Phosphatidylinositol-Specific Phospholipase C of Bacillus cereus: Cloning, Sequencing, and Relationship to Other Phospholipases

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The phosphatidylinositol (PI)-specific phospholipase C (PLC) of Bacillus cereus was cloned into Escherichia coli by using monoclonal antibody probes raised against the purified protein. The enzyme is specific for hydrolysis of the membrane lipid PI and PI-glycan-containing membrane anchors, which are important structural components of one class of membrane proteins. The protein expressed in E. coli comigrated with B. cereus PI-PLC in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as detected by immunoblotting, and conferred PI-PLC activity on the host. This enzyme activity was inhibited by PI-PLC-specific monoclonal antibodies. The nucleotide sequence of the PI-PLC gene suggests that this secreted bacterial protein is synthesized as a larger precursor with a 31-amino-acid N-terminal extension to the mature enzyme of 298 amino acids. From analysis of coding and flanking sequences of the gene, we conclude that the PI-PLC gene does not reside next to the gene cluster of the other two secreted phospholipases C on the bacterial chromosome. The deduced amino acid sequence of the B. cereus PI-PLC contains a stretch of significant similarity to the glycosylphosphatidylinositol-specific PLC of Trypanosoma brucei. The conserved peptide is proposed to play a role in the function of these enzymes.

Phosphoinositide-specific phospholipases C (PLCs) are important enzymes in the regulation of the cellular metabolism of eucaryotic cells. These enzymes have been shown to generate intracellular second messenger molecules in response to the binding of Ca2+-mobilizing hormones and certain growth factors to their target cells (1). Similarly, a PLC specific for the hydrolysis of phosphatidylinositol (PI)-derived glycolipids has been implicated in the mediation of insulin effects in hepatocytes and other tissues (25). Several eucaryotic PI-PLC isoenzymes have recently been cloned and sequenced (2, 12, 17, 28-30). The proteins appear to be highly conserved between species (rat and bovine), whereas isoenzymes in the same cell type display only limited homology. Two polypeptide stretches, denoted regions X and Y (24), which are conserved between isoenzymes have been suggested to play a role in the catalytic mechanism of these enzymes (17, 29, 30). The PI-PLCs from eucaryotic sources are not yet amenable to extensive biophysical and enzymological study because current purification schemes for these enzymes are fairly complex and yield only small amounts of protein.

In contrast, milligram quantities of enzyme can be prepared from cultures of Bacillus cereus (15, 18, 33) and from Bacillus thuringiensis (15, 21), Staphylococcus aureus (19), and Clostridium novyi (32), which secrete the enzyme across the bacterial membrane into the culture medium. These PI-PLCs recognize the inositol-phosphate structure present in PI, but do not hydrolyze the more highly phosphorylated derivatives of PI, PI-4-phosphate and PI-4,5-bisphosphate, which are involved in the signal transduction of calcium-mobilizing hormones. However, bacterial PI-PLCs have been reported to show insulinlike effects when incubated with insulin-sensitive membranes preparations or cells, such as hepatocyte membranes (25), myocytes (26), and hepatoma cells (22). Furthermore, the PI-PLC from B. cereus was shown to facilitate the release of some proteins from the surface of their resident membranes through hydrolysis of a novel PI-glycan-ethanolamine-containing anchor (GP1 anchor) similar to enzymes isolated from trypanosomes (12) and rat liver (9). In recent years, an increasing number of proteins, including acetylcholinesterase, alkaline phosphatase, several lymphocyte surface antigens (Thy-1 and Thy-3), and trypanosomal coat proteins (variant surface glycoprotein), have been found to be covalently linked to membranes by this PI-PLC-sensitive membrane anchor (8). These observations suggest that the study of bacterial PI-PLCs might contribute to our understanding of the role of their eucaryotic counterparts. The PI-PLC from B. cereus is specific for PI and PI-derived glycolipids and facilitates hydrolysis between the glycerol and the lipid head group to generate inositol-phosphate (and derivatives thereof) and diacylglycerol. The Bacillus enzyme has been characterized in terms of substrate specificity (15, 18), lipid-protein interactions (31), and its effect on PI-glycan-linked membrane proteins (reviewed in reference 20). However, a detailed correlation of enzyme function with structure was precluded by the lack of amino acid sequence data available.

As a step toward the investigation of structure-function relationships of PI-PLCs, we report the molecular cloning and nucleotide sequencing of the gene for the PI-PLC of B. cereus and expression of the protein in Escherichia coli. Amino acid sequence comparisons with other phospholipases, including eucaryotic PI-PLCs, are discussed.

MATERIALS AND METHODS

Materials. [α-35S]dATP (1,000 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, Ill. Sequenase 2.0 was obtained from U.S. Biochemical Corp., Cleveland, Ohio. Nitrocellulose filters were obtained from Schleicher & Schuell, Inc., Keene, N.H. Additional chemicals were procured from commercial sources and were of the highest grade available.

Bacterial strains and media. B. cereus ATCC 6464 was used to isolate the PI-PLC gene. E. coli host strains for
bacteriophage infection (BB4) and transformation (XL1-blue), bacteriophage λZAP, and helper phage (R408) were supplied by Stratagene Cloning Systems, San Diego, Calif. Media and transformation procedures were as detailed by the supplier. Transformants were grown on Luria broth (LB) plates, supplemented with tetracycline (25 μg/ml) and ampicillin (100 μg/ml) (LB-tet-amp).

**Assays.** Protein was determined by the Bradford (3) microassay procedure, with Bio-Rad dye reagent and bovine serum albumin as a standard.

**Cloning of the PI-PLC gene of B. cereus.** A commercial library of B. cereus chromosomal DNA in λZAP was prepared by Stratagene. EcoRI linkers were ligated to 5- to 10-kilobase (kb) fragments of chromosomal DNA obtained by shearing after methylation of internal EcoRI restriction sequences. After digestion with EcoRI, the fragments were ligated into λZAP. The resulting library of B. cereus DNA contained about 1.5 x 10⁶ recombinants. Phage clones were screened after infection of E. coli BB4 cells with eight PI-PLC-specific monoclonal antibodies and an alkaline phosphatase-conjugated secondary antibody (Stratagene) as recommended by the manufacturer and essentially as described by Huynh et al. (14). Plasmid DNA was prepared as described by Davis et al. (5). Partial deletions of the phagemid inserts with exonuclease III and mung bean nuclease were performed as recommended by the supplier (Stratagene).

**Characterization of PI-PLC activity in transformants.** E. coli transformed with the PI-PLC gene was grown to the stationary phase in LB-tet-amp and assayed for PI-PLC enzyme activity as described previously (33). Incubations with inhibitory monoclonal antibody A72-24 were performed for 30 min at room temperature after removal of the cells by centrifugation.

**Immunoblot analysis of B. cereus PI-PLC expressed in E. coli.** Cultures of transformed XL1-blue cells were grown at 37°C in LB-tet-amp for 44 h, well into the stationary phase. The enzyme activity detected under these conditions in the culture supernatant was precipitated with ammonium sulfate (at 90% saturation). The protein precipitate was redissolved in 0.5 x Tris-buffered saline and dialyzed against the same buffer. The yellow-brown material was then concentrated by centrifugation through Centricon 10 membrane (molecular weight cutoff, 10,000; Amicon Corp., Lexington, Mass.) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide mini-slab gel (Idea Scientific Co., Corvallis, Oreg.). Gel electrophoresis, staining, autoradiography, and immunostaining were performed by standard procedures.

**Edman degradation of cyanogen bromide fragments of B. cereus PI-PLC.** The PI-PLC gene (pA2-A55) contained about 1.5 x 10⁶ recombinants. Phage clones were grown in LB-tet-amp plates, and single colonies were transferred to liquid culture (LB-tet-amp), grown to high density, and assayed for PI-PLC enzyme activity. The two phagemids pA2 and pB2 conferred PI-PLC activity on XL1-blue cells, whereas no PI-PLC activity was measurable in cultures of untransformed E. coli cells transformed with pBluescript carrying no inserts.

To reduce the size of the insert, we isolated plasmid DNA corresponding to pA2 and partially deleted the plasmid from 8.5 kb to 4.9 kb by using exonuclease III and mung bean nuclease. The smallest plasmid which, after transformation into E. coli, still conferred PI-PLC activity on the host cell was designated pA2-A55. Double-stranded plasmid DNA from this clone was used for sequencing of the PI-PLC gene.

**Nucleotide and deduced amino acid sequence of PI-PLC.** The PI-PLC gene was sequenced by a primer-directed strategy in which primers were used that corresponded to the 5'-terminal section of already known nucleotide sequences. Sequencing inside the PI-PLC gene was started by using two degenerate oligonucleotides derived from the amino acid sequences of the enzyme as determined by Edman degradation of the N terminus of or of cyanogen bromide fragments of the protein (Fig. 1). Thereby, we identified coding sequences corresponding to the N terminus of the protein. Subsequent primers were synthesized to extend further into the insert and to generate overlapping pieces of sequence information covering the entire insert. By using this strategy, we read almost the entire length of the gene on both strands. Figure 1 shows the nucleotide sequence of the PI-PLC gene and the
FIG. 1. Nucleotide sequence and deduced amino acid sequence. The nucleotide sequence of the B. cereus PI-PLC gene and flanking regions and the translation of the gene in one-letter amino acid code are shown. Numbering of the nucleotides is shown to the left; numbering of the amino acids is shown to the right. Translation starts from the ATG at position 836 and continues until the first stop codon is encountered at nucleotide 1823 (TAA) to give a product consisting of a 31-amino-acid presequence and the mature PI-PLC of 298 amino acid residues. The first residues of the presequence (amino acid -31) and the mature protein sequence (amino acid +1) are marked by triangles. Nucleotide alterations in the PI-PLC gene of the related B. thuringiensis (11) between nucleotides 776 and 1844 in our numbering scheme are indicated above the nucleotide sequence. Peptide sequences obtained by Edman degradation of CNBr fragments are underlined. An inverted repeat near the 3' end of the gene is indicated by lines over the nucleotide sequence. Beyond nucleotide 1984, the sequence corresponds to that of the pBluescript vector. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession number M28549.
deduced amino acid sequence of the protein. The first termination codon encountered in the identified open reading frame was at position 1823. This open reading frame codes for a precursor protein containing a presequence of 31 amino acid residues at the N terminus of the mature protein. The mature protein consists of 298 amino acids. Molecular weights of 34,466 and 38,116 were calculated for the mature PI-PLC protein and the precursor, respectively. This is in excellent agreement with the apparent molecular weight of the enzyme (35,000) determined by SDS-PAGE (33). The deduced amino acid sequence and the experimentally determined sequence segments were in agreement. The leader peptide (prepeptide) conforms to the consensus structure (34) bearing a hydrophobic core of 16 amino acids flanked by charged residues. The signal sequence and mature enzyme are connected by a stretch of four relatively small residues [Val(-3) ... Ala(+1)]. The small residue size and the propensity to form a turn structure have been suggested to be structural requirements of a signal peptidase I recognition site (7). An inverted repeat structure which might serve as factor-independent transcription termination signal (4) was identified 13 nucleotides downstream from the termination codon.

Expression of B. cereus PI-PLC in E. coli. E. coli XL1-blue cells (Stratagene) were transformed with pA2, pB2, or pA2-Δ55, grown to saturation in LB-tet-amp broth, and assayed for PI-PLC enzyme activity (Table 1). The enzyme activity was not dependent on induction of the lac operon by isopropyl-β-D-thiogalactopyranoside (IPTG). This result suggests that the transcription-translation machinery of E. coli recognizes the regulatory sequences leading to expression of the PI-PLC gene in B. cereus. Enzyme activity produced by the transformants was inhibited by incubation of aliquots of the culture medium with monoclonal antibody A72-24, as was observed to be the case with the enzyme secreted from B. cereus (A. Kuppe, K. K. Hedberg, and O. H. Griffith, unpublished observation). We investigated by immunoblotting the biosynthesis of B. cereus PI-PLC in transformed E. coli cells. Polypeptides released from the cells were precipitated, separated by SDS-PAGE, and electroblotted to Immobilon PVDF membrane, and the blot was tested with the mixture of monoclonal antibodies used for cloning of the gene. A single polypeptide of apparent molecular weight 35,000 was recognized by the antibodies (Fig. 2). This polypeptide comigrates with the purified B. cereus enzyme in SDS-PAGE, indicating that processing of the precursor protein in E. coli and B. cereus leads to similar protein products.

**DISCUSSION**

This paper reports on the cloning and sequencing of the gene encoding the B. cereus PI-PLC. The gene was isolated from a genomic DNA library and prepared in the vector λZAP, and active enzyme was expressed in E. coli upon transformation with gene-carrying plasmid. Translated sequences were identified by comparison with stretches of amino acid sequence obtained by Edman degradation. The enzyme is probably synthesized as a larger precursor before being secreted across the bacterial membrane. Similar results have been reported for the two other secreted B. cereus PLCs (16, 35).

![FIG. 2. Immunoblot analysis of PI-PLC expressed in E. coli. E. coli XL1-blue cells carrying pA2-Δ55 were grown, and proteins were precipitated from the culture supernatant, separated by SDS-PAGE (12% acrylamide), and electroblotted as described in Materials and Methods. The blots containing polypeptides from pA2-Δ55 (lane a) untransformed XL1-blue (lane b), and purified PI-PLC (lane c) were probed with the mixture of eight monoclonal antibodies used for cloning of the gene.](image)

![FIG. 3. Sequence alignment with T. brucei GPI-PLC. The compared polypeptides are residues 47 to 107 of B. cereus PI-PLC (upper line) and residues 77 to 137 of the T. brucei GPI-PLC (12). Identical amino acids and conservative replacement are indicated by bars and dots, respectively.](image)
those differences (Leu-23, Ser-27, and Lys-135) were verified between the translations from these two PI-PLCs. Three of the eight amino acid substitutions are evident from B. thuringiensis homology to the PI-PLC as translated from the cloned gene. Alignment of PI-PLC with protein sequence data bank for entries showing sequence similarity to the peptide sequence of PI-PLC as translated from the cloned gene. The three PLCs are therefore unlikely to be part of the same transcriptional unit.

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We searched the National Biomedical Research Foundation Protein Sequence Data Bank for entries showing sequence similarity to the peptide sequence of PI-PLC as translated from the cloned gene. Alignment of PI-PLC with the PC-PLC (16) and SM-PLC (35) of B. cereus did not reveal overall or local regions of high similarity. However, the recently published nucleotide sequence of the PI-PLC from B. thuringiensis (11) displayed extensive sequence homology to the B. cereus enzyme at both the nucleotide and amino acid levels. Eight amino acid substitutions are evident between the translations from these two PI-PLCs. Three of those differences (Leu-23, Ser-27, and Lys-135) were verified by Edman degradation of the intact proteins purified from B. cereus and B. thuringiensis cultures or by sequencing of CNBr fragments of the B. cereus enzyme.

A comparison of the B. cereus PI-PLC sequence with several recently cloned eukaryotic PI-PLCs (2, 12, 17, 28-30) indicates that the trypanosomal enzyme (GPI-PLC) specific for hydrolysis of the variant surface glycoprotein from its PI-glycan-containing membrane anchor is most closely related to the B. cereus enzyme. The two enzymes are of comparable sizes (358 versus 298 residues) and align in the N-terminal third to give the highest scores of similarity. Two peptide stretches (Fig. 3), separated by 14 amino acid residues in both proteins, gave 37 to 38% sequence identity (up to 50% if conservative replacements were permitted) with no gaps in the alignment. A small central core (from Arg-56 to Arg-69 [14 residues]) of sequence was found to be conserved between the enzymes. In addition to the hydrolysis of PI, B. cereus PI-PLC has been shown to catalyze the release of certain membrane proteins from PI-glycan membrane anchor similar to trypanosomal GPI-PLC. The conserved peptide therefore might play an important role in the function of these lipases. The sequence similarity discussed here extends over two fairly short segments of polypeptide only. This is not surprising, however, because the genus Bacillus is not evolutionarily close to trypanosomes. A conservation of protein structure is therefore likely to extend over only small regions or individual residues crucial in catalysis or substrate binding or both. Furthermore, the conserved stretches of the trypanosomal phospholipase and the B. cereus enzyme also show some similarity to region X discussed by Khee et al. (24), which is one of two polypeptide

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stretches shown to be conserved between several PI-PLC isoenzymes cloned from rat and beef brain (17, 29, 30). We noticed a relatively high degree of similarity (26% identical, 52% including conservative replacements) of the B. cereus enzyme with sequences of other eucaryotic PI-PLCs in positions which are conserved between the eucaryotic enzy-mes (Fig. 4, boxed sequences). This observation further supports the hypothesis that the active site of PI-PLCs might reside in the conserved peptide X.

How does the conserved polypeptide stretch fit into the single high-resolution structure model of a PLC available to date (13)? Hough et al. (13) have examined a cocrystal of the enzyme with sequences of other eucaryotic PI-PLCs in positions which are conserved between the eucaryotic enzymes (Fig. 4, boxed sequences). This observation further supports the hypothesis that the active site of PI-PLCs might reside in the conserved peptide X.

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LITERATURE CITED


