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Enzymatic characterization of *Chlamydophila pneumoniae* phospholipase D

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SUMMARY

Chlamydophila pneumoniae, an aetiological agent of respiratory infection, is also thought to play an immuno-pathogenetic role in atherosclerosis by contributing to inflammation and plaque instability. Phospholipase D (PLD) is an enzyme involved in lipid metabolism and may have a direct or indirect impact on virulence and the inflammatory response. Some aspects of the developmental cycle of *C. pneumoniae* suggest a direct implication of its PLD (CpPLD) in the pathogenesis, specifically by affecting the regulation of lipid metabolism and lipid exchange between *C. pneumoniae* and host cells. Our previous studies disclosed a specific anti-CpPLD antibody response in patients with acute coronary syndromes chronically infected with *C. pneumoniae*, and demonstrated that this antigen is a factor able to drive the inflammatory process in atherosclerosis. Due to the intriguing aspects of the CpPLD, the present study investigated CpPLD enzymatic activity of the protein and the two domains that include one HKD motif each polypeptide. Our results showed that CpPLD was able to synthesize the cardiolipin (CL) but unable to hydrolyze phospholipids. It was also observed that each single HKD motif has an independent CL synthetase activity. This enzymatic activity of CpPLD could be important in the inflammatory process within the atherothrombotic events.

KEY WORDS: Chlamydophila pneumoniae, Phospholipase D, Cardiolipin synthase, Motif HKD, Recombinant protein.

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INTRODUCTION

Chlamydophila pneumoniae is an obligate intracellular eubacteria with a biphasic developmental cycle and has been shown to infect and multiply in endothelial cells, smooth muscle cells, monocytes, macrophages and lymphocytes (Grayston *et al.,* 1990; Gaydos *et al*., 1996). *C. pneumoniae* has been associated with several acute and chronic respiratory infections, such as chronic bronchitis and chronic obstructive pulmonary disease (COPD), and cardiovascular diseases like coronary heart disease (CHD), acute coronary syndromes (ACS) and acute myocardial infarction (AMI) (Blasi, 2000; Libby *et al*., 2002).

During infection and inflammation, a wide range of alterations can occur, including alterations in lipoprotein metabolism, as represented in changes in lipid and lipoprotein plasma concentrations (Hardardóttir *et al.,* 1994). In particular, oxidized phospholipids have been shown to induce several pro-inflammatory proteins, and it was hypothesized that lipid oxidation products play a role in other chronic inflammatory disorders (Lee *et al*., 2000).

In several adverse conditions, the bacteria can adapt by the expression of a variety of virulence factors and by promoting new physical and metabolic states associated with bacterial persistence.

Phospholipid mediators are certainly key players in inflammation. Much interest in this field has focused on platelet activating factor, the ox-

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idation products of phospholipids and oxLDL (Ninio, 2005). The enzymes responsible for phospholipid metabolism are proteins included in the phospholipase D (PLD) superfamily (Selvy *et al*., 2011).

The PLD superfamily is composed of a group of proteins with various functions, yet all members contain a signature $HXK(X)_4D(X)_6G(X)_2N$ (HKD) motif. This superfamily includes prokaryotic and eukaryotic PLD, bacterial cardiolipin (CL) synthases (CLSs), phosphatidylserine synthases (PSSs), and bacterial endonucleases. PLD, CLSs, and PSSs, all contain two HKD motifs in the active site and catalyze reactions that synthesize or modify phospholipids. PLD catalyzes a phosphatidyl transfer reaction using primary alcohols as nucleophilic acceptors to produce phosphatidyl alcohols. In bacteria, the synthesis of CL is catalyzed by CL synthase through the condensation of two PG molecules, transferring a phosphatidyl group from one PG molecule to another producing CL and glycerol (Schlame, 2008).

Some aspects of the chlamydial developmental cycle suggest a direct implication of PLD enzyme in pathogenesis, particularly with lipid metabolism regulation and lipid exchange between chlamydiae and host cells (Belland *et al*., 2003).

Chlamydia trachomatis expresses several putative PLD proteins, two chromosomal PLDs and five in the variable region of the genome, called plasticity zones (PZ). It has been postulated that these enzymes could hydrolyze phosphatidylcholine, but their substrate specificity *in vivo* is unknown (Nelson *et al*., 2006).

The PLD of *C. pneumoniae* (CpPLD) contains 2 HKD motifs and is not homologous to other PLDs (Figure 1). However, despite some limited sequence similarity (26% of identity with Nuc chain A of *Salmonella typhimurium*, accession number pdb1BYR A), CpPLD preserves only short regions, including the catalytic site that is critical for protein folding (Ciervo *et al*., 2007; Mancini *et al*., 2009).

In our previous investigations, we observed that CpPLD is consistently expressed during the developmental cycle of *C. pneumonia* (early and late chlamydial development), and consequently CpPLD is sensed by the host as an antigenic target during infection by/exposure to this microorganism (Ciervo *et al*., 2007).

We have also generated recombinant CpPLD (rCpPLD) and have shown its specific antibody response in ACS patients with *C. pneumoniae* chronic infection (Mancini *et al*., 2009). Moreover, we demonstrated that this antigen is a factor able to drive the inflammatory process in atherosclerosis (Benagiano *et al.,* 2012).

In recognition of the putative critical role of CpPLD in the metabolism of *C. pneumoniae,* the aim of this study was to determine CpPLD enzymatic activity of the protein and the two domains (CpPLD-HKD1 and CpPLD-HKD2) that include one HKD motif each polypeptide.

MATERIALS AND METHODS

Cloning of cppld gene, cppld-HKD1 and cppld-HKD2 domains

DNA was obtained from purified elementary bodies of *C. pneumoniae* strain Parola by the commercial kit Nucleospin Tissue kit (Macherey-Nagel GmbH, Düren, Germany), using the standard protocol adapted for bacteria (Ciervo *et al*., 2007). The system used for the gene cloning and expression was Champion™ pET200 Directional TOPO® Expression Kit (Life Technologies, Monza, Italy). Appropriate sets of oligonucleotide primers were designed for the PCR amplification of *cppld* gene, *cppld*-HKD1 and *cppld*-HKD2 domains (Table 1). For *cppld* and *cppld*-KHD1 the nucleotide sequence

TABLE 1 - *Primers used for the gene cloning.*

Gene (bp)	*Protein (aa-aa)*	*Primer sequence (5'→ 3')*
Cppld (969 bp)	CpPLD (31-353)	CACCGACACTTTTAAGACTTTTTTAAAG GGGTCAGCTAATCACGCTGCTTCTTGCTCTT
Cppld-HKD1 (552 bp)	CpPLD-HKD1 (31-214)	CACCGACACTTTTAAGACTTTTTTAAAG AGCAAA TCAAGCAACTTGGATGGTT
Cppld-HKD2 (417 bp)	CpPLD-HKD2 (215-353)	CACCATGTTTGCTCTGACCCACTC GGGTCAGCTAATCACGCTGCTTCTTGCTCTT

encoding for the signal peptide was removed. PCR products were cloned in the pET200/D-TOPO vector (Life Technologies, Monza, Italy) and recombinant plasmids were used to transform competent cells of *Escherichia coli* BL21 (DE3) One Shot® (Life Technologies, Monza, Italy). Transformants were selected by nucleotide sequence analysis and the correct recombinant plasmids were used for protein expression.

Expression and purification of rCpPLD, rCpPLD-HKD1 and rCpPLD-HKD2

For the expression of the recombinant proteins, bacteria were exponentially grown in Luria broth at 37°C and induced with 0.5 mM IPTG for 1, 2 and 3 h to set up the time course of protein expression. Cells were harvested by centrifugation (6000 x g, 4°C). Aliquots of bacterial pellet and 1ml of supernatant sample for each point of induction were precipitated overnight at -20°C with three volumes of ice-cold acetone, and were analyzed by western blot.

For large scale production of protein purification, 1L of bacterial culture induced for 1 h was centrifuged and the pellet was resuspended in 20 ml of ice-cold lysis buffer (50 mM Tris-HCl, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8) in the presence of protease inhibitors (complete cocktail tablets, Roche Diagnostics, GmbH, Germany) and sonicated in ice. After sonication 1% SDS was added. The bacterial lysate was gently mixed at room temperature for 1h, and subsequently subjected to centrifugation at 15,000 x g for 30 min to remove cellular debris. The supernatant was filtered through a 0.45 µm pore size membrane and dialyzed overnight in 10mM phosphate-buffered saline (PBS), pH 7.0 with protease inhibitors at room temperature. The rCpPLDs carrying the His6 tag at their N-terminus were purified in native condition by nickel-nitrilotriacetic acid-agarose (Ni-NTA) affinity chromatography.

The dialyzed supernatant was supplemented with Ni-NTA resin and gently mixed at 25° C for 2 h. The lysate-Ni-NTA mixture was loaded into a 15-ml column and washed with 50 mM Tris–HCl, pH 8, 300 mM NaCl, 25 mM imidazole. The fusion protein was eluted from the column with 300 mM imidazole. All recombinant proteins were extensively dialyzed at 4°C in PBS, and stored at -70°C until used. The protein concentration was determined by the method of Bradford (Bradford, 1976).

Gel electrophoresis and western blot analysis

Purified rCpPLDs proteins were separated on 12% or 20% polyacrylamide gel (SDS-PAGE). Gels were stained with Comassie stain (BioRad Laboratories, GmbH, Germany) or transferred to a nitrocellulose membrane (Amersham).

Western blots were performed using a commercial mouse monoclonal antibody recognizing the His6-tag epitope (1:3000; Qiagen, GmbH, Germany). An anti-mouse IgG preparation conjugated to alkaline phosphatase (Sigma Chemical Co.) was used as secondary antibody at a concentration of 1:8000.

Phospholipase D and CL synthetase assays for enzymatic activity detection

Phospholipase D activity was performed *in vitro* using the Amplex® Red Phospholipase D assay kit (Life Technologies, Monza, Italy**)**, according to the manufacturer's protocol. A concentration of 50 mU/ml of purified PLD from *Streptomyces chromofuscus* (Sigma-Aldrich) and 200 µg of BSA (Sigma-Aldrich) were used as positive and negative controls, respectively. Fluorescence was measured with a fluorescence microplate reader using excitation at 530 and fluorescence detection at 590. Data were given as the mean ± SD of six determinations performed on three different experiments.

The activity of the CL synthetase of rCpPLDs was detected as previously described (De Siervo and Salton, 1973) using a modified protocol. The assay mixture contained 0.3 mM -phosphatidylglycerol [1-(3-sn-Phosphatidyl)-rac-glycerol] (Sigma-Aldrich, Milan, Italy), 200 mM tris-hydrochloride buffer, pH 7.0, and 0.25% Triton X-100 with 25, 50, 100, 200 and 500 µg for each protein and 200 µg of albumin from bovine serum (BSA) as negative control.

Solution and sample were added to produce a final volume of 0.2 ml. Samples were incubated at 37°C for 60 min, and then the reaction was terminated by the addition of chloroform, methanol, and water 65:25:4.

Mixtures were incubated at 4°C overnight, and later were vortexed for 10 s to extract lipids and then subjected to a brief centrifugation. The

upper phase was recovered and the chloroform phase evaporated. The lipids were dissolved in chloroform-methanol 10 ul (2:1, v/v) and separated by thin layer chromatography (TLC).

Thin layer chromatography

The TLC of phospholipids was performed using silica gel G plates (Merck-Millipore, Rome, Italy) with the following solvent systems: chloroform/methanol/water (65:25:4). The plate was washed the day before with isopopanol and then air-dried.

The plate was activated before use by heating for 40 min at 100°C and cooled. The eluent solution was chloroform, methanol and water 65:25:4 and 0.25% KCl.

The phospholipids, CL and phosphatidylglycerol (Sigma-Aldrich, Milan, Italy) were revealed using the molybdenum blue spray reagent (Sigma-Aldrich, Milan, Italy) (Dittmer and Lester, 1964).

RESULTS

Expression and purification of recombinant rCpPLDs proteins

In order to assess the enzymatic activity of the HKD groups of the CpPLD, we decided to investigate the whole CpPLD and the two HKD motifs individually, through the production of the 2 recombinant epitopes: rCpPLD-HKD1 (from aa 31 to aa213) and rCpPLD-HKD2 (from aa 214 to aa 353).

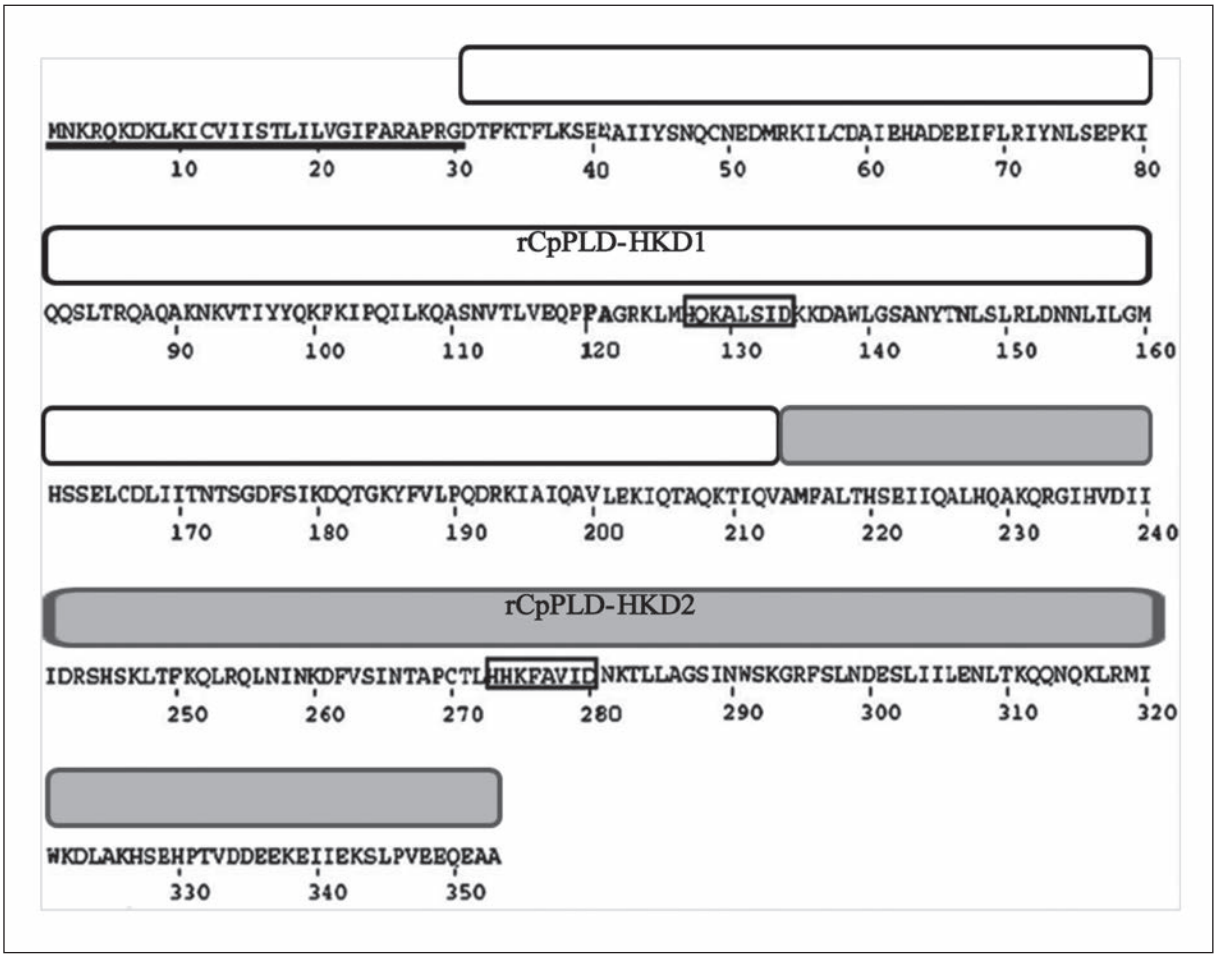

FIGURE 1 - *Secondary structure of CpPLD. The signal peptide is underlined and the 2 HKD motifs are boxed. The two rCpPLD-HKD1 and rCpPLD-HKD2 protein domains are also represented.*

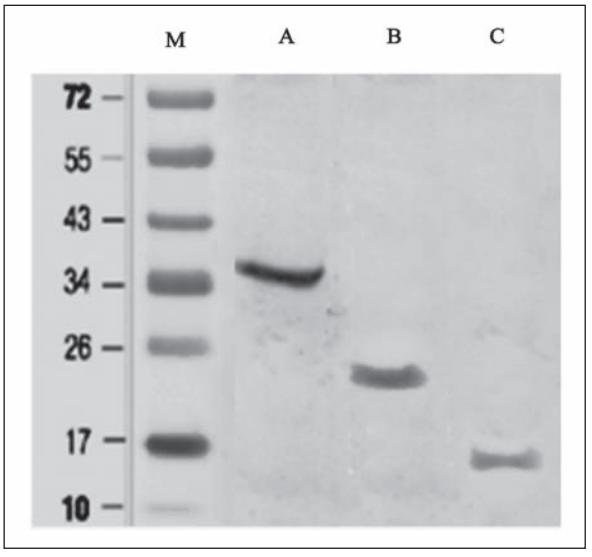

FIGURE 2 - *SDS PAGE Comassie stained of purified rCpPLDs. (A) rCpPLD, (B) rCpPLD-HKD1 and (C) rCpPLD-HKD2. The molecular weight protein standards (M) expressed in kDa are shown on the left.*

To prevent proteolytic digestion of the rCpPLD (locus tag CPn_0329) and rCpPLD-HKD1 proteins, the signal peptide consisting of the first 30 aminoacids was removed (see Figure 1). A time course of the recombinant protein expression in *E. coli*, at 1, 2 and 3 h after induction with 0.5 mM IPTG was assessed by western blot analysis of the cell lysate, using a commercial antibody recognizing the six histidine tail (His6-tag epitope) of the recombinant products. The proteins were seen to be mostly expressed during the first two hours of induction (data not shown).

Purification of the rCpPLDs by nickel affinity chromatography generated specific protein bands: the whole protein at 37 kDa, and the two recombinant epitopes rCpPLD-HKD1 and rCpPLD-HKD2 corresponding to 21 kDa and 16 kDa, respectively (Figure 2). These findings were also confirmed by western blot analyses through the commercial antibody His6-tag epitope (data not shown).

Enzymatic activity of the rCpPLD, rCpPLD-HKD1 and rCpPLD-HKD2

Purified rCpPLDs were analyzed with the Phospholipase D kit assay. The kit can be used to assay PLD enzymes with a near-neutral pH optimum. The PLD from *Streptomyces chromofuscus* was used as a positive control to check the PLD activity, while the BSA was utilized as a negative control. Different protein concentrations of rCpPLDs (100 µg, 200 µg, 500 µg, and 1000 µg) were analyzed in the PLD assay. Because the assay was continuous, the fluorescence was measured at multiple time points (30 min, 45, min, 60 min). In this assay any specific PLD enzymatic activity was detected with all recombinant proteins independently from their concentrations. Figure 3 reports the PLD assay at concentration of 200 µg for each recombinant protein as evidence of the PLD activity evaluation. The inability of all rCpPLDs to convert the phosphatidylcholine to choline indicated that the phosphatidylcholine was not the substrate for the rCpPLDs.

The CL synthase assay was carried out using PG as a substrate and 25 µg, 50 µg, 100 µg, 200 µg and 500 µg for each protein.

The total activity of the purified proteins was measured using TLC analyses. The TLC plate

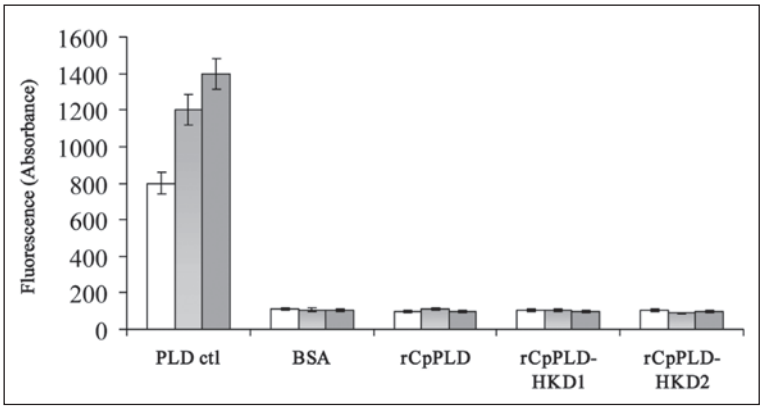

FIGURE 3 - *PLD activity measurement of rCpPLD, rCpPLD-HKD1 and rCpPLD-HKD2. A concentration of 200 µg, for each recombinant protein was used, while a 50 mU/ml of purified PLD from Streptomyces chromofuscus (PLD ctl) and 200 µg of purified BSA were utilized as positive and negative controls, respectively. The fluorescence was measured at 30 min (white), 45 min (grey), and 60 min (dark grey) for each reaction. Results are the means ± SD from six determinations.*

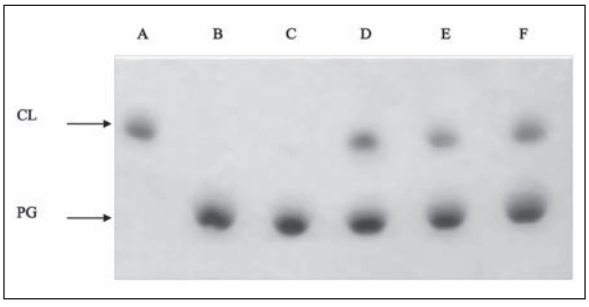

FIGURE 4 - *TLC of CL synthetase enzymatic assay at concentration of 200* µg for each protein. *The phospholipids, CL and PG were revealed by using molybdenum blue spray reagent. Lines: (A) CL standard, (B) PG standard; (C) PG standard + BSA, (D) PG standard + rCpPLD, (E) PG standard + rCpPLD-HKD1; (F) PG standard + rCpPLD-HKD2.*

spots show a lipid formation identified as CL by chromatography with a CL standard in the presence of rCpPLDs and the PG, at each protein concentration.
Figure 4 reports the CL synthase *"in vitro"* assay at a concentration of 200 µg for each rCpPLDs and BSA, as negative control. The TLC assay reveals a CL lipid co-migration for each recombinant protein in the presence of PG as substrate, while no CL migration was observed for the BSA. This finding demonstrates that all rCpPLDs show a CL synthase enzymatic activity independently from their concentration. Effectively, the whole protein and the two HKD domains were able to catalyze the CL reaction synthesis from PG.

DISCUSSION

Many bacterial PLA, PLC and PLD have been studied and are considered virulence factors for several microbial species that cause disparate disease syndromes (Schmiel and Miller, 1999).
All chlamydiae sequenced to date maintain at least one ancestral chromosomal PLD. Nevertheless, *C. trachomatis* (Carlson *et al*., 2005) and *C. muridarum* (Read *et al*., 2000) encode multiple PLD orthologs within the PZ hypervariable region, which is involved in the pathogenesis and in the specific tissue tropism (Nelson *et al*., 2005). The PZ region is absent in *C. pneumoniae, C. caviae* and in *parachlamydiae* (Read *et al*., 2003).
Overall, chlamydial PLDs could play a critical role during persistent infection, and in particular in those infections where lipid metabolism is affected and lipids are exchanged and modified with host cells. It is notorious that chlamydiae incorporate a number of lipids from the host cell pool, including phosphatidylcholine, CL and cholesterol (Nelson *et al*., 2007).
The CL synthase catalyzes CL biosynthesis from phosphatidylglycerol and is a PLD-type enzyme that shares the same key residues forming the catalytic site of these enzymes (Schlame, 2008). Bacteria contain variable amounts of CL depending on their physiologic state. Under certain conditions associated with growth reduction, the CL may increase to become the principal phospholipid (Shibuya, 1992).
The involvement of *C. Pneumoniae* in chronic diseases may be correlated to its ability to induce persistent forms in which the pathogen remains viable, but is not cultivable (Polkinghorne *et al*., 2006).
A study conducted in *Bacillus subtilis* demonstrated that CL is indispensable in the exponential phase and during vegetative growth (Kawai *et al*., 2004). This finding may support the hypothesis that CL could be an important constituent for chlamydial aberrant forms typical of chronic infection. From this point of view, the CpPLD protein might have a role in the progression of atherosclerosis and the sequelae of inflammation due to the interaction/development of foam cells (Ninio, 2005).
To address the issue of the possible role of CpPLD in the pathogenesis of chlamydial infection and its chronic persistent status, it was considered a priority to study of *cppld* gene expression and localization throughout the *C. pneumoniae* developmental cycle, and more important was CpPLD recognition by immune sera of the majority of ACS subjects with chronic *C. pneumoniae* infection. This aspect could reflect a concrete possibility that *C. pneumoniae* chronic or recurrent infections may lead to continuous stimulation of immune responses to CpPLD (Ciervo *et al*., 2007; Mancini *et al*., 2009). Moreover, CpPLD is able to drive Th17 inflammation within the atherosclerotic plaque, corroborating the importance of CpPLD in the progression of atherosclerosis with possible plaque rupture (Benagiano *et al*., 2012).

Another important aspect was also the characterization of the enzymatic activity of CpPLD. The crucial question in this regards was its possible function: phospholipase degradation or CL synthase. For this reason, our attention focused on the qualitative aspect of the enzymatic reaction and not on the quantitative observation that involves an enzymatic kinetics, that could be the object of future studies.

Our study directly demonstrated the CL enzymatic activity of rCpPLD, rCpPLD-HKD1 and rCpPLD-HKD2, and the catalytic conversion of PG to CL. On the other hand, our studies showed that the rCpPLDs were able to synthesize CL but not phospholipase degradation. This aspect may be important for the evaluation of the strain-specificity of chlamydial disease and the ability of *C. pneumoniae* to persist within the host eliciting potent and chronic inflammatory responses critical in the pathology of atherosclerosis (Hackstadt *et al.*, 1995; Carabeo *et al.*, 2003).

Sequence comparisons reveal a marked similarity between CpPLD and other cardiolipin synthase CLs and previous studies highlighted the importance of both HKD motifs for CL synthesis and catalytic activity (Selvy *et al.,* 2011; Schlame, 2008).

Here it was observed that each single HKD motif, HKD-1 or HKD-2, could have an independent CL synthetase activity, but obviously a HKD homodimer formation for both portions cannot be excluded.

It was shown that HeLa cells infected with *C. trachomatis* provided evidence of alteration in glycerophospholipid, PG and CL, increasing the ex novo synthesis of PG and CL in mitochondrial remodelling membrane, also linked to an elevation in mitochondrial metabolism role in this phenomenon (Hatch and McClarty, 1998).

CL is one of the principle phospholipids in the mammalian heart, a tissue particularly sensitive to oxidative stress and mitochondrial dysfunction. Modifications or alterations of CL by oxidative mechanisms are associated with cardiac disorders including ischaemia and heart failure. In addition, the development of anti-CL antibodies is associated with the onset of thrombosis (Shi, 2010).

C. pneumoniae chronic infection and in particular the catalytic process of CpPLD could play an important role in the pathological remodelling of CL in mitochondrial cardiac dysfunction and subsequent heart injury. Furthermore, the enzymatic activity of CpPLD may be important in the progression of atherothrombotic events and may represent a therapeutic target for the prevention and treatment of the disease.

Overall, much more work is needed to elucidate the biological proprieties of CpPLD protein in the *C. pneumoniae* life cycle and its possible pathogenetic role in host interaction, including immune modulatory activity, induction/progression of inflammation, and its potentially deleterious effects on the vessel wall.

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Author disclosure statement

No competing financial interests exist.

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