Cloning and Characterization of a Nonhemolytic Phospholipase C Gene from Burkholderia pseudomallei

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We cloned and characterized a phosphatidylcholine-hydrolyzing phospholipase C (PC-PLC) gene from Burkholderia pseudomallei. DNA sequence analysis of the gene indicated an open reading frame coding for 700 amino acids with a 34-amino-acid signal peptide. When cleaved, this yields a secreted 73-kDa mature protein. The deduced amino acid sequence exhibited 48% similarity to that of a nonhemolytic PLC from Pseudomonas aeruginosa. The expressed PC-PLC was heat stable, nonhemolytic for sheep erythrocytes, and active between pH 2 and 8. Western blot analysis with sera from melioidosis patients indicated that they produced immunoglobulin M antibodies against this PC-PLC protein.

Melioidosis is a potentially fatal disease that is endemic in northern Australia and in Southeast Asia (particularly in northeastern Thailand) (5, 6). The average incidence of human melioidosis in Ubon Ratchatani, northeastern Thailand during the period 1987 through 1991 was 4.4 cases per 100,000 inhabitants (22). The causative agent is a gram-negative bacillus, Burkholderia pseudomallei (formerly known as Pseudomonas pseudomallei) (25). The most common clinical manifestation of this disease is pneumonia. In severe cases, patients die within 48 h of the onset of symptoms (1).

Phospholipases of the C type (PLC) are enzymes which cleave the phosphodiester bond of phospholipids to yield diacylglycerol and a water-soluble phosphate ester. B. pseudomallei, unlike most gram-negative bacteria, produces a PLC which results in a zone of opalescence around colonies grown on egg yolk emulsion-supplemented agar (2, 9, 23). Previous studies have implicated PLCs as virulence factors involved in infections by pathogenic bacteria such as Listeria monocytogenes (16, 21), Clostridium perfringens (19), and Pseudomonas aeruginosa (14, 15). In L. monocytogenes pathogenesis, two different PLC enzymes play a role in escape of the pathogen from the phagosomal membrane and invasion of adjacent cells (16, 21).

This study characterized the gene encoding a nonhemolytic PLC from a strain of B. pseudomallei. The biological properties of the PLC and its similarity to a PLC from P. aeruginosa were examined.

Cloning and expression of the B. pseudomallei PLC gene. B. pseudomallei SpfI was isolated from the sputum of a patient by the Division of Bacteriology, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand. DNA was subsequently isolated by the method of Ausubel et al. (3). The isolated genomic DNA was digested with restriction enzymes (EcoRI, PstI, SacI, SacII, and XhoI), and the digests were hybridized with a PLC-specific probe. The PLC-specific probe (600 bp) was generated by using genomic DNA of P. aeruginosa as the template with PCR primers 5’ CGAC ATTCCCTACTAC 3’ (PLC-L) and 5’ CGCCGGCGGTGCT GAC 3’ (PLC-R), designed from the P. aeruginosa hemolytic PLC gene (GenBank accession no. M13047), nucleotide positions 455 to 470 and 1164 to 1179, respectively. The PCR was carried out on a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) for 35 cycles of melting (94°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 2 min). The PCR product was labeled with fluorescein-dUTP according to protocol of the Fluorescein Gene Images labeling system (Amersham International plc, Little Chalfont, Buckinghamshire, England). Hybridization was performed at 62°C with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (20) in hybridization buffer for 16 h. The stringent wash performed with 0.5× SSC containing 0.1% sodium dodecyl sulfate at 62°C, and the hybridized probe was detected by using the Fluorescein Gene Images detection system (Amersham). A 4.4-kb EcoRI DNA fragment of B. pseudomallei that hybridized with the probe was inserted into the EcoRI site of the pKSI (―) vector (Stratagene, Heidelberg, Germany) and introduced into Escherichia coli DH5α. The recombinant plasmid obtained was designated pSN-1, and the cloned fragment was designated SN-1. A restriction map of SN-1 is shown in Fig. 1. The assay used for detection of phosphatidylcholine-hydrolyzing PLC (PC-PLC) activity has been described previously (4, 10) and is based on enzymatic hydrolysis of p-nitrophenylphosphorylcholine (NPPC; Sigma Chemical Company) to liberate phosphatidylcholine and the yellow chromogenic compound p-nitrophenol. Only samples with PC-PLC activity turned bright yellow after incubation with the NPPC substrate (0.25 M Tris-HCl [pH 7.2], 6% glycerol, 1.0 mM ZnCl2, and 0.01 M NPPC). NPPC hydrolysis was detected in culture supernatants and cell lysates of E. coli carrying pSN-1 (data not shown), indicating that pSN-1 carried the PC-PLC gene from B. pseudomallei. PC-PLC activity was first detected at the beginning of exponential growth (2 h), and it increased continuously thereafter until the stationary phase (16 h) (data not shown). In every assay, positive and negative controls (E. coli harboring plasmids pDR540 and pKSI(―)), respectively) were included. Plasmid pDR540 (14) contained the gene encoding the hemolytic PLC from P. aeruginosa and was kindly provided by M. L. Vasili (University of Colorado Health Sciences Center, Denver).

The cloning vector pSN-1 contained an isopropyl-β-D-thio-galactopyranoside (IPTG)-inducible promoter. When induced by IPTG, the culture supernatants from E. coli harboring pSN-1 showed PC-PLC activity. Activity was also present with-
out IPTG induction (data not shown), suggesting that the SN-1 insert carried a promoter that was recognized by the E. coli RNA polymerase. When pSN-1 plasmid subclones were constructed and assayed for PC-PLC activity, only pSN-1a with insert SN-1a exhibited PC-PLC activity (Fig. 1). Thus, it appeared that the PC-PLC gene was located between EcoRV and EcoRI restriction sites.

DNA sequence analysis. The nucleotide sequence of the 3.6-kb EcoRV-EcoRI fragment SN-1a containing the PC-PLC gene was determined by using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Inc., Foster City, Calif.). Analysis of the data revealed an open reading frame of 2,100 bp which encoded a protein of 700 amino acids. A potential ribosome binding site, AGGAAG, was identified 7 bp upstream of an ATG codon. The gene for PC-PLC on fragment SN-1a had a G+C content of 68%, which closely resembled that estimated for chromosomal DNA from B. pseudomallei (69%) (18). A putative signal peptide sequence (34 amino acids in length) with a polar C-terminal region that ended with the sequence Ala-Leu-Ala, 9 amino acids after the hydrophobic core, was identified. Signal peptidase cleavage was expected to occur at this position.

Comparison of the putative amino acid sequence encoded by the PC-PLC open reading frame with amino acid sequence data deposited in the GenBank database revealed 48 and 44% similarity to the nonhemolytic and the hemolytic PLCs from P. aeruginosa, respectively.

Further comparison of the P. aeruginosa (13) and B. pseudomallei nonhemolytic PLCs revealed several similar properties. The entire proposed signal peptide from B. pseudomallei comprised 34 amino acids, close to the 35-amino-acid signal peptide in P. aeruginosa (13) and longer than that usual pro-caryotic signal sequences of 20 or 23 residues (8, 24). The B. pseudomallei sequence also contains the amino acid phenylalanine, as does the P. aeruginosa sequence but usually not other pro-caryotic signal sequences (13). In contrast, the predicted pI values of B. pseudomallei and P. aeruginosa nonhemolytic PLCs were quite different. That of P. aeruginosa was 8.8 (basic protein) (13), whereas that of B. pseudomallei was 6.7 (acidic protein). The difference could be explained by the smaller number of lysine and arginine residues in the B. pseudomallei PC-PLC (data not shown).

Biological properties of the PC-PLC protein. PLCs can be classified as hemolytic or nonhemolytic depending on their ability to lyse sheep erythrocytes. The hemolytic activity was tested as previously described (12). Culture supernatants and cell lysates of E. coli harboring pSN-1a did not lyse sheep erythrocytes but did hydrolyze NPPC to liberate a yellow chromogen (data not shown), indicating that pSN-1a encoded a nonhemolytic PC-PLC. The result also suggested that this enzyme cannot hydrolyze sphingomyelin because the major phospholipid components of the outer leaflet of the erythrocyte membrane are phosphatidylcholine and sphingomyelin.

FIG. 1. Restriction map of the plasmid clone pSN-1 and its derivatives. The presence (+) or absence (−) of PC-PLC activity is also indicated. Plasmids pSN-1a, -1b, -1c, and -1d were derived from pSN-1 by restriction enzyme digestion at the sites indicated in the map (in nucleotides) and cloned into pKSII(−).

FIG. 2. (A) Agarose gel electrophoresis of genomic DNA from various PLC-producing bacteria. (B) Southern blot of DNA from panel A, probed with the labeled 4.4-kb DNA insert from pSN-1. Lanes: 2, B. pseudomallei; 3, B. mallei; 4, B. cepacia; 5, P. aeruginosa; 6, L. monocytogenes; 7, C. perfringens; 8, Bacillus cereus. A standard lambda/HindIII DNA marker and Salmonella paratyphi A genomic DNA (negative control) were included in lanes 1 and 9, respectively. The positions of molecular size markers are shown on the left of both panels.
macrophages for years (17). The mechanism by which it survives within human phagocytes is not known. In L. monocytogenes, phosphatidylinositol-hydrolyzing PLC and PC-PLC have been shown to be virulence factors involved in intracellular survival and cell-to-cell spread (16, 21). B. pseudomallei PC-PLC might play a similar role. The demonstration that PC-PLC of B. pseudomallei is expressed in melioidosis patients and is active even under acidic conditions provides sufficient grounds for further studies on its possible role as a virulence factor.

Nucleotide sequence accession number. The complete nucleotide sequence of the PC-PLC gene of B. pseudomallei has been deposited in the GenBank database under accession no. AF107252.

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