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Molecular Cloning of a Putative Gene Encoding Phospholipase B (*plbA*) from *Aspergillus nidulans*

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The phospholipase B (PLB) families are enzymes sharing phospholipase (PL), lysophospholipase (LPL) and lysophospholipase-transacylase (LPTA) activities. In this study, we report the putative gene encoding phospholipase B (*plbA*) containing lipase motifs was cloned for the first time from the filamentous fungus, *Aspergillus nidulans*. *plbA* was isolated from *A. nidulans* genomic DNA library using a PCR-amplified probe, which is designed on the basis of sequence information derived from the conserved lipase regions of various PLBs. The deduced product of *plbA* is of 626 amino acids. From the assigned sequence, PlbA showed 72% identity with *Penicillium notatum* PLB but have low similarity with phospholipase A of other organisms.

Key words: *Aspergillus nidulans*, fungi, Phospholipase B, PLB, *plbA*

Phospholipases comprise a large group of structurally and functionally diverse enzymes that hydrolyze one or more ester linkages in glycerophospholipids. Phospholipases are classified according to the specificity of the ester linkage that is cleaved, including Phospholipase A (PLA), B (PLB), C (PLC), D (PLD) and Lysophospholipase (LPL), although the nomenclature can at times be confusing because many phospholipases have multiple enzymatic activities especially in PLB and LPL [4, 10, 20].

PLB is the designation given to phospholipases that catalyze the hydrolysis of fatty acids from both the sn-1 and sn-2 positions of glycerophospholipids. However, most of enzymatically characterized PLB proteins have been shown to possess LPL activity, which cleaves fatty acids from lysophospholipids [8]. Also, PLB enzymes isolated from fungi possess a transacylase activity that catalyzes the synthesis of phospholipids from lysophospholipids [4, 15].

PLB enzyme activities have been described in eukaryotes from fungi to mammals [11, 19]. Fungal PLB and LPL proteins exhibit a limited degree of sequence homology to members of the cytosolic phospholipase A_2 ($cPLA_2$) family of proteins. Several of which have been shown to possess the LPL and transacylase activities common to fungal PLB enzymes [5, 10, 13].

The physiological functions of fungal PLB enzymes are largely unknown. Three closely related members of the fungal PLB family, encoded by the PLB1, PLB2, and PLB3 genes, have been described in the budding yeast, *Saccharomyces cerevisiae* [3, 8, 12], but physiological function is still unknown. PLBs (LPLs) are considered to be important virulence factors for many microorganisms including *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Rickettsia rickettsii*, *Toxoplasma gondii* and *Entamoeba histolytica* [4, 18]. Specifically in the pathogenic dimorphic fungus *Candida albicans* and *Cryptococcus neoformans*, PLBs are considered to be virulence factors [1, 2, 9]. But the functions of PLBs (LPLs) still remain unclear in non-pathogenic filamentous fungi.

We, therefore, decided to identify gene(s) and cDNA(s) responsible of this activity in order to determine their potential role in the *Aspergillus nidulans*. In this paper, we describe the isolation of a DNA and cDNA for the PLB from *A. nidulans* and assign the primary structure of the protein moiety of this enzyme.

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB193027.

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Materials and Methods

Strains

A. nidulans FGSC 4 (*biA1*) were used in this study. Complete medium YG (0.5% yeast extract, 1% glucose, and 0.1% trace elements) and minimal medium (MM) for *A. nidulans* were prepared essentially according to the methods described by Rowlands and Tuner [14].

Library screening

To find the conserved regions of Phospholipase B, the sequences of already published data of PLB and LPL from various organisms were aligned. From the result of alignment, the forward primer PLBfor, GGNGGNGGNTANCGG, correspond to the highly conserved amino acid sequences of human PLB and LPL, GGGxR, reverse primer PLBrer, GANCCAGCCNCNTGC, correspond to the conserved sequences of human LPL, GGGxL, were constructed. PCR was performed against a genomic DNA of *A. nidulans* FGSC 4. A PCR-generated 140-bp fragment was inserted into T-vector (Promega, Madison, WI) and its nucleotide sequence was determined. The amplified fragment was used as a probe for screening of sub-genomic library of *A. nidulans*. Prior to the preparation of the sub-genomic library, Southern analysis was performed against genomic DNA of *A. nidulans* FGSC4 using the PCR-amplified fragment as a probe. Based on the result of Southern analysis, *Pst*I-digested genomic DNA around 5-kb was purified, ligated into pUC18 and used for construction of a sub-genomic library in *Escherichia coli*. Approximately 10^5 *E. coli* transformants were screened with DIG colony hybridization system (Roche, Mannheim, Germany) and three positive clones were isolated. A plasmid harbored these clones were designated as pPLB1 and sequenced.

5'- and 3'-Rapid Amplification of cDNA Ends (RACE)

cDNA of a gene encoding *plb*A was generated by 5'- and 3'-RACE with the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer's instruction. cDNAs were reverse transcribed from poly(A)+ mRNA obtained from the total RNA of *A. nidulans* FGSC4 that was grown in minimal medium at 37°C for 72 h. cDNA synthesis primer used were provided with the kit. The PCR amplification was carried out using an adaptor primer (AP-1; 5'-CCATCCTAATACGACTCACTATAG-GGC-3') provided with the kit and gene-specific forward (GAGATATTCGAGTGGCGC, 3'-RACE) or reverse primers (GTAGCCGACGGCCGGAAT, 5'-RACE). The PCR was performed according to the kit manufacture manual.

Results and Discussion

Isolation of a gene encoding Phospholipase B (*plbA*) of *A. nidulans*

To isolate a gene encoding PLB from *A. nidulans*, we surveyed database from the various fungus and mammalian PLB employed the sequence information of the conserved lipase domains. A set of primers, PLBfor and PLBrer, was designed. Using these primers, a fragment of approximately 140-bp long was amplified by PCR from the *A. nidulans* genomic DNA. Since the nucleotide sequence of this fragment showed highest similarity with fungus PLB, specifically *Pennisilium* and *Neurospora* PLB, we used this fragment as a probe for the screening of sub-genomic library (see MATERIALS AND METHODS). From approximately 10^5 *E. coli* transformants with the library, three positive clones were isolated. The plasmids harbored in these clones were extracted and the 5 kb inserted fragments of these plasmids were sequenced. From the sequencing analysis, it was shown that these three fragments were same.

Positions of introns and the starting codon of *plbA* were determined with the RACE experiment. *plbA* has no intron and an ORF of 1,881 bp that codes for a protein of 626 amino acids, of which estimated mass is 80 kDa.

Characterization of *plbA*

Comparison with the deduced amino acid sequence of *plbA* with two fungal PLB reveals lipase like catalytic domains with two consensus GGGxR, GxSGS motifs existed (Fig. 1). Thus, we designated this *plbA*. Moreover PlbA have 72% and 65% similiarity with *Pennicillium* and *Neurospora crassa* PLB.

Structural analysis by the simple modular architecture research tool (SMART) revealed that the gene product of *plb*A (PlbA) does not have any specific domain. Phylogenetic analysis revealed that the PlbA has the highest similarity with fungal PLB while there is a long phylogenetic distance between PLA_2 as indicated in Fig. 2. A hydropathy plot derived from the Kyte-Dolittle algorithm indicated that PlbA does not contain any membrane-

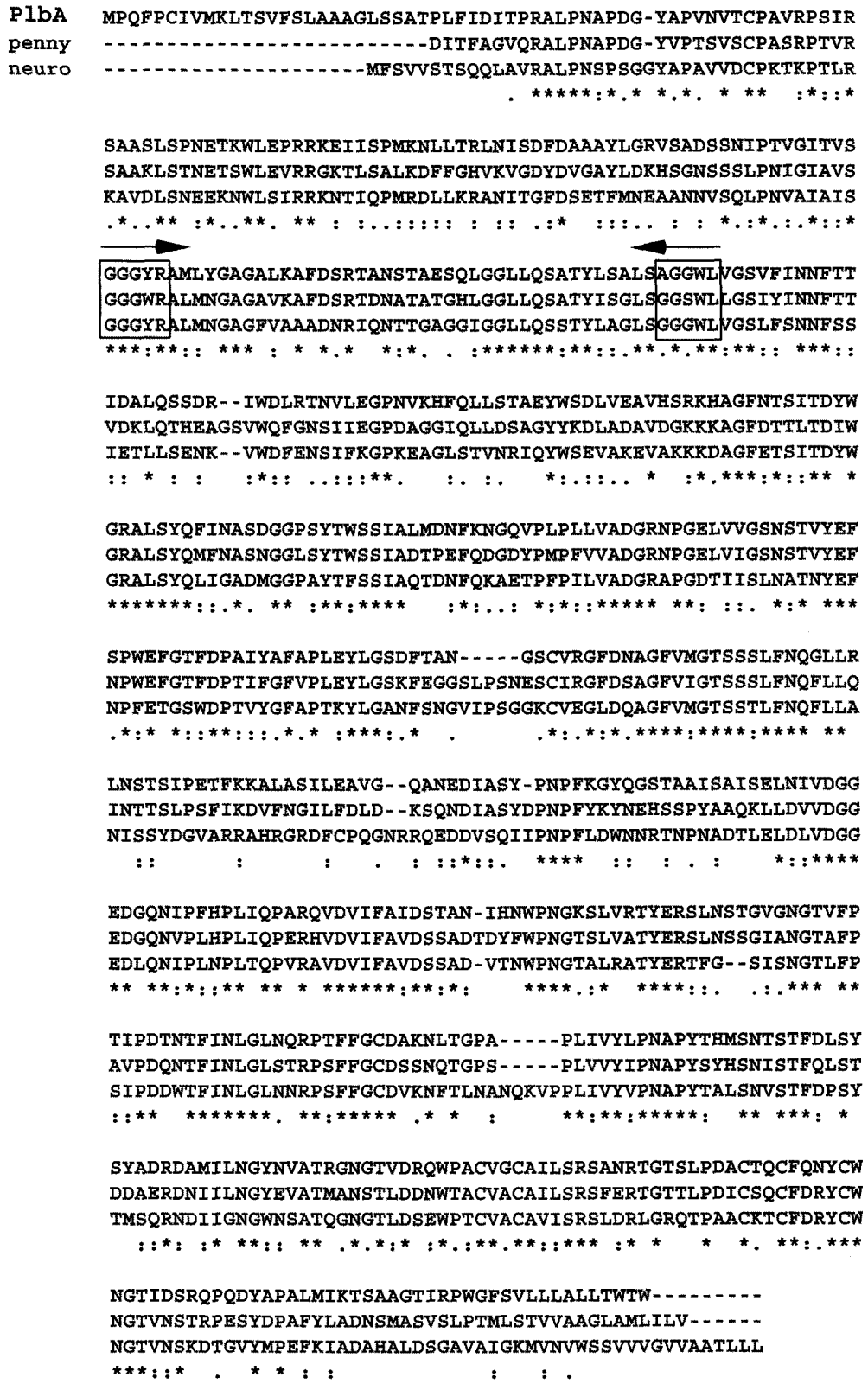

Fig. 1. Alignment of *plbA* with other fungal Phospholipase Bs. Alignment of amino acid sequences of PlbA isoforms (aligned with Clustal X). Identical and conserved amino acids among sequences are indicated by asterisks and dots, respectively. PlbA, *A. nidulans* Phospholipase B; penny, *Pennicillium chrysogenum* Phospholipase B; neuro; *Neurospora crassa* Phospholipase B. Arrows indicated the primers used for PCR.

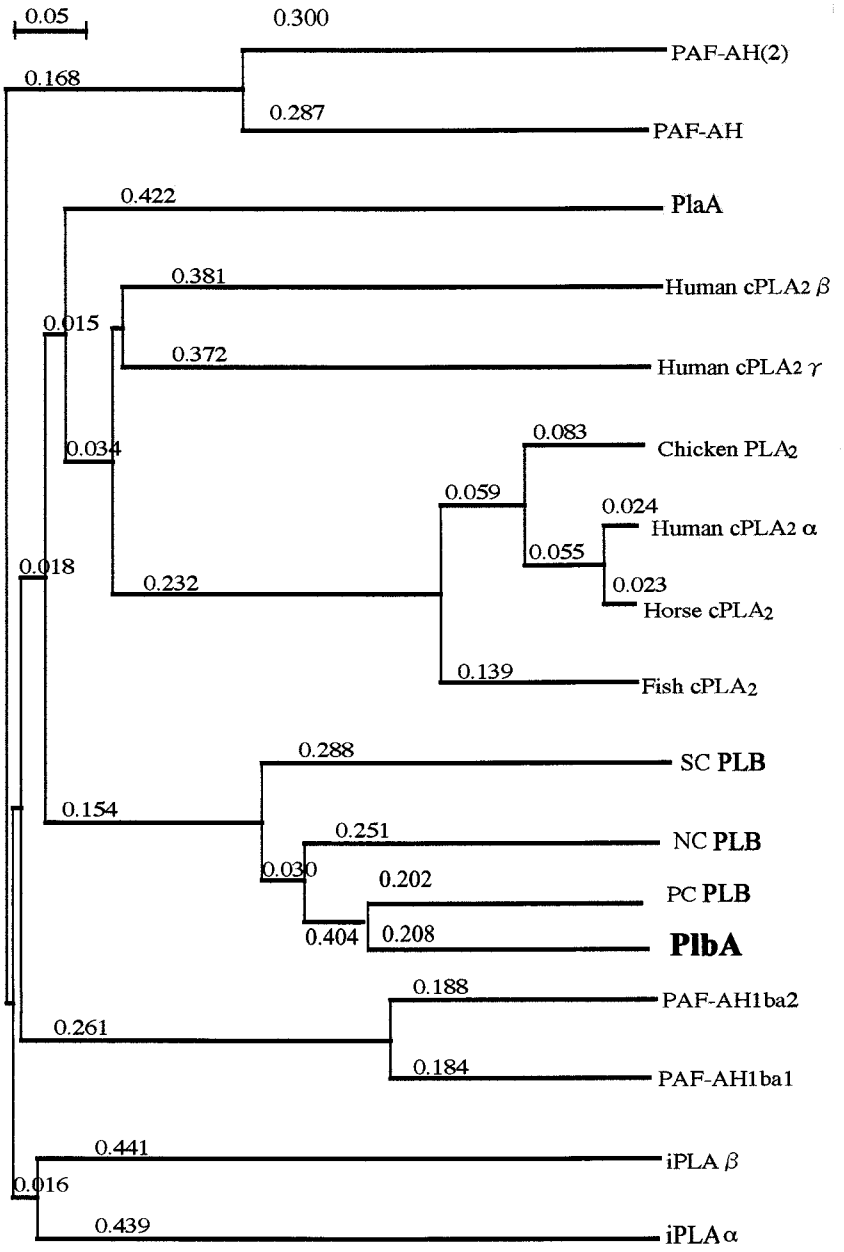

Fig. 2. Phylogenetic tree of PlbA from various organisms. Phylogenetic tree was constructed by using Clustal X program. Horizontal distance corresponds to genetic distance. The abbreviations used and GenBank accession numbers are as follows: PAF-AH(2), Human/bovine Platelet activating factor-AH(2), Q99487; PAF-AH, Human Platelet activating factor-AH, Q13093; PlaA, *A. nidulans* $cPLA_2$ AB101663; Human $cPLA_2\beta$, AAD32135; Human $cPLA_2g$, AAC32823; Chicken, Gallus $cPLA_2$, U10329-1; Human $cPLA_2\alpha$, M68874-1; Horse, *Equus* $cPLA_2$, AF092539-1; Fish, *Danio rerio* cPLA2, U10330-1; SC PLB, *Saccharomyces cerevisiae* Phospholipase B, AF045574_1; NC PLB, *Neurospora crassa* Phospholipase B, AF045575_1; PC PLB, *Pennicillium chrysogenum* Phospholipase B, X60348-1; PAF-AH1b a1, Human brain PAF-AH1b a1, Q15102; PAF-AH1b a2, Human brain PAF-AH1b a2, Q29459; iPLαa, P388D1 $iPLA_2\alpha$, AAD41722; iPLAβ, Human B-lympocytes calcium independent $PLA_2\beta$, BAA94997.

spanning regions but hydrophobic residues appear to be in the C-terminus, suggesting that the C-terminal region have a possibility with membrane attachment (Fig. 3). And PlbA does not have insertion sequence found in *Penicillium notatum* PLB [11, 15].

Finally we have isolated putative Phospholipase B coding gene *plbA* from non-pathogenic filamentous fungus *A. nidulans* for the first time. The deduced product of *plb*A is of 626 amino acids and *plb*A have 72% identity with *Penicillium notatum* PLB. One possibility is that PlbA is involved in regulation of the production of lipid or lipid derived second messengers through by anchoring hydro-

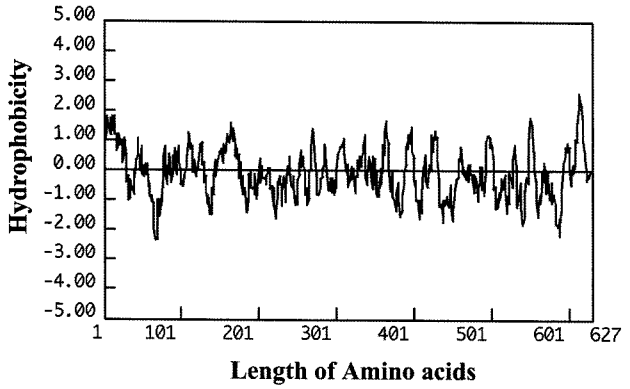

Fig. 3. Hydropathy plot analysis of *plbA*. Hydropathy plot profiles was illustrated by the method of Kyte & Doolittle.

phobic C-terminus, perhaps in concert with other phospholipid/lipid-modifying enzymes.

Gene knockout studies will be required to fully understand the potential role of PlbA in this organism. We are trying to reveal the physiological function of PlbA in *A. nidulans* in the near future.

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초록국문

사상성 진균 *Aspergillus nidulans*의 Phospholipase B 유전자(*plb A*)의 클로닝

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Phospholipase B(PLB) families는 phospholipase(PL), lysophospholipase(LPL) 그리고 lysophospholipase-transacylase(LPTA) 의 활성을 공유하고 있는 효소이다. 본 연구에서는, 사상성 진균인*Aspergillus nidulans*에서 최초로 lipase motifs를 보유하고 있는 단백질 Phospholipase B를 인코딩하는 유전자(*plb A*)를 클로닝하였다. *plb A*는 다양한 PLB효소들의 lipase 보존영역들의 염기서열 정보에 근거하여 제작한 probe를 이용하여 *A. nidulans* genomic DNA library로 부터 분리하였다. Phospholipase B 유전자의 염기서열을 결정한 결과 626아미노산으로 구성된 단백질을 코드하고 있었다. PlbA는 *Penicillium notatum*의 PLB와는 72%의 높은 상동성을 나타내었으나, 다른 생물유래의 PLA와는 낮은 상동성을 나타내었다.