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► **To cite this version:**

Da-Kang Shen, Ali Dehghan Noodeh, Abdolhassan Kazemi, Renée Grillot, Geoff Robson, et al.. Characterisation and expression of phospholipases B from the opportunistic fungus *Aspergillus fumigatus*. FEMS Microbiology Letters, 2004, 239 (1), pp.87 - 93. 10.1016/j.femsle.2004.08.019 . hal-01612756

HAL Id: hal-01612756

<https://hal.science/hal-01612756>

Submitted on 13 Nov 2020

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1 **Characterisation and expression of phospholipases B from the**
2 **opportunistic fungus *Aspergillus fumigatus***

3
4 Da-Kang Shen ^a, Ali Dehghan Noodeh ^b, Abdolhassan Kazemi ^b, Renée Grillot ^{a,c}, Geoff
5 Robson ^b, Jean-François Brugère ^{a,*}

6
7 *^aLaboratoire Interactions Cellulaires Parasite-Hôte - ICPH, Faculté de Médecine et*
8 *Pharmacie, Université Joseph Fourier, F-38706 La Tronche, France*

9 *^bSchool of Biological Sciences, 1.800 Stopford Building, University of Manchester,*
10 *Manchester M13 9T, UK*

11 *^cLaboratoire de Parasitologie et Mycologie, CHU de Grenoble, 38700 La Tronche, France*

12

13 * Corresponding author. Tel.: +33 (0)4 76 63 74 73; Fax: +33 (0)4 76 63 74 73.

14 *E-mail address: Jean-Francois.Brugere@ujf-grenoble.fr (J.-F. Brugère).*

15

16 **Keywords:** Aspergillosis, lysophospholipase, PLB, pathogenic fungus, virulence
17 factor

18

1 **Abstract**

2 The phospholipase B family (PLB) are enzymes sharing phospholipase (PL),
3 lysophospholipase (LPL) and lysophospholipase-transacylase (LPTA) activities. They have
4 been shown to be important virulence factors in several human fungal pathogens including
5 *Candida albicans* and *Cryptococcus neoformans*. *Aspergillus fumigatus*, a human
6 opportunistic fungal pathogen leading to a high rate of mortality in immunosuppressed
7 patients is known to possess an extracellular phospholipase B activity. In this paper, we
8 report the molecular characterisation of 3 PLB genes from *A. fumigatus* (*afplb*) using
9 degenerate primers in PCR amplification and data from the *A. fumigatus* genome project.
10 They are expressed at 37°C, and two of them (*afplb1* and *afplb3*) are induced by lecithin.
11 They encode proteins of 633, 588 and 630 amino acids respectively, presenting together a T-
12 Coffee score of 81. They also possess the amino acid triad responsible for enzymatic activity
13 in the mammalian cytosolic PLA₂ and other fungal PLBs. AfPLB1 and afPLB3 are secreted
14 with a cleaved signal peptide. The complete cDNA sequences were obtained by RACE-PCR
15 for the two secreted afPLBs and probably account for the extracellular phospholipase
16 activity previously reported in the culture media of *A. fumigatus*.

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1. Introduction

Aspergillus fumigatus is one of the most prevalent opportunistic human fungal pathogens, the number of cases having increased together with the number of immunocompromised individuals. The most serious form of *Aspergillus* infection, invasive aspergillosis, has been found in 4% of all patients dying in a modern European teaching hospital [1]. Pathogenesis factors are thought to include toxins, proteins facilitating adhesion, and hydrolases acting on host cell components (reviewed in [2,3]). These include proteases [4] and probably phospholipases. Phospholipases are a heterogeneous group of enzymes that are able to hydrolyse one or more ester linkages in glycerophospholipids and include phospholipase A (PLA), B (PLB), C (PLC) and D (PLD). Phospholipase activity can destabilize host membranes, lyse cells and release lipid second messengers [5]. They are considered to be important virulence factors for many microorganisms including *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Rickettsia rickettsii*, *Toxoplasma gondii* and *Entamoeba histolytica* (review in [6]). Phospholipases and particularly PLBs are also considered to be virulence factors for pathogenic fungi including *Candida albicans* [7] and *Cryptococcus neoformans* [8]. In fungi, these PLBs possess 3 different enzymatic activities, the hydrolase activity of phospholipase, lysophospholipase (LPL) and a lysophospholipase transacylase (LPTA) activity [7,9]. Extracellular phospholipase activities have been detected in *in-vitro* cultures of *A. fumigatus* [10,11], with a predominance of PLB activities [12] supporting the presence of gene(s) coding for secreted PLB(s). We therefore decided to identify gene(s) and cDNA(s) responsible of this activity in order to determine their potential role in the pathogenesis of *A. fumigatus*.

2. Materials and methods

2.1. *Strains, media and culture conditions*

A. fumigatus CBS14489 and AF10 (ATCC 90240) were maintained at 4°C on Sabouraud chloramphenicol agar (bioMérieux, France) and subcultured at 25°C or 37°C up to 48 hours with constant shaking (200 rpm) in modified Czapeck broth (Cz₂) with or without 0.5% (w/v) 3-Sn-phosphatidylcholine from egg yolk (Fluka, Switzerland). Cz₂ consists of 0.1% (w/v) yeast extract, 0.3% (w/v) saccharose, 0.3% (w/v) potassium nitrate, 0.1% (w/v) di-potassium hydrogen phosphate, 0.05% (w/v) potassium chloride, 0.05% (w/v) magnesium sulphate and 0.001% (w/v) iron (II) sulphate.

2.2. *DNA and RNA extraction*

Genomic DNA was extracted as described on the *Aspergillus* website (www.aspergillus.man.ac.uk). Total RNA was extracted using a TRI REAGENT™ kit (Euromedex, France) according to the manufacturer's instructions. Isolation of mRNA was accomplished by using "Dynabeads® mRNA DIRECT™ kit" (Dynal, Norway) on total RNA according to the manufacturer's instructions.

2.3. *Cloning and characterisation of afplb genes*

Degenerate primers for phospholipase B [13] named degPLB Fw and Rv (table 1) were used in PCR amplification. The final optimised conditions were 4 mM MgCl₂ and an annealing temperature of 48°C for 3 min. Cloning of PCR products were performed either in pGEM®-T easy (Promega) or pT-Adv (Invitrogen) according to the manufacturer's instructions. *Xho*I-restricted genomic DNA or *Sal*I-restricted DNA was self-ligated with T4 DNA ligase and used for determination of adjacent sequences by Inverse PCR [14]

1 respectively for *afplb1* and *afplb2*. The sequence of the primers used (invPLB1 and 2, Fw
2 and Rv) is given in Table 1.

3 4 **2.4. Expression of *afplbs* and full-length cDNA cloning and sequencing**

5 Potential expression of PLB(s) was evaluated by RT-PCR on total RNAs
6 (ProSTAR™ First-strand RT-PCR, Stratagene) using an annealing temperature of 58°C.
7 Primers used in PCR (RT-PLBx primers) are shown in Table 1. Amplification of a part of
8 the cDNA of the housekeeping genes actin and β -tubulin was used as a positive control and
9 to normalise the expression levels of the PLB genes between the cultures with and without
10 lecithin. Quantitative-PCR (Q-PCR) was performed on a BioRad i-Cycler IQ system using
11 the IQ SYBR Green supermix kit (BioRad) according to the manufacturer's instructions,
12 relatively to β -actin mRNAs. Primers used are indicated in Table 1.

13 5'- and 3' regions of each cDNA were amplified by RACE-PCR (Rapid
14 Amplification of cDNA Ends) accordingly to the manufacturer's instructions (GeneRacer™
15 Kit Invitrogen) using a set of gene specific primers (5'- or 3'-RACE PLBx, Table 1), with
16 two rounds of PCR, one of 30 cycles with an annealing temperature of 65°C and another 30-
17 cycles at 68°C. For each gene, the 5' and the 3' primers are complementary, therefore
18 permitting full-length cDNA sequences to be assembled after sequencing (GENOME
19 express, Meylan, France).

20 21 **2.5. Sequence analysis and sequence accession numbers**

22 Preliminary genomic sequence data was obtained from The Institute for Genomic
23 Research (TIGR) website at www.tigr.org. Bioinformatics analyses were performed either
24 at the French bioinformatics server "Infobiogen" (www.infobiogen.fr) or at the ExPASy
25 Molecular Biology Server (<http://us.expasy.org>). For alignments and phylogenic analysis,

1 the following PLBs sequences were used: YMR008C (scPLB1), YMR006C (scPLB2),
2 YOL011W (scPLB3), AAF65220 (cnPLB1), CAC86376 (cnPLBa), AAF61964 (cnPLBb),
3 EAL17514 (cnPLBx), Q9UWF6 (caPLB1), O93795 (caPLB2), Q9UVX1 (caPLB3),
4 EAK98600 (caPLB5), Q8TG07 (cgPLB1), Q8TG06 (cgPLB2), P78854 (spPLB1), O13857
5 (spPLB2), Q08108 (spPLB3), Q9P327 (spPLB4), Q9Y7N6 (spPLB5), Q9UTH5 (spPLB6),
6 EAA64795 (anPLBx1), EAA61850 (anPLBx2), O42790 (ncPLB), EAA34954 (ncPLBx),
7 BAD08699 (mgPLBa), BAD08698 (mgPLBb), EAA56932 (mgPLBx), EAK81777
8 (umPLBx).

9 The nucleotide sequence data reported in this paper are listed in the GenBank
10 nucleotide sequence database under accession numbers [AF223004](#) / [AF223005](#) respectively
11 for partial genomic sequences of *afplb1* and *afplb2*, and [AY376592](#) / [AY376593](#) respectively
12 for the complete cDNA sequences of *afplb1* and *afplb3*.

13

14 **3. Results**

15 **3.1. Determination of three *plb* genes in the *A. fumigatus* genome**

16 Degenerate primers designed by Sugiyama et al. [13] for the cloning of a second
17 phospholipase B gene from *Candida albicans* were used in order to amplify a partial region
18 of *plb* gene(s) from genomic DNA of *A. fumigatus*. A band of ~550 bp was obtained,
19 corresponding after sequencing to two different sequences of 545 and 542 nt, sharing 65.9%
20 identity and having a strong homology to fungal PLBs. These two partial sequences were
21 therefore named *afplb1* (Genbank accession number [AF223004](#)) and *afplb2* (Genbank
22 accession number [AF223005](#)). An inverse-PCR [14] on genomic DNA digested respectively
23 by *XhoI* and *SalI* and self-ligated permitted to obtain new PCR products of ~1.8 kb and ~1.1
24 kb leading to sequences of 2197 bp and of 1529 bp respectively for *afplb1* and *afplb2* when

1 assembled (data not shown). However, a BLASTX analysis [15] revealed that while the
2 entire 3' ends of *afplb1* and *afplb2* were cloned, the 5' regions were missing.

3 At the same time, the “*Aspergillus fumigatus* genome project” was initiated and
4 preliminary data were available for the scientific community (<http://www.tigr.org>) from
5 The Institute for Genomic Research (TIGR, USA) and the Wellcome Trust Sanger Institute
6 (UK), the two main centres in this international collaboration [16]. We therefore decided to
7 perform a BLAST search of our sequences to attempt to obtain the 5' regions. For each
8 sequence, a nearly perfect match was obtained confirming these genes were present as a
9 single copy, which was also confirmed by Southern analysis (data not shown). In addition, a
10 third gene with high homology was identified indicating that the same number of *plb* genes
11 was present in *A. fumigatus* genome and in *S. cerevisiae* genome.

13 **3.2. Expression and cDNA sequences of *afplbs***

14 The main site of infection of *A. fumigatus* before invasion is the lung, in which the
15 surfactant is principally composed of dipalmitoyl phosphatidylcholine, also the preferred
16 substrate of the cryptococcal PLB [17]. We therefore investigated the influence of lecithin
17 on the expression of *afplb* genes by quantifying their mRNAs from *A. fumigatus* cultured at
18 37°C with or without lecithin. Real-time PCR indicates that the three *afplbs* are expressed
19 and that *afplb1* and *afplb3* are up-regulated by lecithin (respectively 5-fold and 300-fold)
20 whereas *afplb2* expression is not affected (Table 2).

21 A preliminary bioinformatics study on the predicted amino acid sequence of PLB1,
22 PLB2 and PLB3 showed the presence of an N-terminal prepro sequence for PLB1 and PLB3
23 (but not PLB2) indicating that they were likely to be secreted and therefore potentially
24 involved in the pathogenesis of *A. fumigatus*. We therefore cloned the full-length cDNAs of
25 *afplb1* and *afplb3* by RACE-PCR using mRNA from *A. fumigatus* cultured at 37°C in the

1 presence of lecithin. Assembling of the 5' and 3' part of each sequence revealed an mRNA
2 of 2,125 nt for *afplb1* and of 2,027 nt for *afplb3*, excluding the poly(A) tail, deposited in
3 Genbank respectively under the accession numbers [AY376592](#) and [AY376593](#). These
4 sequences were aligned and compared with genomic data. The ORF for *afplb2* was deduced
5 from genomic data using bioinformatics tools (GeneFinder, TblastX) and sequence
6 alignment against other fungal *plbs*. The features of these mRNAs are summarized in Table
7 3. *Afplb1* and *afplb2* are composed of 3 exons, against one for *afplb3* and the introns are
8 relatively small (varying from 58 to 132 nt) and fit with the consensus sequence usually
9 observed (GT/YAG). Search of the putative translation initiation codon (AUG) beginning
10 the longest ORF sharing homology with other fungal PLBs for *afplb1* and *afplb3* reveals
11 that the 5' UTR is 63-nt and 34-nt long respectively for *afplb1* and *afplb3*. The stop codon
12 used is OCH for *afplb1* and AMB for *afplb2* and *afplb3*. The 3' UTR is 160-nt and 101-nt
13 long respectively for *afplb1* and *afplb3*, so resulting of an ORF size of respectively 1899 nt,
14 1764 nt and 1890 nt for *afplb1*, *afplb2* and *afplb3*.

15

16 **3.3. Analysis of the predicted proteins sequences**

17 The ORFs determined above lead to proteins of 633 amino acids for afPLB1, 588
18 amino acids for afPLB2 and of 630 amino acids for afPLB3. Their theoretic mass and pI are
19 indicated in Table 3. An hydropathy analysis performed according to Kyte and Doolittle
20 [18] revealed two stretches of hydrophobic amino acids at both the N- and C-terminus
21 having a score superior of 2 (Table 3) only for afPLB1 and afPLB3, but not for afPLB2
22 which is predicted to be localized in the nucleus as indicated by a PSORT II analysis (60.9
23 %, k=23, [19]). In addition to the hydrophobic N-tails of afPLB1 and afPLB3, a secretion
24 signal peptide is detected with a cleavage site between position 20 and 21 (VSG-AP) for
25 afPLB1 and position 16 and 17 (ATA-TP) for afPLB3, using SignalP V2.0 with predictions

1 trained on eukaryotes using neural networks [20]. Moreover, a PSORT II analysis reveals
2 that these two proteins could be either extracellular (including the cell wall) or GPI
3 anchored, with the same probability (Table 3). This last hypothesis is due to the presence of
4 (1) a hydrophobic tail, (2) a N-terminal peptide for secretion, (3) a S/T rich region, and (4) a
5 potential ω site (GPI attachment site) with a G [21], elements absent in caPLBs. These
6 signal peptides are indicated in the Figure 1, together with the important number of N-
7 glycosylation sites present in these two secreted proteins (18 for afPLB1 and 14 for
8 afPLB3).

9 An alignment of the sequence of these 3 proteins indicates a high degree of identities
10 (from 59 % to 63 %), confirmed with a score of 81 using T-Coffee software [22]. Moreover,
11 some residues of the cytosolic mammalian PLA₂ (a triad comprising R₂₀₀, S₂₂₈ and D₅₄₉
12 described as essential for the catalytic activity [23]) are also conserved in these three afPLBs
13 (amino acids boxed in Fig. 1), at position 121/105/112 for R, 160/144/151 for S and
14 412/395/405 for D, respectively for afPLB1, afPLB2 and afPLB3. This is also the case for
15 other PLBs from pathogenic fungi *C. albicans* (caPLB1 and caPLB2) and of *C. neoformans*
16 (cnPLB1) (data not shown).

17 In order to identify orthologues in other fungi, a phylogenic analysis was performed
18 using PLBs from yeasts and filamentous fungi. The tree is presented in figure 2 and shows
19 that afPLBs are more closely related together than with PLBs from other genus: orthologous
20 genes are only found in the *Aspergillus nidulans* genome (*afplb1* and *anplb1*, *afplb3* and
21 *anplb2*), but not at the present time in other fungal genomes. A duplication of a common
22 ancestor gene gave two genes, one which evolved into afPLB2, the other which duplicated
23 into two other genes (afPLB1 and afPLB3): these events seems to be quite recent as they are
24 restricted to the *Aspergilli*.

1 **4. Discussion**

2 In this present study, we identified and cloned three genes coding for PLB from *A.*
3 *fumigatus*, as it is in the genome of *S. cerevisiae*. However, the genomic distribution seems
4 to be different between these two organisms. In *S. cerevisiae*, 2 of the 3 *plb* genes (*scplb1*
5 and *scplb2*) are linked on the chromosome XIII around the position 280,000. This is not the
6 case for *afplb* genes as deduced from the partial assembly of genomic sequence data.
7 Moreover, the phylogenic analysis indicates that the *afplbs* genes are probably the result of
8 gene duplications that are independent of those observed in other fungi, with the exception
9 of *A. nidulans*.

10 Our cDNA sequences for *afplb1* and *afplb3* fit well with those from the genome
11 project, with some minor changes: for *afplb1*, a transition (33 T→C), a transversion (1982
12 A→T) and a single-base deletion (2048 ΔT) are observed, and are all localized in
13 untranslated regions. Five single-base modifications are detected for *afplb3*, including 4
14 transitions (A→G in positions 391, 1658, 1974 and 1886) and one transversion (242 C→G).
15 This should lead to 3 amino-acids differences (E₇₀→Q; D₅₄₂→N; S₆₁₈→G), the two other
16 modifications being in an untranslated region (position 1974) or silent (position 391: G₁₃₉).
17 It remains to be determined if these differences are sequencing errors, or more likely, genetic
18 differences between the two different strains used in the genome project and in this study.
19 Moreover, no canonical sequences of polyadenylation (AATAAA) were present in the 3'
20 part of the cDNAs and no consensus sequence for either a "TATA box" nor "CAAT box"
21 could be identified in the 5' upstream regions.

22 These genes encode PLBs which share 58 to 63 % identity at the protein level and
23 have similar properties to other fungal PLBs determined so far, as well for their size
24 (varying from 605 aa for caPLB1 [7] to 637 aa for cnPLB1 [8]), and for their acidic pI or
25 their high number of N-glycosylation sites. Interestingly, de-glycosylation of the PLB from

1 *C. neoformans* using PNGase F leads to an almost total loss of enzyme activity [9]. They
2 also possess the three amino acids responsible for their enzymatic activity. Two of them
3 (afPLB1 and afPLB3) are non-cytoplasmic and possess an N-terminal signal peptide that
4 should target the protein either to the plasma membrane or to the extracellular compartment
5 (including an attachment to the cell wall). One or both proteins may therefore account for
6 the extracellular PLB activity determined by Birch et al. [10,11] and would degrade the
7 phospholipids present in high concentrations in lung surfactant. Interestingly, the expression
8 of both afPLB1 and afPLB3 was induced by lecithin, a constituent of human lung surfactant.
9 Extracellular PLB activity has previously been correlated with virulence in both *C. albicans*
10 and *C. neoformans* [24,25] and PLB knockout strains have been shown to significantly
11 reduce virulence by impeding penetration of host cells [7,8]. Moreover, it was shown
12 recently that extracellular PLB activity is different in environmental isolates of *A. fumigatus*
13 compared to clinical isolates [11], but surprisingly is higher. Thus, although PLB appears to
14 be important in the pathogenicity of *C. albicans* and *C. neoformans*, it is less clear what role
15 extracellular PLB may play in the pathogenicity of *A. fumigatus*. This may reflect the fact
16 that while PLB appears to be the only secreted phospholipase in *C. albicans* and *C.*
17 *neoformans*, *A. fumigatus* is also reported to secrete PLC [10,11]. However, we can not
18 exclude a participation in virulence due to different substrate specificities.

19

20 In conclusion, we have demonstrated that *A. fumigatus* encodes three PLB genes that
21 are predicted to encode two secreted PLB proteins and to be upregulated in the presence of
22 lecithin. Gene knockout studies will be required to fully understand the potential role of PLB
23 in the pathogenicity of *A. fumigatus*.

24

25 **Acknowledgements**

1 Sequencing of *Aspergillus fumigatus* was funded by the National Institute of Allergy and
2 Infectious Disease U01 AI 48830 to David Denning and William Nierman, the Wellcome
3 Trust, and Fondo de Invescicagiones Sanitarias. DKS was supported by a grant from
4 “Région Rhône Alpes” and from the Shanghai 2nd Medical University. HK and AN were
5 supported by the Iranian Ministry of Health.
6 JFB thanks Marie-France Cesbron-Delauw and Hervé Pelloux for assistance in sequencing,
7 and Eric Peyretailade for assistance in phylogenic analysis.

8

9 **References**

- 10 [1] Vogeser, M., Haas, A., Aust, D. and Ruckdeschel, G. (1997). Postmortem analysis of
11 invasive aspergillosis in a tertiary care hospital. *Eur. J. Clin. Microbiol. Infect. Dis.*
12 16, 1 6.
- 13 [2] Latge, J. P. (1999). *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* 12,
14 310 350.
- 15 [3] Tomee, J. F. and Kauffman, H. F. (2000). Putative virulence factors of *Aspergillus*
16 *fumigatus*. *Clin. Exp. Allergy* 30, 476 484.
- 17 [4] Monod, M., Capoccia, S., Lechenne, B., Zaugg, C., Holdom, M. and Jousson, O.
18 (2002). Secreted proteases from pathogenic fungi. *Int. J. Med. Microbiol.* 292, 405
19 419.
- 20 [5] Salyers, A. and Witt, D. (1994). Virulence factors that damage the host, p. 47 62. In
21 A. Salyers and D. Witt (ed.), *Bacterial pathogenesis: a molecular approach*. ASM
22 Press, Washington, D. C.
- 23 [6] Ghannoum, M. A. (2000). Potential role of phospholipases in virulence and fungal
24 pathogenesis. *Clin. Microbiol. Rev.* 13, 122 143.

- 1 [7] Leidich, S. D., Ibrahim, A. S., Fu, Y., Koul, A., Jessup, C., Vitullo, J., Fonzi, W.,
2 Mirbod, F., Nakashima, S., Nozawa, Y. and Ghannoum, M. A. (1998). Cloning and
3 disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of
4 *Candida albicans*. J. Biol. Chem. 273, 26078 26086.
- 5 [8] Cox, G. M., McDade, H. C., Chen, S. C, Tucker, S. C., Gottfredsson, M., Wright, L.
6 C., Sorrell, T. C., Leidich, S. D., Casadevall, A., Ghannoum, M. A. and Perfect, J. R.
7 (2001). Extracellular phospholipase activity is a virulence factor for *Cryptococcus*
8 *neoformans*. Mol. Microbiol. 39, 166 175.
- 9 [9] Chen, S. C., Wright, L. C., Golding, J. C. and Sorrell, T. C. (2000). Purification and
10 characterisation of secretory phospholipase B, lysophospholipase and
11 lysophospholipase/transacylase from a virulent strain of the pathogenic fungus
12 *Cryptococcus neoformans*. Biochem. J. 347, 431 439.
- 13 [10] Birch, M., Robson, G., Law, D. L. and Denning, D. W. (1996). Evidence of multiple
14 extracellular phospholipase activities of *Aspergillus fumigatus*. Infect. Immun. 64,
15 751 755.
- 16 [11] Birch, M., Denning, D. W. and Robson, G. D. (2004). Comparison of extracellular
17 phospholipase activities in clinical and environmental *Aspergillus fumigatus* isolates.
18 Med. Mycol. 42, 81 86.
- 19 [12] Koul, A., Jessup, C. J., Deluca, D. J., Elnicky, C. J., Nunez, M., Washburn, R. G. and
20 Ghannoum, M. A. (1998). Gen. Meet. Am. Soc. Microbiol. abstr. F 78.
- 21 [13] Sugiyama, Y., Nakashima, S., Mirbod, F., Kanoh, H., Kitajima, Y., Ghannoum, M.
22 A. and Nozawa, Y. (1999). Molecular cloning of a second phospholipase B gene,
23 caPLB2 from *Candida albicans*. Med. Mycol. 37, 61 67.
- 24 [14] Ochman, H., Gerber, A. S. and Hartl, D. L. (1988). Genetic applications of an inverse
25 polymerase chain reaction. Genetics 120, 621 623.

- 1 [15] Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1999). Basic
2 local alignment search tool. *J. Mol. Biol.* 215, 403 410.
- 3 [16] Denning, D. W., Anderson, M. J., Turner, G., Latge, J. P. and Bennett, J. W. (2002).
4 Sequencing the *Aspergillus fumigatus* genome. *Lancet Infect. Dis.* 2, 251 253.
- 5 [17] Santangelo, R. T., Nouri-Sorkhabi, M. H., Sorrell, T. C., Cagney, M., Chen, S. C.,
6 Kuchel, P. W. and Wright, L. C. (1999). Biochemical and functional characterisation
7 of secreted phospholipase activities from *Cryptococcus neoformans* in their naturally
8 occurring state. *J. Med. Microbiol.* 48, 731 740.
- 9 [18] Kyte, J. and Doolittle, R. F. (1982). A simple method for displaying the hydrophobic
10 character of a protein. *J. Mol. Biol.* 157, 105 132.
- 11 [19] Horton, P. and Nakai, K. (1997). Better prediction of protein cellular localization
12 sites with the k nearest neighbors classifier. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 5,
13 147 152.
- 14 [20] Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997). A neural network
15 method for identification of prokaryotic and eukaryotic signal peptides and
16 prediction of their cleavage sites. *Int. J. Neural. Syst.* 8, 581 599.
- 17 [21] Hamada, K., Terashima, H., Arisawa, M., Yabuki, N. and Kitada, K. (1999). Amino
18 acid residues in the omega-minus region participate in cellular localization of yeast
19 glycosylphosphatidylinositol-attached proteins. *J. Bacteriol.* 181, 3886 3889.
- 20 [22] Notredame, C., Higgins, D. and Heringa, J. (2000). T-Coffee: A novel method for
21 multiple sequence alignments. *J. Mol. Biol.* 302, 205 217.
- 22 [23] Pickard, R. T., Chiou X. G., Striffler, B. A., DeFelippis, M. R., Hyslop, P. A., Tebbe,
23 A. L., Yee, Y. K., Reynolds, L. J., Dennis, E. A., Kramer, R. M. and Sharp, J. D.
24 (1996). Identification of essential residues for the catalytic function of 85-kDa

- 1 cytosolic phospholipase A2. Probing the role of histidine, aspartic acid, cysteine, and
2 arginine. *J. Biol. Chem.* 271, 19225-19231.
- 3 [24] Ibrahim, A. S., Mirbod, F., Filler, S. G., Banno, Y., Cole, G. T., Kitajima, Y.,
4 Edwards, J. E. Jr, Nozawa, Y. and Ghannoum, M. A. (1995). Evidence implicating
5 phospholipase as a virulence factor of *Candida albicans*. *Infect. Immun.* 63, 1993
6 1998.
- 7 [25] Vidotto, V., Leone, R., Sinicco, A., Ito-Kuwa, S. and Criseo, G. (1998). Comparison
8 of phospholipase production in *Cryptococcus neoformans* isolates from AIDS
9 patients and bird droppings. *Mycopathologia* 142, 71-76.
- 10

- 1 Table 1
 2 Names and sequences of primers used in this work

Name	Sequence (5' - 3')
degPLB Fw	GAYGGIGGIGARAAAYCARAA [13]
degPLB Rv	AYIGTICCRTTCCARCARTA [13]
invPLB1 Fw	CGCACACAGCTCTCATTACTTGGG
invPLB1 Rv	GGGAATACATCACGTGCTTATGAGGC
invPLB2 Fw	CTGGTAGTCGTGTCCGCTGACGAGTCGAC
invPLB2 Rv	TGAGAATGGGTACGATGTGGCAACACTGGG
RT-PLB1 Fw	ATACCACTGCACCCGTTGA
RT-PLB1 Rv	GGGAATTGCAGGAAAGGAA
RT-PLB2 Fw	CTCGATCCCCTTCTTCAGC
RT-PLB2 Rv	GCCAGTCGCGTTTGAACTA
RT-PLB3 Fw	TGCCAGACGTGAACACCTT
RT-PLB3 Rv	CCGCGTCACCACGTTATAC
RT- β ACT Fw	TGATTGGTATGGGCCAGAA
RT- β ACT Rv	CGTAGAGGGAGAGAACGGC
Q-PCR PLB1 Fw	TGTTGGCTTTGTTCATGGGTA
Q-PCR PLB1 Rv	GATGTCGGTGAAGACGGATT
Q-PCR PLB2 Fw	CAGGCATTTCTGCAGATGAA
Q-PCR PLB2 Rv	GGCTCGCATAGCGATAGAAC
Q-PCR PLB3 Fw	CCAATGGAGCTGGAGCTATC
Q-PCR PLB3 Rv	TGCAGTAGACCACCGAGATG
Q-PCR ACT Fw	TGCTCCTCCTGAGCGTAAAT
Q-PCR ACT Rv	ACATCTGCTGGAAGGTGGAC
Q-PCR β -TUB Fw	ACTTCCGCAATGGACGTTAC
Q-PCR β -TUB Rv	GGATGTTGTTGGGAATCCAC
5'-RACE PLB1	GTTCCTAGATACTCGAGGGGCGCA
3'-RACE PLB1	TGCGCCCCTCGAGTATCTAGGAAC
5'-RACE PLB3	GGTGGAGTTGCGGTACCCGTAGAA
3'-RACE PLB3	TTCTACGGGTACCGCAACTCCACC

1 Table 2
2 Influence of lecithin on gene expression of afPLBs^a

3

Gene	Expression level ^a		Fold induction
	- phospholipid	+ phospholipid	
<i>afplb1</i>	0.3 ± 0.1	1.4 ± 0.2	5
<i>afplb2</i>	1.0 ± 0.2	1.0 ± 0.2	0
<i>afplb3</i>	< 0.0001	0.04 ± 0.01	> 300

4

5 ^aThe expression of *afplbs* mRNA was determined by Real Time PCR after extraction of
6 mRNAs from *A. fumigatus* cultured at 37°C for 48 hours in Cz2 medium supplemented with
7 (+) or without (-) 0.2 % (w/v) lecithin. Results (means of six replicates) were normalized to
8 β-actin and are given relative to its expression. The experiment was performed twice with
9 similar results.

10

11

1 Table 3

2 Main features of the sequences of the cDNA and of the deduced protein of afPLBs

		afPLB1	afPLB2	afPLB3
cDNA	cDNA size	4826	4951	4925
	exons	3	3	1
	size of exons	114 nt	ND	4925
		657 nt	566 nt	
		1354 nt	ND	
	introns	2	2	0
	size of introns	102 nt	132 nt	-
		58 nt	59 nt	
	(genomic)- +1 ^a	(t)-A	ND	(c)-A
	5' UTR length	63 nt	ND	34 nt
	3' UTR length	160 nt	ND	101 nt
	ORF size	1899 nt	1764 nt	1890 nt
	STOP codon	UAA	UAG	UAG
	Protein	Predicted size ^b	633 aa	588 aa
Predicted mass ^b		68,143 Da	63,370 Da	67,448 Da
Theoretical pI ^b		4.59	5.11	5.22
Hydrophobicity ^c		N and C ter	none	N and C ter
Secretion signal ^d		Yes	No	Yes
Cleavage sequence ^d		..VSG ₂₀ / A ₂₁ P..	-	..ATA ₁₆ / T ₁₇ P..
Resulting mass ^b		66,202 Da	-	65,893 Da
Resulting pI ^b		4.55	-	5.14
Localization ^e		EC / PM	N	EC / PM
Probability ^e (%)		34.8 / 34.8	60.9	34.8 / 34.8
N-glycosylation ^f		18	-	14

3 ^aThe genomic nucleotide preceding the transcription initiation site (+1) is indicated in

4 bracket when determined.

1 ^bPhysical properties of deduced proteins (size, mass, pI) were determined using ProtParam
2 at the ExPASy web site.

3 ^cHydropathy analysis was performed accordingly to Kyte and Doolittle [18].

4 ^dSecretion signal and cleavage sequence were determined using SignalP v2.0 [20].

5 ^eLocalization is deduced from a PSORT II analysis [19]. EC stands for a extracellular
6 position (including a cell wall localization), N for a nuclear position and PM for a plasma
7 membrane localisation.

8 ^fN-glycosylation sites (N-{p}-[ST]) were determined using NetNGlyc 1.0 program.

9

10

11 LEGENDS of FIGURE:

12

13 Fig. 1. Alignment of the three afPLBs. The secretion signal peptide of afPLB1 and afPLB3
14 is indicated in bold and italicized. N-Glycosylation sites (N-{p}-[ST]) are indicated in bold.
15 Amino-acids of the catalytic triad identified in mammalian cytosolic PLA₂ are boxed and
16 indicated by an arrow above them.

17

18 Fig. 2. Phylogenic tree of various fungal PLBs. The tree (unrooted tree) was drawn with
19 TreeView, after a ClustalW alignment and a Phylip analysis using the Neighbor-joining
20 method (x, hypothetical protein; af, *Aspergillus fumigatus*; an, *Aspergillus nidulans*, ca,
21 *Candida albicans*; cg, *Candida glabratra*; cn, *Cryptococcus neoformans*; mg, *Magnaporthe*
22 *grisea*; nc, *Neurospora crassa*; um, *Ustilago maydis*). See Materials and Methods for
23 accession numbers.

FIGURE 1

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afPLB1 MKTTTVACAVAGLLFSCVSGAPDPVHVEIQQRALPNAPDGYTPSTVGC PASRPTIRSAASLSPNETSWLETRRGKTT SAMKDFFNHVKIQDFDAAGYIDR
afPLB2 -----MYKNRVELTTTAPVN---RALPNAPDGYTPQGETCPSKRPSIRNATALSSAETSWLKARRNNTKDALKAFLSRVLDLGSFNGSDYIAN
afPLB3 -----MKALLSLLTAVAVATATPLDLSLRALPNAPDGYTPAKVSCPATRPSIRGAGSLSPNETSWLEIRRKNTVEPMTDLLGRLNL-GFDAAGYIDR

afPLB1 HSSNSSDLPNIGIAVSGGGYRALMNGAGAIAKAFDSRTPNSTSAGQLGGLLQSATYLSGLSGGSWLVGSIYINNFTTISALQTHQKGTWVQFQNSIFEGPD
afPLB2 HSANASALPNIGIAVSGGGYRALMNGGALQAFDNRTTNSTHSGQLGGLLQSATYLSGLSGGSWLVGSIYMNNFSDVSSLQ--DNGSVWQFQDSIFSGPT
afPLB3 VSSNASLNPNI AIAVSGGGYRALTNGAGAIAKAFDSRTQGSTQSGHLGGLLQSATYVSGLSGGWLVGSVYLNFTT IADLQSGDHGNVWQFSTSILEGPK

afPLB1 GGSIQILDSATYYRDISNAVSGKSDAGYPTSITDYWGRALSYQMIN--ATNGGPSYTWSSIALTDAFQKAEMPMLVADGRYPGELLISSNATVYEFNP
afPLB2 QSTTWDIGTVEYYSQLLGAVD GKS NAGYEVSIITDYWGRSLSYQLIN--ASEGGVGYTWSSIALSKDFQAGTMPPLVIADGRAPGEILVPANTTVFEFNP
afPLB3 AKHLQFLSTADYWKDLLKAVD GKS DAGFNLSLTLDYWGRALSYQFINDRGTGNGGLSYTWSSIALTDPFRRGEMPLPILVADGRNPGELLIGSNSTVYEFNP

afPLB1 WEFGTFDPTVFGFAPLEYLGTKFNGGSVPSNESCVRGFDNVGFVGMTSSTLNFQFLLQINSTALPDWLKSVFTDILKDIGENDE DIAQYAPNPFYHFSNT
afPLB2 WEFGSWDKLSAFVSLEFLGSNFSKGTLATGEKCVRGFDNAGFIMGTSSSLFNQAF LQMNTDAPSVVKDAISAILGKIGSENNDIAVYKPNPFYRYASQ
afPLB3 WEFGSFDPSIFGFAPLEYLGS RFDNGQLPRGEP CVRGFDNAGFVGMTSSTLNFQFILRLNKTDLPLD LAKDVFSKILTAIGRDGDDIAVYGPNPFYGYRNS

afPLB1 TNPSAAELELDLVGGEDLQNIPLHPLIQPERHVDVIFAVDSSADTTYSWPNGTALVATYERSLNSSGIANGTSFPAIPDQNTFVNKGLNTRPTFFGCNS
afPLB2 SKYTSSP-SLTLVGGEDLQNIPLDPLLQQRHVDVILA V DSSADTTTRWPNGTSLVATYERNVDSSQRNSSLPFPSPVDQNTFVNLGLNTRPTFFGCNS
afPLB3 TAAYSRSRELDVVGGEDGQNIPLHPLIQPV R HVDVIFAVDSSADG PYSWPNGSALVATYERSLNSSGIANGTVFPAVPDVNTFVNLGLNTRPTFFGCDFP

afPLB1 SNTTGPSPLIVYLPNYPYTAYSNFTSTFPQPDYTEQERDSTILNGYDVVTMGNSTRDGNWSTCVGCALLSRSLERTNTNVPEICKQCFQRYCWDGSLNSTTP
afPLB2 SNATG-APLVVYIPNAPYIYPSNVSTFDLQYNTSERNALIE NGYDVATLGNGTVDSNWPA CLACAILSRSFERTNTTVPKTCSTCFKTYCWNGTINATTP
afPLB3 ANLSAPAPLVVYLPNAPYSTHSNFTSTFQLAYSDSERDEIITNGYNVVTRGDATVDKSWPSCVGCALLQRSMYRTNTSMPAVNCSCFKEYCWNGTVDKSTP

afPLB1 AGYEPVTILDSAASGIIPSISTVAMAVVFAAWTIF----
afPLB2 GDYYPTLKLH-----
afPLB3 RTYEPTLLLGSTSTNAAYTQGVTLVLSILAVGVAMGMTA

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