Brief Definitive Report

Listeria monocytogenes Mutants Lacking Phosphatidylinositol-specific Phospholipase C Are Avirulent

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Summary

A number of bacterial species secrete phosphatidylinositol-specific phospholipase C (PI-PLC). In this report, we show that the facultative intracellular bacterial pathogen, *Listeria monocytogenes*, contains a gene, *plc*A, predicting a polypeptide with 31% amino acid identity to a *Bacillus thuringiensis* PI-PLC. Accordingly, *L. monocytogenes* secretes PI-PLC activity, while a mutant with a transposon insertion in *plc*A lacks detectable PI-PLC activity. In addition, expression of *plc*A in *B. subtilis* resulted in secretion of PI-PLC activity. The *L. monocytogenes* PI-PLC–defective mutant was three logs less virulent for mice and failed to grow in host tissues. The mutant was also defective for in vitro growth in mouse peritoneal macrophages. These results strongly suggest that PI-PLC is an essential determinant of *L. monocytogenes* pathogenesis. Whether the PI-PLC acts on a bacterial or host substrate remains to be determined.

Listeria monocytogenes, a facultative intracellular bacterial pathogen, has been extensively studied as a model for infections that are resolved by cell-mediated immunity. Recently, the cell biology of listerial intracellular parasitism has been documented and shown to involve entry of bacteria into the host-cell cytoplasm and cell-to-cell spread (1). Until now, the only proven virulence determinant of *L. monocytogenes* was a secreted pore-forming hemolysin, called listeriolysin O, which has been shown to mediate lysis of the host phagosome (1–3). In a recent study, we reported the isolation of a class of transposon mutants that were normal for hemolysin production and growth in tissue culture cell lines yet were defective for cell-to-cell spread (4). These mutants had insertions in a previously reported open reading frame (5), which we now designate *plc*A. In this report, we show that *plc*A encodes a secreted phosphatidylinositol-specific phospholipase C (PI-PLC), the loss of which results in the inability of the mutant bacterium to propagate in host tissues.

Materials and Methods

Bacterial Strains. *L. monocytogenes* 10403S (6) was the wild-type parental strain in these studies. Strain DP-L1054 is a Tn*917*-LTV3 insertion mutant isolated as a small-plaque former in tissue culture cells (4). Strain DP-L1044 contains a Tn*917*-LTV3 insertion within the listeriolysin O structural gene (4).

Construction of Bacillus subtilis Strain Expressing Phosphatidylinositol-Specific Phospholipase C. The *L. monocytogenes plc*A gene was cloned into an asporogenic strain of *B. subtilis* under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, essentially as described (2). The *plc*A gene, bases 279–1349 (5), was amplified from *L. monocytogenes* 10403S chromosomal DNA using PCR, and ligated into the IPTG-inducible promoter using SalI- and XbaI-generated DNA ends. The SalI and XbaI restriction sites of the PCR-amplified product were created as noncomplementary ends of the amplification primers. PCR primers used were 5′-GGG-TCGACTAGGAATAATATATGTTAGTTG-3′ and 5′-GGTCTAGA-TTCTAGTCCTGCTGTCC-3′ (Genosys Biotechnologies, Inc., The Woodlands, TX).

Assay for Phosphatidyl-inositol-Specific Phospholipase C Activity. Culture supernatants of *L. monocytogenes* strains were prepared by overnight growth to stationary phase (OD_{600} = 2.1) in brain heart infusion (BHI) broth at 38°C with aeration and from *B. subtilis* by growth at 37°C with aeration to early stationary phase (OD_{600} = 1.5) in BHI supplemented with 1 mM IPTG. Aliquots of supernatant fluid were then assayed for PI hydrolysis activity essentially as described (7) except that [^{3}H-inositol] PI, 0.045 μCi, 17.4 Ci/mmol (Amersham Corp., Arlington Heights, IL), and 13.5 μg PI (Sigma Chemical Co., St. Louis, MO) were sonicated in 100 μl of 40 mM Tris-HCl, pH 7.2, and 0.2% deoxycholate (per assay), and the PI-sonicate was then incubated with culture supernatant in a final volume of 200 μl at 37°C.

Chromatography of Phosphatidylinositol Hydrolysis Products. The lipid product of PI (Bovine liver; Sigma Chemical Co.) hydrolysis was chromatographed on Silica gel 60 (E. Merck, Darmstadt, FRG) TLC plates in petroleum ether/ethyl ether/acetic acid, 50:50:1 (vol/vol/vol), and also in hexane/ethyl ether/formic acid 9:6:0.4 (vol/vol/vol). The water-soluble product of PI hydrolysis obtained after partitioning the chloroform-methanol phase with either water or 4% TCA was chromatographed on cellulose (E. Merck) TLC plates in *n*-propanol/NH_4OH/H_2O; 5:4:1 (vol/vol/vol) or in 95% ethanol/13.5 M NH_4OH, 3:2 (vol/vol). The acidified product was also chromatographed on an anion exchange column (8).

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LD_{50} and Growth in Liver and Spleen. LD_{50}s were determined after intravenous injection of BALB/c mice with bacteria grown overnight in Trypticase soy broth as described (6). Bacteria were diluted in PBS, pH 7.4, and 10^6 viable cells in 0.2 ml PBS were injected intravenously into 20 female $CB6F_1$ mice, 9 wk-of-age (Trudeau Institute Animal Breeding Facility, Saranac Lake, NY). After 0.5, 6, 24, and 48 h, groups of five mice were killed, and enumeration of *L. monocytogenes* in whole spleens and livers was performed as described (9).

Growth in Resident Peritoneal Macrophages. In vitro growth in resident peritoneal macrophages was performed as described (10).

Materials. Bacillus thuringiensis PI-PLC was the kind gift of Martin G. Low. *B. cereus* PI-PLC and IPTG were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. DL-*myo*-inositol 1,2-cyclic monophosphate and inositol-1-*P* were obtained from Sigma Chemical Co.

Results

Expression of Listeria monocytogenes Phosphatidylinositol-specific Phospholipase C. Comparison of the amino acid sequence of PlcA with sequences in the protein Identification Resource database (Release 24.0) using the FASTP algorithm (11) revealed extensive homology (31% identity) with a *B. thuringiensis* PI-PLC (12) (Fig. 1). The sequence homology between *plc*A and the *B. thuringiensis* PI-PLC suggested that *L. monocytogenes* may secrete a PI-PLC. Indeed, the wild-type strain, 10403S, secreted an activity that efficiently hydrolyzed PI, whereas the *plc*A mutant, DP-L1054, showed no secreted activity (Table 1). To demonstrate that *plc*A directly encoded PI-PLC, the entire *plc*A gene was cloned into *B. subtilis* which subsequently gained the ability to secrete PI-PLC into culture media (Table 1). Thus, *plc*A was necessary and sufficient for secretion of PI-PLC.

Confirmation of PLC activity was based on identification of the products of PI hydrolysis. The released diacyglycerol revealed identical TLC mobility with the product of PI hydrolysis by PI-PLC from *B. thuringiensis* and *B. cereus*, and similar mobility to that of diolein. The water-soluble product migrated with DL-*myo*-inositol 1,2-cyclic monophosphate on cellulose TLC in two solvent systems. After acidification, the water-soluble product migrated with inositol-1-*P* on cellulose TLC and showed the same elution pattern as inositol-1-*P* upon anion exchange chromatography, consistent with initial formation of the cyclic monophosphate derivative (8). These results conclusively demonstrate that the *L. monocytogenes* enzyme is a PLC.

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L  MYKNYLQRTLVLLLC-FILYFFTFPLGGK---AYS----LNNWNKPIKNSVTT 45
B  MSNKKLI--LKLFICSTIFITFVFALHDKRVVAASSVNELENWSK-------- 43

L  KQWMSALPDTTNLAALSIPGTHDTMSYNGDITWTLTKPLAQ----TQTMSLYQ 94
B  --WMQPIPDNIPLARISIPGTHDS------GTFKLQNPIKQVWGMTQEYDFRY 88

L  QLEAGIRYIDIR---AKDN-LNIYHGPIFLNASLSGVLETITQFLKKNPKETI 143
B  QMDHGARIFDIRGRLTDDNTIVLHHGPLYLYVTLHEFINEAKQFLKDNPSETI 141

L  IMRLKDEQNSNDSFDYRIQPLINIYKDYFYTTPRTDTSNKIPTLKDVRGKILL 196
B  IMSLKKE--YEDMKGAEGSFSSTFEKNYFVDPIFLKTEGNI-KLGDARGKIVL 191

L  LSENHTKKPLVINSRKFGMQFGAP---------NQ---VIQDDYNGPSVKTKF 237
B  L-----KRYSGSNESGGYNNFYWPDNETFTTTVNQNVNVTVQDKYKVNYDEKV 239

L  KEIVQTAYQASKADNKLFLNH--ISATSLT------FTPRQYAAALN----NK 278
B  KSIKDTM--DETMNNSEDLNHLYINFTSLSSGGTAWNSPYYYASYINPEIAND 290

L  VEQFVLNLTSEKVRGLGILIMDF-PEK---QTIKNIIKNNKFN  317
B  IKQ--KNPT-----RVGWVIQDYINEKWSPLLYQEVIRANKSLIKE  329
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Figure 1. Sequence comparison of *L. monocytogenes* PlcA polypeptide (*L*) and *B. thuringiensis* PI-PLC (*B*). A solid line indicates residue identity and a colon indicates a conservative substitution (GA, EDQN, ST, RKH, LIMVA, FYW). The *L. monocytogenes* PI-PLC sequence predicted by *plc*A uses a putative translational start site at base number 1247 in the sequence reported by Mengaud et al. (5).

Growth of Listeria monocytogenes Strains In Vivo. The PI-PLC mutant, DP-L1054, showed a severe defect in its ability to grow in a murine model of infection (Fig. 2). After intravenous inoculation, ~90% of both the mutant and the parental strains were killed in the liver, presumably in the Kupffer cells, during the first 6 h of infection. However, unlike the parental strain, which then grew logarithmically, the mutant showed no evidence of growth. In the spleen, the mutant showed reproducible evidence for a limited amount of growth, although this was considerably less than that seen for the parental strain. Importantly, this lack of growth in

Table 1. *Activity of Phosphatidylinositol Hydrolysis and LD_{50} in Mice*

Strain (species)	Genotype	Activity*	LD_{50}
		U ± SD	
10403S (*L. monocytogenes*)	Parental	2,635 ± 196	1.7×10^4
DP-L1054 (*L. monocytogenes*)	*plc*A::Tn*917*-LTV3	UD‡	1.4×10^7
DP-B1066 (*B. subtilis*)	Parental	UD	ND
DP-B1340 (*B. subtilis*)	+*plc*A	2,706 ± 214	ND

* Mean activity ± SD from triplicate assays, in units of nanomoles of product formed per hour per milliliter of supernatant. 1 ml of supernatant represented 2×10^9 CFU of *L. monocytogenes* or 2×10^8 CFU of *B. subtilis*.
‡ Undetectable.

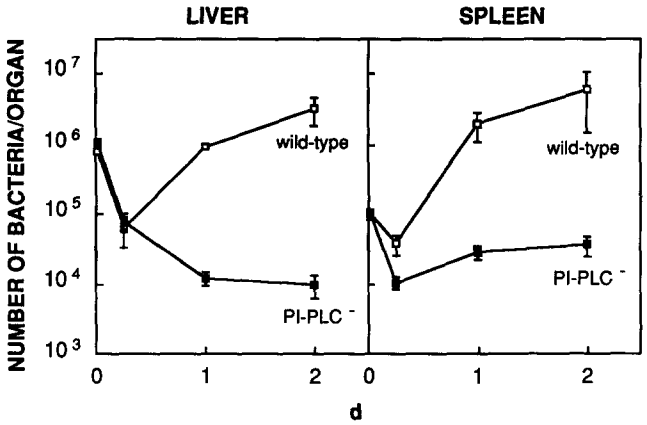

Figure 2. Growth of wild-type *L. monocytogenes* and DP-L1054 (PI-PLC⁻) in murine liver and spleen. Data points and error bars represent the mean and SD of the number of viable bacteria recovered from whole spleen or liver from five mice.

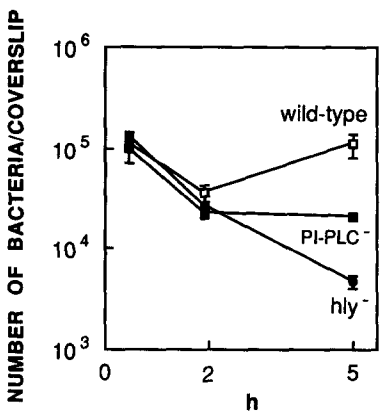

Figure 3. Growth of wild-type *L. monocytogenes*, DP-L1054 (PI-PLC⁻), and hemolysin-minus mutant DP-L1044 (hly⁻) in murine resident peritoneal macrophages. Data points and error bars represent the mean and SD of the number of viable bacteria recovered from three coverslips.

host tissues was reflected in a three-log increase in LD_{50} (Table 1).

Growth of L. monocytogenes in Mouse Peritoneal Macrophages. Growth of both the parental and mutant strains in vivo was mirrored in vitro in primary cultures of mouse resident peritoneal macrophages (Fig. 3). As previously reported (10) for the parental strain, ~70% of the infecting bacteria were killed by the macrophages followed by subsequent intracellular growth of the survivors. In contrast, a hemolysin-minus mutant, which fails to lyse the phagosomal membrane, showed a continual decline in viable count (Fig. 3). The PI-PLC mutant, DP-L1054, had an intermediate level of viability, but showed no evidence for bacterial growth (Fig. 3). However, microscopic examination of monolayers 5 h after infection with DP-L1054 revealed rare foci of infection resulting from growth of the bacteria in the cytoplasm (data not shown). The most likely explanation for these results is that the PI-PLC mutant was somewhat defective in escaping from the host phagosome although not to the extent seen in the hemolysin-minus mutant.

Discussion

A number of bacterial species secrete PI-PLCs, including *Staphylococcus aureus*, *Clostridium novyi*, *Bacillus cereus*, and *B. thuringiensis* (13). These enzymes have proven to be valuable reagents for the identification and characterization of a family of eukaryotic membrane proteins anchored to the cell surface by PI-glycan (13). However, a role for PI-PLC in bacterial growth or pathogenicity was previously unknown. The results of this study strongly suggest that the PI-PLC secreted by *L. monocytogenes* is an essential determinant of pathogenicity since a mutant with a transposon insertion in the structural gene encoding PI-PLC had reduced virulence and failed to grow in host tissues. Since *plcA* is monocistronic (5) it is unlikely that the mutation directly affects expression of other genes. In addition, an independent mutant with a transposon insertion in *plcA* had identical characteristics in tissue culture and the same increase in LD_{50} as DP-L1054. However, absolute proof for a role of PI-PLC in pathogenesis awaits fulfillment of a molecular version of Koch's postulates, by reintroducing *plcA* into DP-L1054 and showing that virulence is restored (14).

The major question that arises from this study is, what is the role of PI-PLC in pathogenicity and, more specifically, what is the biological substrate(s) for this enzyme? The results presented here indicated that PI-PLC is required for growth in host tissues which may reflect a role in lysis of the macrophage vacuole and, as previously shown (4), subsequent cell-to-cell spread. It is possible that PI-PLC acts in concert with hemolysin and perhaps a distinct PLC secreted by *L. monocytogenes* (15), to mediate entry into the host cytoplasm and to solubilize host membrane(s) during cell-to-cell spread. Thus, the biological substrates for *L. monocytogenes* PI-PLC may be host PI or PI-glycan in the phagosomal membrane and PI or its phosphorylated derivatives in the cytoplasmic membrane. Recognition of the latter substrates is an intriguing possibility because of their roles in signal transduction in eukaryotic cells (13). However, other bacterial PI-PLCs, although capable of hydrolyzing PI and PI-glycan, do not hydrolyze phosphorylated derivatives of PI. The precise substrate-specificity of the *L. monocytogenes* PI-PLC awaits its purification.

Lastly, it is formally possible that the listerial PI-PLC is acting on a bacterial substrate such as a PI-anchored bacterial protein. *L. monocytogenes* has been reported to lack PI (16), and there is no precedence for PI-anchored proteins in prokaryotes. Nevertheless, recent evidence has been provided for the presence of PI-anchored polysaccharides, the lipoarabinomannan and lipomannan in *Mycobacterium tuberculosis* (17). In addition, it has been pointed out that a number of surface proteins from Gram-positive cocci have a conserved hexapeptide with homology to the proposed cleavage and PI-glycan addition site found in some eukaryotic PI-glycan anchored proteins (e.g., the cellular adhesion molecules N-CAM and LFA-3) (18). Thus, there may be a prokaryotic version of a PI-anchor. Whether the *L. monocytogenes* PI-PLC acts on a host or bacterial substrate awaits further study.

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