bacterium tuberculosis prevented only the disease that they were able to induce (*23*). Anti-clonotypic responses induced by the inoculates in the same strain of rats were specific for the given T cells and did not cross-react with each other, suggesting recognition of a clonotypic epitope within the TCR. Furthermore, recognition of clonotypic epitopes of the TCR V_β chain of human MBP-reactive T cells leads to specific cytolysis of the target T cells (*10*). The clarification of the target antigen within the TCR will allow future development of "vaccines" that incorporate a peptide matching the target sequences.

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13. The MBP-reactive T cell lines were cloned at 0.3 cell per well by limiting dilution with 10^5 irradiated autologous feeders and PHA (2 μg/ml). Cultures were refreshed with medium containing recombinant interleukin-2 (rIL-2; 5 U/ml) every 3 days. After 12 to 14 days, growing clones were first examined for their reactivity to three fragments of MBP, regions 1 to 37, 45 to 89 and 90 to 170, and subsequently tested with 11 peptides of MBP. Cells (10^4) of each clone were cultured with 10^5 irradiated autologous APCs per well, to which each fragment (10 μg/ml) or each peptide (2 μg/ml) was added. Cells were cultured for 72 hours and pulsed with [^{3}H]thymidine during the last 16 hours of culture and then harvested with an automated harvester to measure tritiated thymidine uptake. The same procedure was used in the other proliferation assays mentioned elsewhere in this report.
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16. The MBP-reactive T cell clones were activated with irradiated MBP-pulsed autologous WMNC at an APC to T cell ratio of 10 and supplemented with rIL-2. Activated MBP-reactive T cells were isolated by Ficoll gradient separation, when necessary, and washed three times with sterile phosphate-buffered saline (PBS) before irradiation (8000 rads, ^{60}Co source). The inoculates were resuspended in 1 ml of PBS and injected subcutaneously (0.5 ml per arm). The number of cells used for vaccination was chosen by an extrapolation of vaccine doses effective in experimental animals on the basis of relative skin surface areas (*24*). All subjects were monitored for changes in counts of granulocytes, lymphocytes, monocytes, and platelets and in prothrombin times, serum glutamic-oxalacetic and glutamic-pyruvic transaminase (SGOP and SGPT), uric acid, blood urea nitrogen, and creatinine at 2-month intervals throughout the inoculation procedure.
17. To assess the proliferative responses, we cultured 5×10^4 fresh WMNC per well in triplicate with 5×10^4 irradiated inoculates or concurrently prepared nonspecific T cell blasts for 72 hours. As a control, WMNC and irradiated inoculates or T cell blasts were cultured alone. Cell proliferation was measured by the proliferation assays (*13*). To estimate the frequency of antigen-reactive T cells, we plated out WMNC at 2×10^5, 10^5, and 0.5×10^5 cells per well for MBP stimulation (MBP, 40 μg/ml) or plated out at 4×10^4, 2×10^4, and 10^4 cells per well for TT stimulation (TT, 2.5 Lf/ml), respectively (60 wells for each concentration). The concentration range was predetermined to allow for sensitive detection (*14*). Cultures were then restimulated with MBP- or TT-pulsed WMNC as a source of APC, and rIL-2 was added at 5 U/ml. After 1 week, each culture was split and tested for specific proliferation to MBP or TT in a proliferation assay. A T cell line was defined as specific when cpm of the wells containing MBP- or TT-pulsed APC/cpm of control wells exceeded 3, and Δcpm was greater than 1000. The frequency of antigen-reactive T cells was estimated by Poisson statistics when the frequency distribution followed the "single-hit" rule (*25*). In cases of "multiple-hit" distribution, the frequency was calculated by dividing the number of specific wells by the total amount of WMNC plated at a concentration that yielded the highest number of specific wells (*14, 15*). For each individual, the same method of calculation was used consistently to compare the frequency changes during the trial.
18. To estimate the frequency of T cells responding to the inoculates, we plated freshly isolated WMNC at 4×10^4 and 2×10^4 cells per well and cultured them with 4×10^4 irradiated inoculates. After 7 days, the cultures were restimulated with the irradiated stimulator T cells and supplemented with rIL-2 (5 U/ml). At day 14, 50% of the cells from each culture were taken out and irradiated at 8000 rads. The irradiated cells were then split into four portions and added in duplicate to culture wells containing 10^4 inoculates or TT-reactive T cells and 10^5 irradiated APCs pulsed with MBP or TT in proliferation assays to measure the inhibitory effect. Percent inhibition was measured as [1 − (proliferation in the presence of irradiated responding T cells as inhibitors/proliferation in the absence of the inhibitor)] × 100%. Cultures exerting more than 60% inhibition on the proliferation of the inoculates were considered as responding cell lines. The frequency was estimated by dividing the number of responding wells by the total WMNC plated (6×10^4 cells). The percentage of responding wells did not exceed 15% in each case.
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Cloning of an *M. tuberculosis* DNA Fragment Associated with Entry and Survival Inside Cells

Sergio Arruda, Gloria Bomfim, Ronald Knights, Tellervo Huima-Byron, Lee W. Riley*

Mycobacterium tuberculosis infects one-third of the world's human population. This widespread infection depends on the organism's ability to escape host defenses by gaining entry and surviving inside the macrophage. DNA sequences of *M. tuberculosis* have been cloned; these confer on a nonpathogenic *Escherichia coli* strain an ability to invade HeLa cells, augment macrophage phagocytosis, and survive for at least 24 hours inside the human macrophage. This capacity to gain entry into mammalian cells and survive inside the macrophage was localized to two distinct loci on the cloned *M. tuberculosis* DNA fragment.

Mycobacterium tuberculosis, the etiologic agent of tuberculosis (TB), produces chronic asymptomatic infection in most people that it infects. It sustains chronic infection by entering and surviving indefinitely inside macrophages. In time, and in response to poorly understood cues, the organism proliferates, escapes the macrophages, and causes active TB in a subset of infected persons who become infectious. Resistance to oxidative killing (*1*), inhibition of phagosome-lysosome fusion (*2*), and formation of the so-called electron-transparent zone (ETZ) that impairs diffusion of lysosomal enzymes (*3, 4*) are some of the mechanisms that may explain the survival of *M. tuberculosis* inside macrophages. However, the specific bacterial factors responsible for these properties have not been characterized.

Mycobacterium tuberculosis can invade HeLa cells, which are not phagocytes (Fig. 1) (*5*). Bacterial factors alone are, there-

S. Arruda, G. Bomfim, R. Knights, L. W. Riley, Division of International Medicine, Cornell University Medical College, New York, NY 10021.
T. Huima-Byron, New York Blood Center, New York, NY 10021.

*To whom correspondence should be addressed.

fore, sufficient for M. *tuberculosis* to gain entry into mammalian cells. To identify the M. *tuberculosis* DNA sequences that encode mammalian cell entry, we constructed a genomic library of M. *tuberculosis* H37Ra strain [American Type Culture Collection (ATCC) 25177] in pBluescript. A nonpathogenic *Escherichia coli* strain XL1-Blue was transformed with the recombinant vectors, and the transformant strains were screened for HeLa cell–invasive clones by a method similar to that described by Isberg and Falkow (*6*, *7*).

One *E. coli* transformant, XL1-Blue(pZX7), which harbored a plasmid (pZX7) containing a 1535-base insert in the Bam HI–Eco RI restriction enzyme sites of the pBluescript vector, was found by the screening procedure to associate consistently with HeLa cells. We confirmed by transmission electron microscopy that this clone entered HeLa cells (Fig. 1). Its internalization by HeLa cells was time-dependent (Fig. 1B), with intracellular organisms visible as early as 3.5 hours after infection. Some phagosomes contained multiple organisms (Fig. 1C), which suggested that the bacteria proliferated intracellularly. Some of the internalized bacilli were surrounded by a distinct ETZ, similar in appearance to the clear zone surrounding M. *tuberculosis* inside HeLa cells (Fig. 1A, arrow). Whether this zone represents the ETZ often seen around other pathogenic intracellular mycobacterial organisms (*3*, *4*, *8*) or is an artifact of the preparation is not clear.

Nonpathogenic *E. coli* XL1-Blue strains containing the vector pBluescript or another pBluescript-derived recombinant vector (pZN7) showed no association with HeLa cells after 7.5 hours. To demonstrate that the invasive phenotype was indeed encoded by the cloned M. *tuberculosis* DNA fragment, we transformed other nonpathogenic *E. coli* strains, specifically HB101, DH5α, and NM522, with pZX7. The constructs HB101(pZX7), DH5α(pZX7), and NM522(pZX7) were invasive for HeLa cells. A spontaneous loss of pZX7 on prolonged storage of XL1-Blue(pZX7) was associated with loss of the invasive phenotype. In addition, we tested four exonuclease III unidirectional deletion subclones of pZX7 and the subclones Bam HI–Pst I (pZX7.1), Pst I–HinD III (pZX7.2), and Bam HI–Eco RI (pZX7.7) for HeLa cell association (*9*) (Fig. 2). The strains of *E. coli* XL1-Blue harboring pZX7.3, pZX7.4, or pZX7.5 associated with HeLa cells in a pattern similar to that for *E. coli* XL1-Blue(pZX7), whereas the other subclones did not.

We infected monolayers of human macrophages with the *E. coli* recombinant clones (Fig. 3) (*10*). After 1 hour of infection, the percentage of cells infected by the recombinant clone (82 ± 8%) was more than five times that of cells infected by XL1-Blue(pBluescript) (15 ± 6%, $P < 0.0001$) (Table 1). This observation suggests that the cloned M. *tuberculosis* DNA sequences facilitate bacterial uptake at quantities above the background phagocytic activity of the macrophage cells. After 24 hours of infection, 12% (± 10%) of the macrophages exposed to XL1-Blue(pBluescript) and 60% (± 13%) of the cells exposed to XL1-Blue(pZX7) were infected ($P < 0.001$). After 24 hours bacilli were more numerous inside the cells and compartmentalized, surrounded by multiple layers of a membrane presumably of host origin (Fig. 3B). No bacteria could be seen inside macrophages infected with *E. coli*(pBluescript) after 24 hours. Culture of the lysate of macrophages that had been infected for 24 hours showed that the intracellular *E. coli* XL1-Blue(pZX7) strains were viable (Table 1).

We compared the capacity of XL1-Blue(pZX7), XL1-Blue(pBluescript), and one HeLa cell–invasive deletional derivative, *E. coli* XL1-Blue(pZX7.3), to infect macrophages (Table 1). At 1 hour of infection, the invasive capacity of *E. coli* XL1-Blue(pZX7.3) was four times that of XL1-Blue(pBluescript) ($P < 0.001$), but by 24

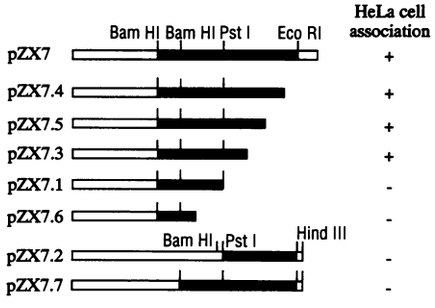

Fig. 2. Unidirectional deletional subclones (pZX7.3, pZX7.4, pZX7.5, and pZX7.6) and Bam HI–Pst I (pZX7.1), Pst I–HinD III (pZX7.2), and Bam HI–Eco RI (pZX7.7) subclones were constructed from the original vector pZX7 [see (*9*)]. The black bars represent the *M. tuberculosis* DNA sequences, and the white bars represent pBluescript sequences. We transferred the subclone vectors into *E. coli* XL1-Blue and then incubated these transformed strains for 6 hours with a HeLa cell monolayer.

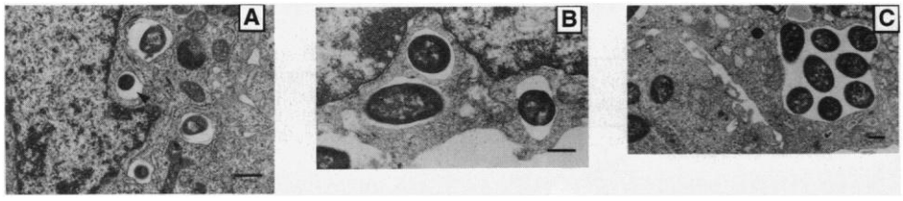

Fig. 1. Thin-section electron micrograph of HeLa cells infected with *M. tuberculosis* strain H37Ra (ATCC 25177) (**A**) and the invasive recombinant strain *E. coli* XL1-Blue(pZX7) (**B** and **C**). An ETZ surrounds *M. tuberculosis* organism [arrowhead in (A)]. The cells were incubated with *M. tuberculosis* strain for 72 hours in (A) and with XL1-Blue(pZX7) for 7.5 hours in (B) and (C). Multiple organisms are visible in (C), suggesting bacterial proliferation inside phagosomes. Bars represent 0.5 μm.

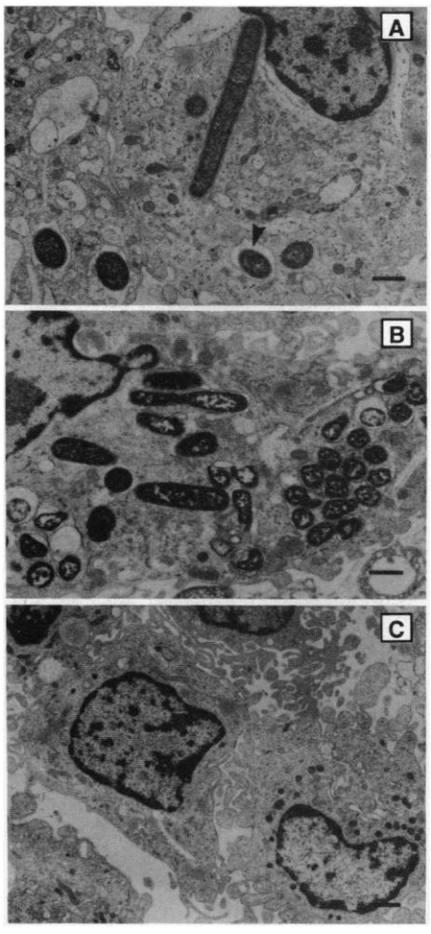

Fig. 3. Thin-section electron micrograph of human macrophages exposed to the invasive recombinant *E. coli* clone XL1-Blue(pZX7) for 3 hours (**A**) and 24 hours (**B**) compared with cells exposed to nonpathogenic *E. coli* XL1-Blue(pBluescript) for 24 hours (**C**). The bacteria become compartmentalized, surrounded by layers of membrane inside the macrophage (B). No bacteria were visible after 24 hours by electron microscopy in macrophages exposed to XL1-Blue(pBluescript). Bars represent 1 μm.

hours the difference was no longer apparent (Table 1). Thus, the DNA sequences associated with HeLa cell invasion are responsible for increased uptake by the macrophage, and the sequences that confer survival within the macrophage are located downstream of those necessary for mammalian cell entry.

The Bam HI–Eco RI DNA fragment comprised 1535 base pairs [European Molecular Biology Laboratory (EMBL) accession number X70901]. The sequence showed no homology with any of the DNA sequences in the database of GenBank (R72.0) or EMBL (R31.0). No obvious prokaryotic promoter consensus sequence could be discerned. If we assume that *M. tuberculosis* uses the common prokaryotic termination codon sequences, amino acid sequence homologies can be identified. A region near the NH_2-terminus of the deduced sequence of one potential open reading frame was found to share (i) 27% identity with an 80-residue NH_2-terminus region of internalin, a protein encoded by *Listeria monocytogenes* that is associated with mammalian cell entry (*11*); (ii) 20% identity with a 145-residue region of the *Ipa*H gene product of the invasiveness plasmid of *Shigella* (*12*); and (iii) 18% identity with a 176-residue region of human β-adaptin, a plasma membrane protein that links clathrin to receptors in coated vesicles which are responsible for receptor-mediated endocytosis (*13, 14*). When aligned against the invasin protein of *Yersinia pseudotuberculosis*, the region associated with cell entry was 19% identical with a 100-residue region near the invasin COOH-terminus (*15*). The functional significance of these alignments is not clear.

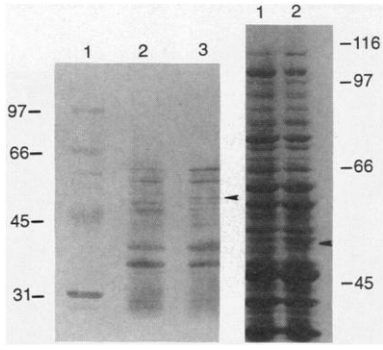

Fig. 4. SDS-PAGE of acetone-precipitated soluble fraction of bacterial cell sonicate. The polypeptides were analyzed in a 9% gel (**left**): molecular size standards (lane 1), *E. coli* XL1-Blue with a vector (pZN7) containing an unrelated *M. tuberculosis* DNA fragment between the Bam HI–Eco RI pBluescript cloning sites (lane 2), and XL1-Blue(pZX7) (lane 3). Analysis in an 8% gel (**right**): XL1-Blue containing a vector (pZX7.8) with a two-base frameshift introduced 12 bases upstream from the Bam HI cloning site in pZX7 (lane 1) and XL1-Blue(pZX7) (lane 2). Molecular sizes are indicated at the far right. We detected a 52-kD polypeptide in the soluble protein fraction of XL1-Blue(pZX7) (arrow). A protein of about 50 kD is expressed by XL1-Blue containing pZX7.8. The expression of the 52-kD protein was always associated with HeLa cell interaction of the recombinant *E. coli* clone.

A soluble fraction of the bacterial cell sonicate of XL1-Blue(pZX7) contained a 52-kD polypeptide that was not detected in the soluble fraction of XL1-Blue with a pBluescript-derived vector (pZN7) harboring an unrelated *M. tuberculosis* DNA fragment (Fig. 4) (*16*). A two-base frameshift, introduced by blunt-end ligation after the 5′ protruding end had been filled with Klenow DNA polymerase at the Xba I site 12 bases upstream from the Bam HI cloning site in pZX7 (confirmed by sequencing), led to loss of association with HeLa cells of the *E. coli* XL1-Blue containing this plasmid (pZX7.8). This clone did not express the 52-kD protein, but a new polypeptide of lower molecular mass was detected in the soluble fraction (Fig. 4). A spontaneous loss of the capacity to associate with HeLa cells after prolonged storage of XL1-Blue(pZX7) was accompanied by loss of the 52-kD protein. Hence, this 52-kD protein is likely to be a product expressed by the cloned *M. tuberculosis* DNA fragment. There were no detectable differences in the bacterial outer membrane polypeptide fractions.

It is not clear if the cloned 1535-bp fragment has more than one open reading frame or if a single gene product mediates both cell invasion and survival inside the macrophage. Drugs designed to target an *M. tuberculosis* product mediating macrophage survival, or vaccines against a product encoding mammalian cell entry, could contribute substantially to the worldwide TB control strategies.

Table 1. Light microscopy and lysate culture studies of human macrophage monolayer cells infected with the HeLa cell–invasive *E. coli* XL1-Blue(pZX7), subclone XL1-Blue(pZX7.3), and noninvasive XL1-Blue(pBluescript). The colony-forming units (CFU) were determined per milliliter of lysate culture. ND, not determined.

Exposure (hours)	Percentage of infected cells (mean ± SEM)			CFU per milliliter of lysate culture (mean ± SEM)	
	pBluescript	pZX7.3	pZX7	pBluescript	pZX7
1	15 ± 6	59 ± 10**	82 ± 8****	ND	ND
3	9 ± 4	ND	55 ± 17	1800 ± 500	3500 ± 1700
8	4 ± 2	ND	35 ± 5	10 ± 5	1600 ± 400
24	12 ± 10	23 ± 8*	60 ± 13***	3 ± 1	1300 ± 200

*$P > 0.05$, compared with pBluescript clone. **$P < 0.001$, compared with pBluescript clone. ***$P < 0.001$, compared with pBluescript or pZX7.3 clones. ****$P < 0.0001$, compared with pBluescript clone, $P < 0.05$ compared with pZX7.3 clone.

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6. The recombinant invasive clones were constructed as follows: *M. tuberculosis* H37Ra strain (ATCC 25177) genome was digested with restriction enzymes Sau3 AI and Eco RI, and the DNA fragments were ligated into the Bam HI–Eco RI restriction sites of a phagemid vector pBluescript II (Stratagene, La Jolla, CA). The recombinant vectors were introduced into *E. coli* XL1-Blue (Stratagene) by electroporation. We screened the recombinant strains for HeLa cell–invasive clones by a method similar to that described by Isberg and Falkow (*7*).
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9. We generated the unidirectional deletion subclones of pZX7 using exonuclease III according to the manufacturer's instruction (Erase-a-Base System, Promega, Madison, WI). The plasmid pZX7 was double-digested with HinD III and Kpn I restriction enzymes downstream from the Eco RI site of the Bam HI–Eco RI DNA insert to generate a 5′ protruding end adjacent to the insert and a four-base 3′ protrusion at the opposite strand to protect it from Exo III digestion. The digested plasmid was mixed with 300 U of Exo III at 37°C, and every 30 s 2.5-μl aliquots of the Exo III digestion were transferred to tubes containing S1 nuclease to remove the remaining single-stranded tails. The S1 nuclease was inactivated by neutralization and heating at 70°C for 10 min. We added Klenow DNA polymerase to create blunt ends which we ligated to circularize the deletion-containing vectors. The ligation mixture was then used to transform the competent *E. coli* XL1-Blue strain by electroporation.
10. Macrophage monolayers were established on glass cover slips at the bottom of polystyrene wells. They were initially infected with ~10 overnight-growth bacteria per macrophage cell for 1 or 2 hours followed by washing with phosphate-buffered saline (pH 7.4) and incubation for an additional 1, 6, or 22 hours. Cultures were performed at 37°C in RPMI-1640 medium (Gibco) with 2% AB heat-inactivated human serum containing gentamicin (10 μg/ml). The gentamicin was included to kill the extracellular bacteria. We washed the macrophage monolayer again and then lysed it with sterile, distilled water. The lysate was plated on tryptic soy agar medium to obtain colony counts. For microscopy, the macrophage monolayer was fixed with 100% methanol, stained with 10% Giemsa stain, and examined by light microscopy or processed for electron microscopy. The monolayer that was infected for 1 hour only was examined by light microscopy immediately after it was washed, fixed, and stained. The macrophage lysate culture and light microscopy results are shown in Table 1. We calculated the

percentage of infected macrophages from counts of infected macrophages per 100 to 200 macrophage cells on a cover slip monolayer. Each *E. coli* strain was tested four to six times for each time point, and the means of the percentages of the cells infected by the *E. coli* recombinant clone and the control strains XL1-Blue(pBluescript) and XL1-Blue(pZX7.3) were compared by Student's *t* test.
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16. Protein fractions analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) were prepared as follows: A 5-ml aliquot of bacterial overnight growth (adjusted to absorbance at 550 nm at optical density 600) in tryptic soy broth containing ampicillin (100 μg/ml) was harvested by centrifugation. We then sonicated the bacterial pellet in 1.5 ml of 10 mM tris-HCl buffer (pH 8.0) containing 5 mM $MgCl_2$. The sonicate was centrifuged for 25 min at 12,000 rpm in a microcentrifuge (Eppendorf model 5415C) at 4°C. Acetone was added to 600 μl of the supernatant in a fresh microcentrifuge tube (60%, v/v), and the mixture was centrifuged for 25 min at 14,000 rpm at 4°C. The pellet was resuspended in 20 μl of distilled water and 20 μl of Laemmli's boiling buffer, heated over boiling water for 5 min, and analyzed by SDS-PAGE. The bacterial debris containing the outer membrane fraction after the first centrifugation was resuspended in 100 μl of water and 100 μl of 15 mM tris-HCl buffer (pH 8.0) containing 7.5 mM $MgCl_2$ and 3% (v/v) Triton X-100 and centrifuged for 25 min at 14,000 rpm. We resuspended the pellet in 25 μl of water and 25 μl of boiling buffer and boiled it and analyzed a 20-μl aliquot of the sample by SDS-PAGE.
17. We thank J. L. Ho, W. D. Johnson, Jr., and M. Stoeckle for helpful suggestions and C. Johnson and B. Rubin for technical assistance. S.A. and G.B. were supported by a fellowship grant from Fogarty International Center (TW00018). L.W.R. is a Cornell Scholar in Biomedical Science and is, in part, supported by the Heiser Program in Leprosy and Tuberculosis and Pew Charitable Trusts.

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High-Resolution Conformation of Gramicidin A in a Lipid Bilayer by Solid-State NMR

R. R. Ketchem, W. Hu, T. A. Cross*

Solid-state nuclear magnetic resonance spectroscopy of uniformly aligned preparations of gramicidin A in lipid bilayers has been used to elucidate a high-resolution dimeric structure of the cation channel conformation solely on the basis of the amino acid sequence and 144 orientational constraints. This initial structure defines the helical pitch as single-stranded, fixes the number of residues per turn at six to seven, specifies the helix sense as right-handed, and identifies the hydrogen bonds. Refinement of this initial structure yields reasonable hydrogen-bonding distances with only minimal changes in the torsion angles.

For more than 50 years since the discovery of gramicidin A (gA), a three-dimensional structure has been sought for the cation channel that this peptide forms in a lipid environment. X-ray diffraction studies of gA crystals (produced by *Bacillus brevis*) derived from organic solvent have resulted in interesting structures (*1*), but these conformations are inconsistent with channel function. The gA sequence is a pattern of 15 alternating L and D amino acids with both end groups blocked. The folding motif was first proposed by Urry (*2*) to have β sheet–type torsion angles. Because of the alternating stereochemistry, all the side chains are on one side of the strand, thereby forcing the polypeptide into a helix, hence a β helix. This motif has been confirmed by solution nuclear magnetic resonance (NMR) in SDS micelles (*3*) and by solid-state NMR in phospholipids (*4–7*). The single-stranded pitch has been demonstrated by low-angle scattering (*8*), and the formation of a dimeric NH_2-terminus to NH_2-terminus junction has been determined by ^{13}C and ^{19}F solution NMR (*9*). Considerable spectroscopic data appears to support 6.3 residues per turn (*3, 7*). The helix has been shown to be right-handed in micelles (*3*) and lipids (*4*). Many computational efforts modeling the gA channel are consistent with this conformation (*10, 11*).

Solid-state NMR takes advantage of the lack of isotropic molecular motions. The orientation dependence of the nuclear spin interaction tensors can be used to generate orientational constraints. If the orientation of the tensor is known with respect to the molecular frame, then the observed frequency from a sample aligned to the magnetic field will constrain the molecular frame orientation. If numerous sites and interactions are analyzed with respect to the same axis, the relative orientation of structural units (minimal units separated by φ, ψ, and ω torsional angles) can be determined. For gA, several research groups have used orientational constraints to study the backbone (*6, 7*) and side chains (*12*). With constraints based on ^{15}N chemical shifts (*4, 5, 13, 14*), ^{15}N-^{1}H and ^{15}N-^{13}C dipolar interactions (*14–16*), and ^{2}H quadrupole interactions (*14*) from uniformly aligned samples of isotopically labeled gA in dimyristoyl phosphatidylcholine (DMPC) bilayers (*17*), we determined the backbone structure and indole side chain conformations (Table 1 and Fig. 1).

The orientation dependence of the axially symmetric ^{15}N-^{1}H and ^{15}N-^{13}C dipolar interactions is

$$\Delta\nu_{obs} = \nu_{\|}(3\cos^2\theta - 1)$$

where $\nu_{\|}$ is the magnitude of the dipolar interaction and θ is the angle between the magnetic field and the unique axis of the dipolar interaction. The dipolar magnitude ($\gamma_N\gamma_C r^{-3}$) can be calculated from a knowledge of the internuclear distance, *r*, and the gyromagnetic ratios for ^{15}N (γ_N) and ^{13}C (γ_C). The N–C peptide bond length is well defined (1.32 Å), and we used the neutron diffraction amide N–H bond length of 1.024 Å (*18*). The unique axis of the dipolar tensors is the internuclear vector; thereby, an observed dipolar coupling constrains the molecular frame with respect to the magnetic field. The ^{15}N chemical shift interaction is represented by an asymmetric tensor. We obtained the tensor element magnitudes from observations of unoriented preparations of single-site ^{15}N-labeled gA. Many of the ^{15}N chemical shift tensors are oriented with respect to the ^{15}N–$^{13}C1$ dipolar interaction (*5, 19*). Thus, the ^{15}N

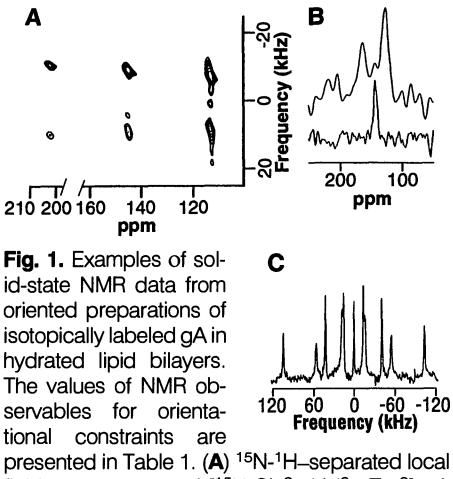

Fig. 1. Examples of solid-state NMR data from oriented preparations of isotopically labeled gA in hydrated lipid bilayers. The values of NMR observables for orientational constraints are presented in Table 1. (**A**) ^{15}N-^{1}H–separated local field spectroscopy of [^{15}N-Gly^2, -Val^8, -Trp^9] gA obtained as in (*30*). (**B**) ^{15}N spectrum at the top is that of [$^{13}C1$–Val^7, ^{15}N-Val^8] gA, and the bottom spectrum is that of [^{15}N-Val^8] gA. The dipolar splitting is symmetric about the chemical shift of 145 ppm. (**C**) Five ^{2}H quadrupole splittings and residual HOD are observed in this spectrum of [$^{2}H_5$–Trp^{13}] gA.

Institute of Molecular Biophysics, National High Magnetic Field Laboratory and Department of Chemistry, Florida State University, Tallahassee, FL 32306.

*To whom correspondence should be addressed.