Identification and Characterization of a Phospholipase D–Superfamily Gene in Rickettsiae

Patricia Renesto, Pierre Dehoux, Edith Gouin, Lhoussine Touqui, Pascale Cossart, and Didier Raoult

The completion of the sequencing of the genomes of both *Rickettsia conorii* and *R. prowazekii* provides the opportunity to identify putative virulence factors within these strictly intracellular pathogens. A role for a phospholipase A₂ (PLA₂) in rickettsial pathogenicity was hypothesized, but the corresponding gene has not been identified. We have identified a gene that encodes a putative phospholipase D (PLD) and that has been detected by Southern blotting in 11 analyzed strains of rickettsiae. The recombinant protein is dimeric and has PLD activity, as demonstrated by its capacity to release \(^{3}H\)-choline from phosphatidyl \(^{3}H\)-choline. This PLD is present in whole rickettsial lysates and likely is a virulence factor, because incubation of rickettsiae with an anti-PLD antibody reduced their cytotoxic activity against Vero cells. This enzyme might account for the activity previously attributed to PLA₂ and might be critical for the intracellular life of these bacteria.

Rickettsiae are emerging or reemerging arthropod-borne human pathogens characterized by their strictly intracellular location [1]. Because of the intrinsic difficulty in working with these bacteria and the lack of adequate methods for their genetic manipulation [2], there is a paucity of information regarding molecular mechanisms involved in their pathogenicity. The sequences of both *Rickettsia prowazekii* [3] and *R. conorii* genomes [4] are now completed, and virulence functions have been assigned to conserved protein families, by comparison with available databases. Among rickettsial genes thus suspected to be critical in causing disease, there is an open-reading frame (RC0909) coding for a protein that is likely to be involved in actin polymerization, as well as some hemolysin and invasin homologs [3, 4]. The presence of a type IV secretion system also is probable [5]. In contrast, no phospholipase-encoding gene was identified. This finding was intriguing, considering that the role for a phospholipase A₂ (PLA₂) in the penetration process has long been proposed for *R. rickettsii* [6, 7] and recently has been extended to *R. conorii* and *R. prowazekii* [8].

Phospholipases are enzymes characterized by well-known, specific active sites and by consensus sequences that are highly conserved within a family. By use of a sequence analysis program to identify all phospholipase signatures, we looked for such motifs among all the rickettsial proteins from the genome databases. This analysis allowed for the identification of only 1 protein that contains regions matching the phospholipase motifs, identified as phospholipase D (PLD). The purpose of our work was to express this protein, evaluate its enzymatic activity, and detect the expression of both mRNA and protein in rickettsiae. The role of the protein in rickettsial pathogenicity also was investigated.

**MATERIALS AND METHODS**

*Rickettsiae.* Rickettsiae were propagated in Vero cells, as described elsewhere [1]. The different strains used in the present study are listed in table 1.

**Sequence analysis.** Sequence analyses were performed on the mainframe computer at the Institut Pasteur.
Table 1. Rickettsial strains studied.

| Group, rickettsia | Strain                     |
|------------------|----------------------------|
| Spotted fever group |                          |
| R. australis     | Phillips                   |
| R. akari         | MK (=Kaplan) ATCC VR-148T  |
| R. bellii        | 369L42-1                   |
| R. canadensis    | 2678, ATCC VR-610T         |
| R. conorii       | Seven (Malish), ATCC VR-613T |
| R. felis         | California, ATCC VR-1525T  |
| R. helvetica     | C9P9, ATCC VR-1375T        |
| R. massilae      | Mtu1, ATCC VR-1376T        |
| R. rickettsii    | Sheila Smith, ATCC VR-149T |
| Typhus group     |                            |
| R. prowazekii    | Brein L, ATCC VR-142T      |
| R. typhi         | Wilmington, ATCC VR-144T   |

Figure 1. Multiple alignment of rickettsiae phospholipase Ds (PLDs) with Salmonella typhimurium PLD. Programs used in the analysis are described in Materials and Methods. Protein identifiers are as follows: RC1270, Rickettsia conorii PLD; RP819, Rickettsia prowazekii PLD; and STNuc, S. typhimurium PLD Q46707. The fourth line shows secondary structures that were inferred from crystal structure of S. typhimurium PLD (pdb 1BYR25), where “h” denotes the helix and “e” denotes the β strand. The open box represents a putative signal peptide, and double arrows represent motifs 1–4 of the PLD superfamily [19, 25]. The canonical HKD motif “HxK(x) 4D(x)6G” is underlined (motifs 3-HKD and 4). Conservative changes are in bold. The arrow indicates where the putative maturation site cleaved.
Figure 2. Phylogenetic tree of bacterial phospholipase D (PLD) superfamily. A global alignment of the 20 bacterial PLD sequences listed in table 2 was performed using CLUSTAL V software [11] and was used to generate an unrooted tree by use of the MEGA2 software package [32]. Nos. refer to bootstrap values for each node.

ACAGAATC-3′ and PLD/SmaI (5′ATAATACCCGGGCGTCGCGCTTTC-3′). For cloning purposes, engineered NcoI and SmaI sites were introduced to the forward and reverse oligonucleotide, respectively. In addition, 5–6 random nucleotides were also added to each end, to ensure efficient digestion. The resulting 532-bp insert was ligated with the pIVEX 2.3 vector (Roche), according to standard DNA cloning procedures [13]. This vector allows the expression of the PLD in phase with a C-terminal histidine (His) tag under the control of a T7 promoter. Cloning was verified by restriction mapping of the plasmid (Qiagen plasmid purification kit) and subsequent analysis on agarose gel. The ultimate proof for the fidelity of PCR and cloning was achieved by sequencing (ABI 310 automated sequencer; Perkin-Elmer). Expression of the protein was performed with the High Yield RTS 500 Escherichia coli Circular Template kit (RTS 500; Roche), on the basis of the principle of continuous exchange cell-free protein synthesis [14]. Reactions were performed at 30°C for 16 h, with a stirrer speed of 120 rpm, with 15 μg of selected plasmid used as the DNA template and in the presence of protease inhibitors (Complete Mini; Boehringer).

**Purification of recombinant His-tagged PLD.** The RTS sample was applied directly on a column previously filled with Ni-Nta resin (Qiagen) and was equilibrated (10 mL of 20 mmol/L Tris, 200 mmol/L NaCl, and 5 mmol/L imidazole; 10 mL of 20 mmol/L Tris, 200 mmol/L NaCl, and 20 mmol/L imidazole; and 5 mL of 20 mmol/L Tris, 200 mmol/L NaCl, and 50 mmol/L imidazole), specifically bound proteins were eluted (5 mL of Tris, 20 mmol/L HCl, 200 mmol/L NaCl, and 500 mmol/L imidazole). Eluted fractions were pooled, dialyzed overnight (50 mmol/L Tris), and concentrated (Amicon), to a final volume of 0.5 mL. His-tagged PLD was revealed in each fraction by use of a Penta-His antibody (Qiagen), and protein content was estimated by the Bio-Rad method.

**Antibodies.** Six-week-old female BALB/c mice (Charles River) were immunized intraperitoneally (ip) with 100 μg of purified PLD emulsified with complete Freund’s adjuvant. Two boosts with the same amount of antigen in incomplete Freund’s adjuvant were injected ip at 15-day intervals, and a third boost was administered intravenously 10 days after the second booster injection. The antiserum was affinity-purified by use of EAH-Sepharose 4B (Pharmacia) coupled to the purified PLD, according to the manufacturer’s instructions. Anti-PLD antibodies were eluted with 0.1 mol/L glycine (pH 2.5), dialyzed against PBS, and stored in PBS/50% glycerol. Serum levels of PLD-specific IgG were determined by ELISA. In brief, 96-well plates (Dynatec) were coated with 100 μL of purified recombinant protein diluted in PBS (10 μg/mL), incubated overnight at 4°C, washed, and blocked with 1% bovine serum albumin for 2 h at 37°C. After 3 washes, serial dilutions of serum were
Table 2. Bacterial genes compared in the phylogenetic analysis.

| Species                                      | GenBank accession no. | Name                  |
|----------------------------------------------|-----------------------|-----------------------|
| Arcanobacterium haemolyticum                 | AAA21882.1            | PLD                   |
| Buchnera aphicolaica                         | P57361                | Cardiolipin synthase  |
| Chlamydia trachomatis                        | F71550                | Probable endonuclease |
| Chlamydia muridarum                          | D81711                | PLD family protein    |
| Chlamydia pneumoniae J138                    | NP_200388             | Putative PLD          |
| Clostridium acetobutylicum                   | NP_348284             | PLD family protein    |
| Corynebacterium pseudotuberculosis           | AAA64910              | PLD                   |
| Corynebacterium ulcerans                     | AAB68401              | PLD                   |
| Escherichia coli                             | P39369                | Hypothetical protein yjhR |
| Helicobacter pylori                          | AAD07391              | Membrane-bound endonuclease |
| Mycoplasma pulmonis                          | NP_326496             | Cardiolipin synthetase|
| Photobacterium damselae                      | AAA27515              | PLD                   |
| Pseudomonas putida                           | P31048                | Putative cardiolipin synthetase |
| Ralstonia solanacearum                       | NP_520573             | Putative endonuclease protein |
| Rickettsia conorii                           | AAL03808              | PLD (this study)      |
| Rickettsia prowazekii                        | CA15244.1             | PLD (this study)      |
| Salmonella typhimurium                       | 1BYRA                 | PLD family member (Nuc) |
| Salmonella typhimurium LT2                   | NP_490494             | Putative PLD          |
| Streptomyces antibioticus                    | BAA03913              | PLD precursor         |
| Yersinia pestis                              | AAC82729              | Ymt murin toxin       |

**NOTE.** PLD, phospholipase D.

Applied to the plate in triplicate, and the plate was incubated at 37°C for 2 h. Goat anti–mouse IgG–peroxidase conjugate (Dako) then was added to each well, and, following 1 h of incubation at 37°C and extensive washings, the substrate (TMB Microwell; KPL) was added, and the absorbance at 450 nm was measured with a microplate reader (Bio-Rad). Under these conditions, the higher dilution of our affinity-purified antibody recognizing the recombinant protein was 1:500. Specificity of the antibody against rickettsial PLD was assessed by immunoblotting.

**Determination of phospholipase activity.** [3H]-PC (1,3 phosphatidyl[N-methyl-3H]choline, 1,2 dipalmitoyl; 86 Ci/mmol) was purchased from Amersham, and unlabeled phosphatidylcholine (PC) was obtained from Sigma. These phospholipids were evaporated to dryness under nitrogen and resuspended in 1 mL of Hanks’ balanced salt solution (final concentrations, 1 mmol/L PC and 10⁶ cpm/mL [3H]-PC). This suspension was sonicated for 5 min at room temperature to generate liposomes (solution A). rPLD (100 μg) was incubated overnight at 37°C in 450 μL of a buffer solution (50 mmol/L Tris, 80 mmol/L KCl, and 5 mmol/L CaCl₂ [pH 8]) to which 50 μL of the solution A was added. As a positive control, a commercial PLD (Type I PLD from cabbage; Sigma; final concentration, 20 U/mL) was incubated with the substrate, in the same conditions. The reaction was stopped by the addition of 1 mL of chloroform/methanol/HCl (50/50/0.5, vol/vol/vol). The amount of radioactivity and the composition of the aqueous phase was analyzed by thin-layer chromatography (TLC), as described elsewhere [15].

**Gel electrophoresis and Western-blot analysis.** Recombinant protein or rickettsiae purified on renographin gradient [16] were separated on 12% polyacrylamide gels and transferred to a nitrocellulose membrane (Amersham). Western-blot analysis was performed using either Penta-His antibody (1:1000; Qiagen) or the mouse PLD antibody (1:500) produced and purified as described above. After washing, the blots were both incubated with peroxidase-labeled anti-mouse antibodies (1:1000; Amersham) and detected by chemiluminescence.

**Inhibition of rickettsiae-induced cytotoxicity on Vero cells.** Vero cells grown in MEM supplemented with 4% fetal calf serum and 2 mmol/L l-glutamine, in microtiter plates, were inoculated with 3000 pfu of rickettsiae/well [17]. To examine whether affinity-purified PLD antibody could inhibit the cytotoxicity of rickettsiae, bacteria purified on sucrose gradient were incubated for 15 min at 4°C, with increasing dilutions of antibody, before incubation with Vero cells. After 7 days of incubation at 37°C in 5% CO₂, the cell culture supernatant was removed, and cell monolayers were incubated for 1 h at 37°C with 50 μL of neutral red dye (0.15% in saline [pH 5.5]). Dye not absorbed by the viable cells was removed by 2 washes with PBS (pH 6.5). Finally, the dye absorbed by the cells was ex-
Figure 3. A, Southern-blot analysis of rickettsial genomic DNA (5 μg each) digested with HindIII and probed with *Rickettsia conorii* phospholipase D (PLD). Lanes 1–9, spotted fever–group rickettsiae: lane 1, *R. akari*; lane 2, *R. australis*; lane 3, *R. bellii*; lane 4, *R. canadensis*; lane 5, *R conorii*; lane 6, *R. felis*; lane 7, *R. helvetica*; lane 8, *R. massiliae*; and lane 9, *R. rickettsii*. Lanes 10 and 11, typhus-group rickettsiae: lane 10, *R. prowazekii*; and lane 11, *R. typhi*. Estimated size markers are noted in kilobase pairs. B, Reverse-transcriptase (RT) polymerase chain reaction (PCR) analysis of a PLD transcript in *R. conorii* and *R. prowazekii*. RNA was purified from *R. conorii* (lane 1) and *R. prowazekii* (lane 2) and was processed with the Superscript One-Step RT-PCR system (Invitrogen) (left panel). Lane 3, RNA was replaced by water as a negative control. The right panel corresponds to the same experiment but with the RT/PLATINIUM Taq mix replaced by PLATINIUM Taq alone (Invitrogen). Molecular size is indicated on the left.

The addition of 100 μL of 10% ethanol in PBS (pH 4.2), and the optical density at 492 nm was measured with a microplate reader (Bio-Rad). Since the purified antibody was dialyzed against PBS/50% glycerol buffer, the results were expressed as the percentages of the cytotoxicity obtained with rickettsiae incubated with the same dilutions of buffer alone.

**RESULTS**

Identification of PLD superfamily protein in *R. conorii* and *R. prowazekii* genomes. A search of the phospholipase sequence, performed with a premade homology database of different phospholipase families [18], failed to identify a PLA₂-encoding gene. However, this analysis identified a putative rickettsial protein belonging to the PLD superfamily [19], which was detected by its 2 active site blocks [18]. This protein, encoded by the gene *RC127* and previously annotated as an unknown protein in the *R. conorii* genome [4], possesses the HKD motif (motif 3), which is conserved in all members of the PLD superfamily and is critical for biochemical activity [19–21]. In fact, the 4 conserved regions (motifs 1–4) defined within the PLD superfamily [19] were identified (figure 1). This gene was also found to be present in the *R. prowazekii* genome (RP819), with a high level of identity (74%). As illustrated in figure 1, the rickettsial *pld* genes encode for proteins with putative signal peptides of 28 aa. These sequences are shorter than those of other proteins of the PLD superfamily and are present in only 1 HKD domain. Most of the enzymes in the initially described PLD superfamily contained 2 copies of this consensus sequence, with few exceptions [19, 20], but BLAST searches of bacterial sequences identified other proteins containing only 1 HKD domain (e.g., *Ralstonia solanacearum*, *Clostridium acetobutylicum*, *Salmonella typhimurium* LT2, and *Helicobacter pylori*). When aligned with known bacterial PLD superfamily proteins to generate an unrooted phylogenetic tree (figure 2; table 2), these PLD proteins clustered with high bootstrap values. Most of the microorganisms found in this cluster are able to colonize eukaryotic cells, but phylogenetic analysis of these proteins is not congruent with that of the 16S rRNA [22].

Detection and transcription of *pld* in *rickettsiae*. Southern-blot analysis of genomic DNA demonstrated that the *pld* gene was present among all *Rickettsia* species tested (figure 3A,
Figure 5. Hydrolysis of phosphatidylcholine by recombinant phospholipase D (PLD). Recombinant or commercial PLD was incubated with [3H]-PC for 24 h. The release of [3H]-choline was determined by thin-layer chromatography analysis, as described in Materials and Methods. In control assays, the substrate was incubated with saline in the same conditions. Choline (C), phosphocholine (PCL), and glycerophosphocholine (GPC) were used as standards for migration. Data are representative results from 3 independent experiments. F, front migration; O, origin migration.

Figure 6. Evidence for phospholipase D (PLD) expression in rickettsiae. Rickettsia conorii (Rco) and R. prowazekii (Rpr) were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane subsequently probed with purified antibodies from recombinant PLD-immunized mice. Recombinant PLD (rPLD) was electrophoresed in parallel and identified with antihistidine antibody. Molecular size is indicated on the left. For both R. conorii and R. prowazekii, PLD mRNAs were successfully amplified by RT-PCR (figure 3B), demonstrating that the pld gene is expressed in these 2 species.

Expression and activity measurement of the rickettsial rPLD. Despite several efforts (transformation of E. coli BL21 (DE3)pLysS or BL21-CodonPlus (both from Invitrogen) and transfection of High Five cells), we failed to express the R. conorii PLD in vivo. In contrast, expression of this rickettsial protein was successful when we used a recently developed in vitro expression system (RTS 500; Roche). As illustrated in figure 4A, 2 bands of 24 and 20 kDa in length were recognized by anti-His antibodies and resulted from protease cleavage. Indeed, the N-terminal sequence of the lower band was SKVST–FTPAG–TKFIANQ (dashes correspond to expected cysteines), indicating a cleavage after the arginine residue. The addition of a protease inhibitor cocktail and a low temperature failed to prevent cleavage (figure 4A, lane 2). To determine whether the R. conorii PLD was monomeric, the purified sample was electrophoresed under nondenaturing conditions. As shown in figure 4B, 5 different molecular weight bands were observed. As with denaturing conditions, the full-length (24 kDa) and the truncated (20 kDa) monomer were present. In addition, 3 other bands, of 48, 44, and 40 kDa in length, respectively, were detected. These bands are likely to correspond to dimers composed of (1) 2 intact monomers (2 × 24 kDa), (2) an intact monomer plus a truncated one (24 kDa + 20 kDa), or (3) 2 truncated monomers (2 × 20 kDa). Finally, we examined whether this protein was endowed with PLD activity. Data showed that incubation of purified rPLD with [3H]-PC led to the generation of water-soluble radioactivity. Further TLC analysis of the water-soluble fraction showed a major radioactive peak, which coincided with that produced by commercial PLD (figure 5). Both peaks comigrated with the choline standard, proving that the recombinant protein exhibits PLD catalytic activity.

Detection of PLD in R. conorii and R. prowazekii and inhibition of rickettsiae-induced cytotoxicity with anti-PLD antibody. A polyclonal antibody directed against rickettsial PLD was obtained after immunization of mice, with the recombinant protein, and affinity-purification of the resulting antiserum. This antibody confirmed that PLD was present in the whole rickettsial lysates (figure 6A). The protein migrated in polyacrylamide gels to the same molecular size as the rPLD (figure 6B). Rickettsial extracts exhibited 2 bands, recognized by the anti-PLD antibodies, with the same molecular size as that observed for recombinant proteins that reacted with anti-His antibodies.

When R. conorii or R. prowazekii was pretreated for 15 min with increasing titers of anti-PLD antibody and then added to Vero cells, cell cytotoxicity measured after 1 week of incubation was significantly inhibited (figure 7). This inhibition was antibody concentration dependent and reached 50% when the 1:20 dilution of antibody was used.

DISCUSSION
The present study has described the first phospholipase found in rickettsiae. In an effort to identify a phospholipase from the rickettsial genome, we detected the HKD motif, the signature of proteins belonging to the PLD superfamily [19, 20]. Southern-blot analysis demonstrated that this gene was present in
all rickettsiae tested and, most probably, in all Rickettsia species. Only 1 HKD motif is present in rickettsial PLDs. In this respect, and as evidenced by phylogenetic analysis, these enzymes are members of a new clade of PLD superfamily proteins.

The most common approach to investigation of the role of a protein is the gene knock out strategy, which, unfortunately, cannot be applied in rickettsiae [2]. Expression cloning is another pathway we have adopted to address the function of the identified protein. RT-PCR indicated that the pld gene was transcribed in both spotted fever–group and typhus–group rickettsiae. This was of importance when considering the difficulties encountered in the expression of this protein. In examining these results, we assumed that this protein was most likely expressed in rickettsiae. To circumvent the problem of toxicity impairing in vivo expression, the pld gene from R. conorii was expressed in vitro. This allowed us to investigate the biochemical and the biological activities of rickettsial PLD, which was shown to be dimeric. Two HKD signature motifs are required for catalytic activity [23, 24], and the 2 HKD domains form an active site. In fact, these enzymes can be active either as bilobed monomers or as dimers [25, 26]. It appears that the rickettsial protein belongs to the second category.

Antibodies directed against PLD were used to demonstrate its expression in both R. conorii and R. prowazekii. Both the full-length and truncated native PLDs were detected, and the truncated protein appeared to be more abundant. This cleavage may correspond to a processing or maturation step. The possible role of this enzyme in virulence is suggested by the capacity of PLD antiserum to reduce the cytotoxic effect induced by either R. conorii or R. prowazekii on Vero cells. The mechanism of this inhibitory effect has not been elucidated, and we are currently designing experiments to investigate when inhibition occurs.

In conclusion, the present study has been the first to identify and characterize a phospholipase in rickettsiae. This enzyme belongs to the PLD protein superfamily. The failure to identify a rickettsial PLA2 from the rickettsial genome associated with the limited specificity of previous experimental analyses raises the issue of whether PLD might exert the function attributed to PLA2 (i.e., a role in escape from the vacuole [6, 7]). Recently, rickettsial PLA2 was described as a possible virulence factor, on the basis of the ability of the p-bromophenacyl bromide to inhibit the release of free fatty acids from the host’s infected cells [8]. We observed that this compound also inhibited hydrolysis of [3H]-PC by PLD (66% inhibition at a concentration of 10 μmol/L). In rickettsiae, PLD has a predicted signal peptide and may be secreted. The best-characterized bacterial phospholipase virulence factors are secreted phospholipase Cs and PLAs. However, secreted PLDs are also likely to be essential virulence determinants [27]. Although further experiments will be required to fully define the role of rickettsial PLD, this protein evidently confers pathogenicity-associated characteristics. Rickettsial PLD shares some homologies with the murine toxin from Yersinia pestis, a dimeric PLD superfamily protein that is able to hydrolyze phosphatidylycholine [23] and that is involved in the survival of Y. pestis in the flea [28]. Rickettsiae are also transmitted to humans by arthropod vectors [1], such as ticks (R. conorii), lice (R. prowazekii), or fleas (R. typhi and R. felis). It is also possible that rickettsial PLD permits survival of rickettsiae within infected arthropods. This gene is also present in the symbiotic Buchnera species, and analysis of the Chlamydia trachomatis genome, another strictly intracellular bacterium, shows the presence of 6 paralogous PLD superfamily proteins [29]. This most probably results from a late gene-duplication event in this cluster. Intracellular parasites have small genomes and an evolutionary tendency toward further genomic reduction [30]. Because such genomes contain a larger proportion of essential genes than larger genomes [31], it may be hypothesized that multiplicity of the pld genes in chlamydiae is of functional importance. Because the association of intracellular bacteria with host cells evolved to optimize the survival, growth, and transmission of bacteria, we speculate that this protein is associated with the intracellular life of these bacteria, whereas its function might be different as bacteria replicate in vacuoles or in cell cytoplasm. Much more work is needed to clarify the normal and pathological roles of rickettsial PLD.

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