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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 Ca^{2+}-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 membrane 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 (GPI 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-^{35}S]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

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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. *Eco*RI linkers were ligated to 5- to 10-kilobase (kb) fragments of chromosomal DNA obtained by shearing after methylation of internal *Eco*RI restriction sequences. After digestion with *Eco*RI, the fragments were ligated into λZAP. The resulting library of *B. cereus* DNA contained about 1.5×10^6 recombinants. Phage clones were screened after infection of *E. coli* BB4 cells with eight PI-PLC-specific monoclonal antibodies and an alkaline phosphatase-conjugated second 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× 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, electroblotting, and immunostaining were performed by standard procedures.

Edman degradation of cyanogen bromide fragments of *B. cereus* PI-PLC. PI-PLC purified from the culture supernatant of *B. cereus* by the method of Volwerk et al. (33) was digested with CNBr as described previously (10). The fragments were then separated by SDS-PAGE (20% acrylamide gel) and electroblotted on an Immobilon PVDF membrane (23). Gas-phase sequencing of the blotted polypeptides was performed by the University of Oregon Biotechnology Laboratory on a protein sequencer (model 470A; Applied Biosystems, Inc., Foster City, Calif.). Phenylthiohydantoin-amino acids were identified with an Applied Biosystems model 120 PTH analyzer.

DNA sequence analysis. Nucleotide sequences were determined by the dideoxy-chain termination method (27), using a primer-directed sequencing strategy and Sequenase 2.0 chemistry (U.S. Biochemical Corp.) on a double-stranded DNA substrate. Oligonucleotide primers were designed to match a stretch of the PI-PLC polypeptide known from amino acid sequencing of the protein or were derived from previously determined sequences. Primers were synthesized by the University of Oregon Biotechnology Laboratory or were from Stratagene (SK-primer).

Sequence analysis and homology search. Sequence plots and homology searches were performed by using University of Wisconsin Genetics Computer Group software as described previously (6).

RESULTS

Cloning strategy. Chromosomal DNA of *B. cereus* was fragmented by shearing, and fragments in the range of 5 to 10 kb were cloned into the *Eco*RI site of the phage vector λZAP. About 30,000 plaques were plated onto two NZYM plates, and filter lifts from the plates were screened with a mixture of eight monoclonal antibodies specific for *B. cereus* PI-PLC. All eight antibodies showed strong recognition of only the PI-PLC polypeptide in immunoblots of *B. cereus* proteins and did not cross-react with lysate of *E. coli* host cells (data not shown). Eight strongly positive plaques were identified on the filters; two of the plaques were subcloned to yield phage clones reproducibly positive in the antibody-screening protocol (14). Assuming that the average insert size is 7.5 kb and the genome of *B. cereus* is on the order of 3×10^6 to 1×10^7 base pairs, a quick calculation shows that a single-copy PI-PLC gene is expected to be identifiable in about 20 plaques in our experiment. This order of magnitude agrees well with the number of positive plaques observed experimentally. In the work described below, we took advantage of the λZAP sequences which facilitate automatic excision of the insert and some additional 3 kb of DNA from the phage to produce a phagemid (pBluescript) for transformation of *E. coli*. *E. coli* XL1-blue cells were transformed to ampicillin resistance with phagemids derived from the two positive phage clones. Transformants were grown on 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* or cells transformed with pBluescript carrying no insert.

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 phagemid which, after transformation into *E. coli*, still conferred PI-PLC activity on the host cell was designated pA2-Δ55. 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 3′-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 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

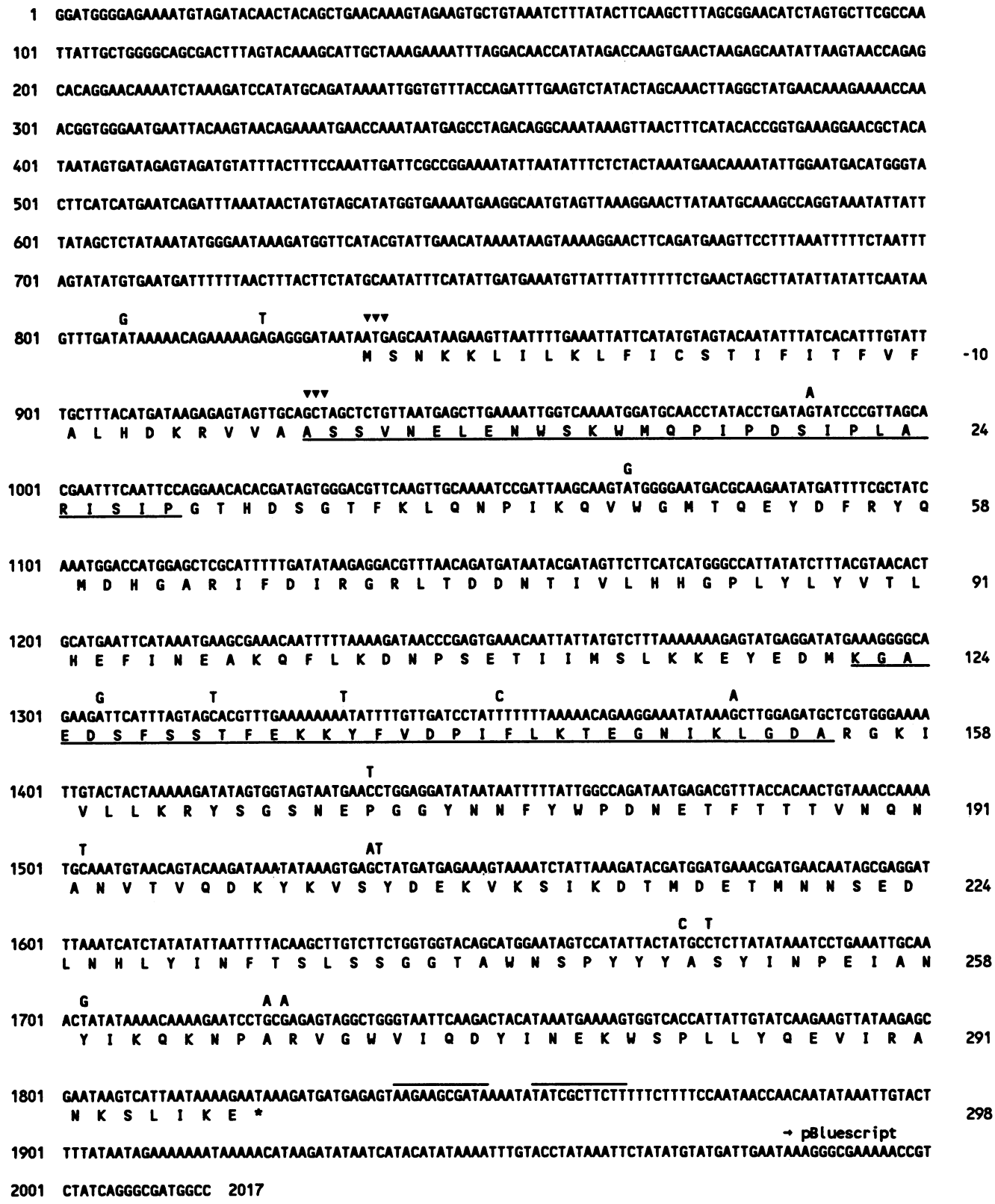

FIG. 1. Nucleotide sequence and deduced amino acid sequence. The nucleotide sequence of the *B. cereus* PI-PLC gene and flanking regions and the translation of the gene in one-letter amino acid code are shown. Numbering of the nucleotides is shown to the left; numbering of the amino acids is shown to 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.

TABLE 1. Expression of PI-PLC activity in transformed *E. coli*[a]

E. coli cells	PI-PLC activity (%)[b]	PI-PLC activity in presence of inhibitory antibody (%)
pA2	94	<5
pB2	100	<5
pA2-Δ55	74	<5
XL1-blue (untransformed)	<5	<5
XL1-blue[c]	<5	<5

[a] *E. coli* XL1-blue cells carrying the pBluescript phagemids were grown in LB-tet-amp broth for 25 h at 37°C with vigorous aeration and assayed for PI-PLC activity in LB-broth. Incubation with inhibitory antibody was carried out for 30 min at room temperature before an enzyme assay was performed.
[b] Normalized to values obtained with pB2.
[c] Transformed with pBluescript without insert.

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 was expressed in these cultures, but was absent from control cultures of untransformed XL1-blue cells and cells transformed with no insert-bearing vector. The expression of 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

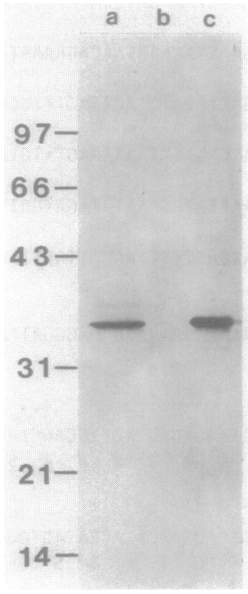

FIG. 2. Immunoblot analysis of PI-PLC expressed in *E. coli*. *E. coli* XL1-blue cells carrying pA2-Δ55 were grown, and proteins were precipitated from the culture supernatant, separated by SDS-PAGE (12% acrylamide), and electroblotted as described in Materials and Methods. The blots containing polypeptides 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.

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).

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47                                                                107
 WGMTQEYDFRYQMDHGARIFDIRGRLTDDNTIVLHHGPLYLYVTLHEFINEAKQFLKDNPS
 ||  |    |   |||.|  |.|  .. |    .       . | | | . ..| ||    |
 WGRCQNLSIRQLLDHGVRYLDLRMNVSPDQENKIYTTHFHISVPLQEVLKDVKDFLTTPAS
77                                                                137
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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.

FIG. 4. Sequence alignment with PI-PLCs involved in signal transduction. The sequence of the *B. cereus* PI-PLC polypeptide (BCER) was matched to an alignment of eucaryotic PI-PLCs (29). Positions in which a consensus between the eucaryotic sequences was observed are boxed. Identity (■) or conservative replacement (○) of amino acid residues of the *B. cereus* enzyme with conserved residues in the eucaryotic proteins is indicated. Sequence alignment of the eucaryotic enzymes was as in reference 29 (RAT1, residues 324 to 414; BOV2, residues 346 to 410; RAT3, residues 321 to 391), except for inclusion of the *Drosophila* norpA protein sequence (FLY; residues 339 to 425 [2]) and the trypanosome GPI-PLC (TRYP; compare Fig. 3) sequences.

Johansen et al. (16) and Yamada et al. (35) have raised the question whether these three enzymes might be synthesized as one transcriptional unit. Their work has demonstrated that the genes coding for the phosphatidylcholine (PC)-preferring phospholipase C (PLC) and the sphingomyelinase (SM-PLC) reside on contiguous stretches of the *B. cereus* chromosome, with the PC-PLC gene terminating only about 75 nucleotides upstream from the first coding sequences of the SM-PLC. To address this question, we translated the entire 2,017-nucleotide stretch of DNA presented in Fig. 1 in all three reading frames and compared these "hypothetical" peptide sequences with the data presented by Johansen et al. (16) and Yamada et al. (35). No significant homologies to the 5′-flanking region of the PI-PLC gene or the 3′-flanking region of the SM-PLC gene were detected in the alignments of these sequences. Hence, we conclude that the PI-PLC gene is located no closer to the gene cluster of the two other PLCs than 550 nucleotides upstream or at least 800 nucleotides downstream. The three PLCs are therefore unlikely to be part of the same transcriptional unit.

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 eucaryotic 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 50% identical (or 65% including conservative changes) 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 anchors 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 Rhee et al. (24), which is one of two polypeptide

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 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.

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 PC-PLC and P_i as a simple analog of an enzyme-substrate complex. The X-ray structure determination suggested that the phosphate group in the crystal is liganded by three Zn^{2+}-ion cofactors of the enzyme, which apparently surround the negatively charged phosphate. In contrast, no requirement for metal ions or presence of divalent ions has been reported for the PI-PLC from *B. cereus* or the trypanosomal GPI-PLC. In these proteins, an interaction of positively charged amino acid side chains (such as in Arg, Lys, and His) might substitute for the metal-ion cofactor in binding of the phospholipid substrate. This binding interaction can be expected to be important in the catalysis of PLCs, because it occurs next to the hydrolyzable bond between phosphate-oxygen and the glycerol group. Interestingly, several residues which are charged at physiological pH are conserved among PI-PLC from *B. cereus*, GPI-PLC from *Trypanosoma brucei*, and other PI-PLCs and might contribute to substrate binding or catalysis.

In summary, we report here the nucleotide and deduced amino acid sequence of the PI-PLC of *B. cereus* as a step toward an investigation of the structure-function relationships of this enzyme. The PI-PLC gene does not form a gene cluster with the two other secreted PLCs. The deduced amino acid sequence aligns with the sequence of the trypanosomal GPI-PLC, suggesting that the two polypeptide segments conserved between the enzymes might be involved in the function of the proteins.

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