit, T. Hammond for production of tobacco plants, X.-H. Jiang and B. J. Sneath for preliminary research from which this work developed, and M. Haus for preparation of the manuscript.

27 January 1992; accepted 27 May 1992

Increased Osteoclast Development After Estrogen Loss: Mediation by Interleukin-6

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Osteoclasts, the cells that resorb bone, develop from hematopoietic precursors of the bone marrow under the control of factors produced in their microenvironment. The cytokine interleukin-6 can promote hematopoiesis and osteoclastogenesis. Interleukin-6 production by bone and marrow stromal cells is suppressed by 17β-estradiol in vitro. In mice, estrogen loss (ovariectomy) increased the number of colony-forming units for granulocytes and macrophages, enhanced osteoclast development in ex vivo cultures of marrow, and increased the number of osteoclasts in trabecular bone. These changes were prevented by 17β-estradiol or an antibody to interleukin-6. Thus, estrogen loss results in an interleukin-6–mediated stimulation of osteoclastogenesis, which suggests a mechanism for the increased bone resorption in postmenopausal osteoporosis.

Loss of ovarian function in animals and humans, such as at menopause, causes a dramatic and precipitous loss of bone that can be prevented by estrogen replacement (*1*). However, the cellular and biochemical changes that mediate the adverse effects of estrogen deficiency on skeletal homeostasis have been difficult to elucidate. Nonetheless, it is now believed that osteoclasts arise from hematopoietic progenitors of the marrow, most likely the colony-forming units for granulocytes and macrophages (CFU-GM) (*2*), and that osteoclast development is under the control of the same paracrine cytokines that are responsible for CFU-GM formation. Among these cytokines, interleukin-6 (IL-6) plays a prominent role in the early stages of both hematopoiesis and osteoclastogenesis (*3*). Moreover, IL-6 production by bone marrow stromal cells and osteoblastic cells, both of which influence osteoclastogenesis, is inhibited by 17β-estradiol in vitro; and 17β-estradiol as well as a neutralizing antibody to IL-6 suppress osteoclast development in cultures of mouse bone cells (*4*). Prompted by this and by evidence for a role of IL-6 in the pathologic bone resorption associated with Paget's disease and multiple myeloma (*5*), we used ovariectomized mice to test the hypothesis that estrogen loss up-regulates osteoclastogenesis through an increase in the production of IL-6 in the microenvironment of the marrow.

Mice were killed at various times after ovariectomy, cells were obtained from the femur or spleen, and short-term cultures were established. The osteoclast progenitors in these ex vivo cultures were quantitated (*6*). In pilot experiments, ovariectomy led to a decrease in uterine weight and to a progressive loss of trabecular bone; and implantation of slow-release pellets containing 17β-estradiol to the ovariectomized animals prevented both of these effects (*7*). The minimum effective amount of 17β-estradiol was 10 μg per 30-g mouse, and this amount of 17β-estradiol was therefore used for estrogen replacement in the present studies.

The number of CFU-GM per femur and spleen was increased approximately twofold in mice killed 14 days after ovariectomy, compared to sham-operated animals (Fig. 1, A and B). This increase was prevented when ovariectomized mice were implanted with estrogen pellets. In parallel studies, the effect of ovariectomy and estrogen re-

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