Identification of a Novel Variant Form of *Aspergillus fumigatus* CalC and Generation of Anti-CalC Monoclonal Antibodies

Shogo Takatsuka¹, Tatsuya Inukai¹, Shun Kawakubo¹,², Takashi Umeyama¹, Masahiro Abe¹, Keigo Ueno¹, Yasutaka Hoshino¹, Yuki Kinjo¹,²,³, Yoshitsugu Miyazaki¹ and Satoshi Yamagoe¹

¹ Department of Chemotherapy and Mycoses, National Institute of Infectious Diseases
² Department of Life Science and Medical Bioscience, Waseda University
³ Department of Bacteriology, The Jikei University School of Medicine
4 Jikei Center for Biofilm Science and Technology, The Jikei University School of Medicine

ABSTRACT

*Aspergillus fumigatus* is a critical human fungal pathogen that infects the host via inhalation of airborne conidia. These conidia then germinate to form filamentous hyphae, which secrete various elements to survive in the host lung. Elements such as proteins secreted by *A. fumigatus* can act as virulence factors in host tissues. Among secreted proteins, we were interested in the thaumatin-like proteins of *A. fumigatus*. In our analysis of the function of thaumatin-like proteins, we found that, like CalA and CalB, CalC has a secreted form. Originally, CalC was predicted to be a GPI-anchored protein, as documented in the *Aspergillus* Genome Database. Here, we report on a novel secreted form of CalC. Furthermore, we established two novel hybridomas, C103 and C306, which recognized CalC. Monoclonal antibodies produced by these hybridomas responded to recombinant CalC produced by the mammalian cell line HEK293T and to the supernatant of cultured *A. fumigatus*. Taken together, our data suggest that calC can be spliced to give rise to a novel secretory form of CalC, which is present in the supernatant of cultured *A. fumigatus*. The hybridomas that we established will be helpful in understanding the biological role of *A. fumigatus* CalC.

Key words: *Aspergillus fumigatus*, monoclonal antibodies, calA, calC, thaumatin

Introduction

*Aspergillus fumigatus*, an opportunistic fungal pathogen, causes invasive aspergillosis in immunocompromised patients⁷. In healthy individuals, *A. fumigatus* causes allergic bronchopulmonary aspergillosis. In all cases, *A. fumigatus* infects the host via inhalation of airborne conidia. These inhaled conidia germinate to form filamentous hyphae, which then secrete various elements to survive in the host lung.

These secretory elements can act as virulence factors or biomarkers.

To identify novel secretory elements of *A. fumigatus*, we narrowed down a list of candidate elements by *in silico* analysis using the *Aspergillus* genome database (AspGD) and FungiDB. During the screening process, we focused on thaumatin-like proteins in *A. fumigatus*. Thaumatin proteins are originally known as sweeteners derived from plants because they bind to G protein-coupled receptors in the taste buds. Besides, a member of the thaumatin-like proteins is present in some fungi, nematodes, and insects, as well as in plants. Recently, it was reported that CalA family members containing the thaumatin-like domain show characteristics of virulence factors such as adhesin (including invasin)²⁷. The CalA family proteins of *A. nidulans* are not only localized to the cell wall but also found in the culture medium⁷. Therefore, in the present study, we focused on one CalA family protein found in *A. fumigatus*, CalC (Afu3g00510), which has been predicted using bioinformatic analysis to show similarity to *A. fumigatus* CalA and CalB²⁷.

In the present paper, we report on a novel secreted form of CalC and note that this protein was detected in the supernatant of cultured HEK293T cells transfected with *calC* cDNA using anti-CalC polyclonal antibodies (pAbs). Furthermore, we...
report our attempt to produce anti-CalC monoclonal antibodies (mAbs) that would react with a native form of CalC by DNA immunization\(^\text{19}\) in order to understand the biological role of CalC. Two newly established hybridomas, C103 and C306, were selected as candidate clones that would recognize CalC. We found that mAbs produced by these hybridomas in fact responded to recombinant CalC produced by HEK293T cells and to the supernatant of cultured \textit{A. fumigatus}.

**Materials and methods**

**cDNA cloning**

\textit{A. fumigatus} MF-13\(^\text{7}\) was grown at 30°C in YPD medium for 2 d. Cells were frozen in liquid nitrogen and then disrupted using SK-mill (Tokken, Japan). RNA was prepared, and cDNA was synthesized using ISOGEN (NIPPON GENE, Japan) and ReverTra Ace (Toyobo, Japan). \textit{calc} cDNA amplification was performed using KOD-plus-DNA polymerase (Toyobo, Japan) and ReverTra Ace (Toyobo, Japan).

The following set of primers was used:

- **CalC-sense:**
  5'-GCCCTCGAGATGTTCTTTTCCAGACT CC-3'
- **CalC-antisense:**
  5'-GCCCTCGAGTCAGCCCAACGTAACCGTAATTC-3'

**3′ RACE-PCR**

\textit{calc} cDNA fragments were isolated from \textit{A. fumigatus} MF-13\(^\text{7}\) by the 3′- rapid amplification of cDNA ends (RACE) method. 3′ RACE was performed using the Full RACE Core Set (TaKaRa, Japan).

The following primers were used:

- **3′-RACE calC-198-sense:**
  5′-CGTAAACAAACTTGACA CCAC-3′
- **3′-RACE calC-374-sense:**
  5′-AATATACCAAAGCCACGC GACAC-3′

**Proteome analysis**

The proteomes of \textit{A. fumigatus} were retrieved from the \textit{Aspergillus} genome database AspGD\(^\text{9}\) (http://www.aspergillusgenome.org/) and FungiDB\(^\text{10}\) (http://fungidb.org/fungidb/) to identify thaumatin-like proteins.

**Generation of \textit{A. fumigatus} CalC-specific pAbs/mAbs**

Rabbit anti-\textit{A. fumigatus} CalC pAb was generated commercially (IWAKI & Co., Ltd., Japan) by immunizing rabbits with recombinant CalC produced in \textit{Escherichia coli} and isolating CalC-specific IgG by recombinant CalC-conjugated affinity column. Male BXSB/MpJ ms Slc-Yaa (Sankyo Lab Service, Japan) mice between 6-8 weeks old were immunized with DNA. Intramuscular injection of plasmids followed by electroporation was performed as previously described\(^\text{15}\).

**SDS-PAGE and western blot analysis**

SDS-PAGE and western blot analysis were performed as previously described\(^\text{15}\) with the following modifications. In brief, the supernatants of HEK293T cells transfected with \textit{A. fumigatus} \textit{calc} cDNA were separated by 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore Japan, Tokyo). Membranes were blocked for 1 h at 37°C with 5% skim milk and washed with 0.05% Tween 20 in PBS (washing buffer); then rabbit anti-CalC pAb (1 µg/mL) was laid on the membranes overnight at 4°C. The membranes were then incubated with peroxidase-conjugated anti-rabbit IgG (1: 15,000 dil, Amersham, USA). The membranes were washed three times with washing buffer; then the positive band was determined with SuperSignal\textsuperscript{TM} West Pico PLUS Chemiluminescent Substrate (Thermo Fisher, USA).

**mAb labeling**

C306 mAb labeling was performed using a Peroxidase Labeling Kit-NH2 (Dojindo, Japan).

**ELISA screening**

ELISA was performed as follows: flat-bottomed 96-well plates (Thermo Fisher, USA) were coated with AF293 supernatants cultured in RPMI1640 for 7 d and blocked with 3% FCS; then the supernatants of cultured hybridoma or 1µg/mL purified mAbs were added to individual wells. Bound Abs were revealed by HRP-conjugated goat anti-mouse IgG Ab in conjunction with TMB substrate (SURMODICS, USA). Absorbance at 450 nm was measured with a microplate reader (FilterMax; MOLECULAR DEVICES, Japan).

**Sandwich ELISA**

Sandwich ELISA was performed as follows: flat-bottomed 96-well plates were coated with 1 µg/mL purified C103 mAbs and blocked with 3% FCS; then the supernatants of HEK293T cells transfected with \textit{A. fumigatus} \textit{calc} cDNA and cultured for 2 d were added to individual wells. Captured CalC was revealed by HRP-conjugated C306 mAbs in conjunction with TMB substrate (SURMODICS, USA). Absorbance at 450 nm was measured with a microplate reader (FilterMax; MOLECULAR DEVICES, Japan). We used anti-OVA mAbs (mouse IgG2b) generated by DNA immunization as isotype-matched control mAbs. Then, we prepared the HRP-conjugated isotype-matched control mAbs using a Peroxidase Labeling Kit-NH2 (Dojindo, Japan) as well as C306-HRP.

**Results**

To identify novel secretory elements of \textit{A. fumigatus}, we focused on \textit{calc}, which, according to AspGD, is predicted to encode a signal sequence, a thaumatin-like domain protein, and a number of glycosylation residues. However, we could not isolate the \textit{calc} cDNA fragments (encoding 290 aa) registered in AspGD; instead, we isolated an unexpected \textit{calc} cDNA that encoded a different carboxyl-terminus (C-terminus). We deduced that this unexpected cDNA was a result of alternative splicing. We used the 3′-RACE method to confirm this. Each of the five \textit{calc} cDNA fragments obtained...
by the 3′-RACE method encoded the same C-terminus, as shown in Fig. 1a. In addition, we compared the splicing sites of \(\text{calC}\) between the registered Afu3g00510 and the newly identified site in the present study (Table 1). This variant cDNA form encodes 200 aa with a signal sequence, a thaumatin-like domain protein, and a number of glycosylation residues, as does the \(\text{calC}\) registered in AspGD. Additionally, we aligned and analyzed the sequences of CalA family members (Fig. 1b). Recently, it was reported that CalA is a secretory protein\(^3\). Likewise, CalB is also predicted to be a secretory protein by AspGD on account of its signal peptide. As shown by sequence alignment analysis, the CalC we isolated was broadly similar to CalA and CalB, but the CalC registered in AspGD contains extra sequences in the C-terminus. It is known that the structure of signal sequences and the functioning of the secretory machinery are well conserved from prokaryotes to eukaryotes\(^{12}\). We finally confirmed that the CalC we isolated was a secretory protein based on detection of CalC in the supernatant of HEK293T cells transfected with a CalC expression vector using anti-CalC pAb (Fig. 1c).

**DNA immunization and hybridoma screening**

Next, we attempted to produce anti-CalC mAbs that would react with the native form of CalC by DNA immunization (Fig. 2a). Two male BXSB/MpJj ms Slc-Yaa mice were immunized simultaneously with plasmids encoding \(\text{calC}\) and \(\text{E. coli}\) GroEL, as described in the Materials and Methods. Antisera from the two mice were raised against recombinant CalC produced in \(\text{E. coli}\). Anti-recombinant CalC IgG titers were detected in a 1:10\(^6\) dilution (Fig. 2b). Then, we established hybridomas prepared by conventional methods using splenocytes from immunized mice and SP2/0 myeloma. In order to select the hybridoma secreting IgG mAbs reactive to CalC, we performed ELISA with the supernatant of cultured \(\text{A. fumigatus}\) Af293 (Af293) and the supernatant of cultured \(\text{C. albicans}\) SC5314 as a negative control (Fig. 2c).

### Table 1. The position of introns CDSs* of CalC

| CDS  | Afu3g00510 | Present study |
|------|------------|---------------|
| 1-588| 1-603      |               |
| 589-842|          |               |
| 843-932|          |               |
| 933-1045|         |               |
| 1046-1240|        |               |

*CDS: coding sequence
Recognition of *A. fumigatus* CalC

As we established two hybridomas, C103 and C306, that secreted mAbs that recognized CalC, we verified whether they could be used for sandwich ELISA. We used C103 as the capture antibody and HRP-conjugated C306 as the detection antibody in a sandwich ELISA to detect recombinant CalC produced by HEK293T cells. Recombinant CalC produced by HEK293T cells could be detected in a dose-dependent manner (Fig. 3a). Furthermore, C103 and C306 reacted with the supernatant of cultured Af293, but not with the supernatant of cultured SC5314 (Fig. 3b).
Discussion

Elements secreted by pathogens can act as virulence factors in host tissues. There have been a number of reports of secretory elements, such as hydrolytic enzymes, mycotoxins, and adhesins (including invasin). Previous studies have reported that CalA exhibits adhesive abilities. So far, however, the biological functions of CalC have not been determined. As with calA, the calC we identified in the present study encoded a thaumatin-like domain protein. Additionally, sequence alignment analysis showed that the CalC we identified was different from the predicted sequence documented in AspGD. We hypothesized that the calC we identified encoded a novel variant form as a result of alternative splicing. In practice, the expression of spliced forms of calC may be changed by altering the culture conditions, culture time, or strains of *A. fumigatus*. Although the CalC was detected as a smear band around 30 kDa by western blotting in Fig.1c, the estimated molecular weight was about 20 kDa. We considered that the smear band was caused by posttranslational modification of CalC as a result of high glycosylation. The CalC registered in AspGD is predicted to be a GPI-anchored protein. However, we showed that the variant form of CalC has the potential to be a secretory protein for the following reasons. First, the variant was a form of CalC registered in AspGD with a deletion at the C-terminus. Second, the C-terminus of the variant is similar to that of other CalA family proteins. Other CalA family proteins were localized not only to the cell wall but also in the culture medium. Third, our results also provided evidence that the CalC we identified was detected in the supernatant of HEK293T cells transfected with CalC using anti-CalC pAb. However, we did not detect an endogenous CalC in the supernatant of *A. fumigatus* Af293 by western blot analysis using anti-CalC pAb (data not shown). This indicates that the expression of endogenous CalC in the supernatant of *A. fumigatus* Af293 might be lower than the detection limit of western blot analysis using anti-CalC pAb. On the other hand, the two newly established hybridomas in the present study reacted with CalC. C103 and C306 recognized recombinant CalC produced by HEK293T cells, but it is unclear whether C103 and C306 recognized the native form of CalC produced by *A. fumigatus*. Since we were not able to prepare the intact CalC produced by *A. fumigatus*, we looked into the possibility that C103 and C306 recognized the supernatant of cultured *A. fumigatus* in the present study. Furthermore, we have confirmed that C103 and C306 do not respond to the supernatant of cultured CalC-deficient *A. fumigatus* (manuscript in preparation). In future studies, it is necessary to evaluate whether C103 and C306 properly recognize the intact CalC produced by *A. fumigatus*.

Taken together, our data suggest that calC can be spliced to give rise to a novel secretory form of CalC. Moreover, if the role of CalC as a virulence factor or biomarker can be established, anti-CalC pAb and mAbs may be useful for neutralizing the function or detecting the protein in various situations. Anti-CalC pAbs and mAbs may be applicable for immunodiagnostic kits or other biological tools in the future.

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Conflicts of interest

All authors declare that they have no competing interests.

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