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Protein targets for broad-spectrum mycosis vaccines: quantitative proteomic analysis of *Aspergillus* and *Coccidioides* and comparisons with other fungal pathogens

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***Aspergillus* species are responsible for most cases of fatal mold infections in immunocompromised patients, particularly in those receiving hematopoietic stem cell transplants. Experimental vaccines in mouse models have demonstrated a promising avenue of approach for the prevention of aspergillosis, as well as infections caused by other fungal pathogens, such as *Coccidioides*, the etiological agent of valley fever (coccidioidomycosis). Here, we investigated the hyphal proteomes of *Aspergillus fumigatus* and *Coccidioides posadasii* via quantitative MS^E mass spectrometry with the objective of developing a vaccine that cross-protects against these and other species of fungi. Several homologous proteins with highly conserved sequences were identified and quantified in *A. fumigatus* and *C. posadasii*. Many abundant proteins from the cell wall of *A. fumigatus* present themselves as possible cross-protective vaccine candidates, due to the high degree of sequence homology to other medically relevant fungal proteins and low homologies to human or murine proteins.**

Keywords: quantitative proteomics; mass spectrometry; panfungal vaccine; *Aspergillus*; *Coccidioides*

Introduction

Pathogenic fungi, particularly the mold *Aspergillus fumigatus*, cause thousands of deaths per year,[1] mostly among immunosuppressed patients, such as cancer patients receiving hematopoietic cell transplants for the treatment of leukemia and other hematologic malignancies.[2] Aspergillosis is not limited to these patients though, as it occurs in other cases such as premature infants,[3] burn victims,[4] AIDS patients,[5] and patients in intensive care settings.[6] *Candida albicans*, as well as many other less prevalent fungal species, can infect the same patient populations.[2] *Coccidioides* spp. are responsible for valley fever (coccidioidomycosis), which is endemic in the southwestern United States, Mexico, and parts of South America, and can infect immunocompetent, but susceptible individuals.[7,8] Current antifungal drugs have limited effectiveness,[9] and *A. fumigatus*[10] and other species[11,12] are becoming resistant to our first-line treatments. Thus, the need exists for better control and prevention of fungal infections.

Experimental vaccines have shown considerable promise for the prevention of fungal infections. In *A. fumigatus* infection models, the protein Asp f3 (Pmp20) has been used to successfully prevent death

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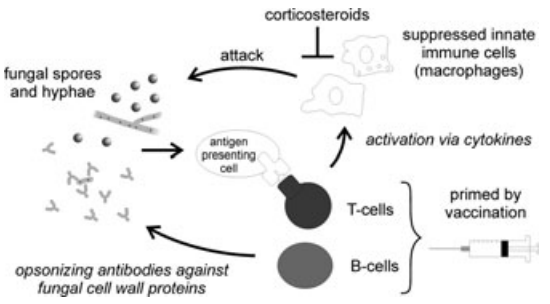


Figure 1. **Proposed mechanism of antifungal T cell–based vaccines under conditions of corticosteroid immunosuppression.**

of mice that were immunosuppressed with corticosteroids.[13,14] Similarly, a homolog of Asp f3, Pmp1 from *Coccidioides posadasii*, was effective in a murine model of coccidioidomycosis.[15] Another coccidioidal protein, Pep1, was also successful in protecting mice against *C. posadasii* challenge,[16] and Pep1 has an *A. fumigatus* homolog as well. Recently, the *A. fumigatus* protein Crf1 was shown to be cross-protective against experimental infections with *A. fumigatus* or *C. albicans*.[17] In addition, extracts from *Saccharomyces cerevisiae*, as well as whole heat-killed yeast cells, were shown in murine models to provide cross-protection against systemic *A. fumigatus* infection,[18,19] coccidioidomycosis,[20] candidiasis,[21] and cryptococcosis.[22]

Vaccine protection in these mouse models appears to function primarily via a Th1 mechanism, in which $CD4^+$ T cells are presented fungal antigens by antigen-presenting cells, such as dendritic cells or other phagocytes (Fig. 1). The activated T cells then stimulate phagocytes (primarily macrophages),[14,17,23] mainly via the cytokine interferon (IFN)-γ.[24] Activation with this cytokine increases the antifungal activity of the phagocytic effector cells and overcomes corticosteroid-induced immunosuppression.[25] Protective T cell transplants have been demonstrated successfully in immunosuppressed mice.[26] For example, protection was transferred from Asp f3–vaccinated animals into nonimmunized mice by transplantation of $CD4^+$ T cells, while transfer of Asp f3–specific antibodies was not protective.[14] However, antibodies could potentially enhance vaccine-derived immunity against fungi,[27,28] particularly when the targeted fungal antigen is located on the cell surface. Antibodies against such cell surface proteins could assist in opsonization of fungal cells by phagocytes, which in turn would enhance the major histocompatibility complex presentation of specific antigens that can be recognized by vaccine-primed T cells (Fig. 1). In contrast, Asp f3 is an intracellular, peroxisomal protein not accessible to humoral antibodies, which may explain the lack of protection observed after transfer of Asp f3–specific antibodies into nonimmunized mice.[14]

An ideal vaccine would be cross protective against multiple species of fungi and would contain conserved homologous antigens (epitopes) in the fungal cell walls that elicit both antibody and T cell responses. Such antigens should also be as foreign to mammals as possible, to enhance their chance of being sufficiently immunogenic and to avoid triggering autoimmunity. To systematically discover promising potential vaccine candidates with these characteristics, we examined the total proteome of *A. fumigatus* using MS^E (mass spectrometry-elevated collision energy), a mass spectrometry technique allowing for label-free quantification of large numbers of proteins in complex samples.[29–33] Proteins identified from different fractions of *A. fumigatus* were quantified, and their subcellular protein localization was predicted. The amino acid sequences of promising candidates were compared to homologous proteins from other medically relevant fungal species, as well as mouse and human. *C. posadasii* hyphal protein extract was MS^E analyzed as well. Among filamentous fungi, *Coccidioides* is closely related to *Aspergillus*, and also in need of a vaccine.[28] Protection by the homologous vaccine candidates Asp f3[14] and Pmp1[15] against *A. fumigatus* and *C. posadasii*, respectively, indicates that *C. posadasii* may be a good target for finding potential vaccine candidates that are cross-protective against *A. fumigatus*.

Methods

Culture conditions

A. fumigatus strain AFCOH1 isolated from a patient at City of Hope (Duarte, California) and *C. posadasii* strain Silveira were used for all experiments. The AFCOH1 strain has continuously proven to be sufficiently virulent in animal models, and mass spectrometric analysis of its proteins has always matched available sequence data with excellent scoring quality. For growth of *A. fumigatus,* either Czapek Dox (CD) medium (a minimal medium, Difco, Detroit, MI) or potato

dextrose (PD) medium (a rich medium, Difco, Detroit) was inoculated with 10^7 conidia/mL at 37 °C. Cultures were harvested in the late growth phase, i.e., after 24 h for PD medium and nine days for CD medium. *C. posadasii* was grown in a broth of 2% glucose and 1% yeast extract for five days at ambient temperature.

Sample preparation

A. fumigatus hyphal sonicate (HS) was prepared by cryogenic grinding of liquid nitrogen frozen dried hyphae with a MM301 ball mill (Retsch, Haan, Germany), followed by resuspension and sonication using a Sonicator 3000 (Misonix, Farmingdale, NY). *C. posadasii* hyphal extracts were prepared by collection of hyphae by filtration, suspension in phosphate buffered saline (PBS) and vortex mixing with glass beads. Homogenates were clarified by centrifugation and supernatants filter sterilized using 0.22 μm syringe filters. *A. fumigatus* culture filtrate (CF) was obtained by filtering spent fungal growth media through 0.22 μm PVDF pores, freezing the culture filtrate in liquid nitrogen, lyophilizing the frozen pellets, and filtering through Amicon centrifugal filter units with 10 kDa molecular weight cutoff (Millipore, Billerica, MA). The retentate was suspended to concentrate CF proteins 300×. Cell wall (CW) extract was obtained by digestion of hyphae for eight hours at 30 °C in a cell wall digestion cocktail containing 10 mg/mL 1-3-β-glucanase (Interspex, San Mateo, CA), 5 mg/mL Driselase (Interspex, San Mateo, CA), and 200 U/mL Lyticase (Sigma, St. Louis, MO), followed by centrifugation and filtration of the supernatant through a Millex PVDF filter with 0.22 μm pores (Millipore, Billerica, MA).

Mass spectrometry

Proteins were precipitated in 10% trichloroacetic acid, denatured with trifluoroethanol, reduced with tris-(2-carboxyethyl)-phosphine, alkylated with iodoacetamide, and digested overnight with trypsin (sequencing grade modified) (Promega, Madison, WI). Human serum albumin or rabbit glycogen phosphorylase was added as a standard to each sample for label-free quantification. MS^E was conducted using a SYNAPT G2 HD Q-TOF mass spectrometer, equipped with a UHPLC and an ion mobility 2D separation unit (Waters, Milford, MA). Data were analyzed and proteins tabulated using the Protein Lynx Global Server (PLGS) (Waters, Milford, MA).

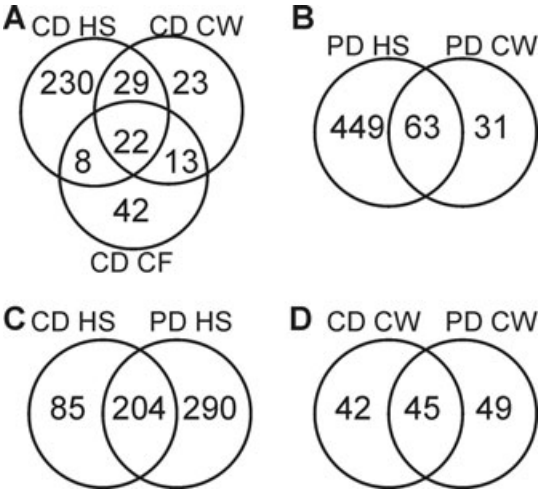


Figure 2. Comparison of proteins detected in different *A. fumigatus* fractions and growth media. Comparisons are among (A) HS, CF, and CW in CD medium, (B) HS and CW in PD medium, (C) HS from CD and PD media, and (D) CW from CD and PD media.

Protein analysis

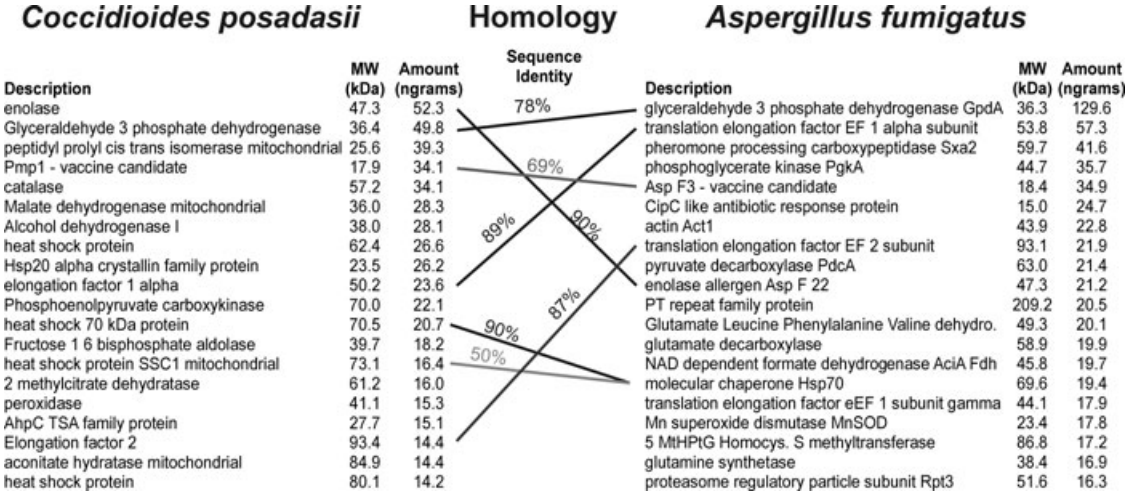
When conducting cross-comparisons of different fungal fractions, proteins with a PLGS score of less than 500 were excluded from the analysis. Scaffold (Proteome Software, Portland, OR) was used for quantification of proteins. For homology analysis, NCBI's BLAST[34] was used to obtain percent identity and percent sequence alignment coverage between different proteins. Protein localization was predicted using the WoLF PSORT tool.[35]

Results

Mass spectrometry of A. fumigatus *proteins*

The 20 most abundant proteins from each *A. fumigatus* fraction were quantified using Scaffold, including HS/CD medium (Table S1), CF/CD medium (Table S2), CW/CD medium (Table S3), HS/PD medium (Table S4), and CW/PD medium (Table S5). Only proteins with WoLF PSORT-predicted extracellular localization are displayed in CW fractions.

In total, 367 proteins were detected in the three fractions of *A. fumigatus* grown in CD medium (Table S6). There were 289 proteins in the HS fraction, 85 in the CF fraction, and 87 in the CW fraction (Fig. 2A). Many proteins detected in the CW and HS fractions, but not CF, do not have predicted extracellular localization, and the bulk of these proteins



Description	MW (kDa)	Amount (ngrams)
enolase	47.3	52.3
Glyceraldehyde 3 phosphate dehydrogenase	36.4	49.8
peptidyl prolyl cis trans isomerase mitochondrial	25.6	39.3
Pmp1 - vaccine candidate	17.9	34.1
catalase	57.2	34.1
Malate dehydrogenase mitochondrial	36.0	28.3
Alcohol dehydrogenase I	38.0	28.1
heat shock protein	62.4	26.6
Hsp20 alpha crystallin family protein	23.5	26.2
elongation factor 1 alpha	50.2	23.6
Phosphoenolpyruvate carboxykinase	70.0	22.1
heat shock 70 kDa protein	70.5	20.7
Fructose 1 6 bisphosphate aldolase	39.7	18.2
heat shock protein SSC1 mitochondrial	73.1	16.4
2 methylcitrate dehydratase	61.2	16.0
peroxidase	41.1	15.3
AhpC TSA family protein	27.7	15.1
Elongation factor 2	93.4	14.4
aconitate hydratase mitochondrial	84.9	14.4
heat shock protein	80.1	14.2

Description	MW (kDa)	Amount (ngrams)
glyceraldehyde 3 phosphate dehydrogenase GpdA	36.3	129.6
translation elongation factor EF 1 alpha subunit	53.8	57.3
pheromone processing carboxypeptidase Sxa2	59.7	41.6
phosphoglycerate kinase PgkA	44.7	35.7
Asp F3 - vaccine candidate	18.4	34.9
CipC like antibiotic response protein	15.0	24.7
actin Act1	43.9	22.8
translation elongation factor EF 2 subunit	93.1	21.9
pyruvate decarboxylase PdcA	63.0	21.4
enolase allergen Asp F 22	47.3	21.2
PT repeat family protein	209.2	20.5
Glutamate Leucine Phenylalanine Valine dehydro.	49.3	20.1
glutamate decarboxylase	58.9	19.9
NAD dependent formate dehydrogenase AciA Fdh	45.8	19.7
molecular chaperone Hsp70	69.6	19.4
translation elongation factor eEF 1 subunit gamma	44.1	17.9
Mn superoxide dismutase MnSOD	23.4	17.8
5 MtHPtG Homocys. S methyltransferase	86.8	17.2
glutamine synthetase	38.4	16.9
proteasome regulatory particle subunit Rpt3	51.6	16.3

Figure 3. Homology between the 20 most abundant *A. fumigatus* and *C. posadasii* proteins. Quantification is per 1 μg of total protein.

in the CW fraction likely are contamination due to imperfect CW extraction methods. Of the most abundant 20 proteins in the CF, only one, XP˙749979.1, a protein of unknown function with cyanovirin-N homology (CVNH) domains, lacked extracellular localization according to WoLF PSORT. This small 11.8 kDa protein may lack a known extracellular signal. Furthermore, an independent study of the secreted proteome of *A. terreus* also identified a homolog of this protein.[36]

With *A. fumigatus* grown in PD medium, 494 proteins were detected in HS and 94 in CW fractions (Table S7); 63 proteins were detected in both fractions (Fig. 2B). Although HS from *A. fumigatus* grown in CD and PD contained many of the same proteins, a considerably greater number of proteins were detected in HS from growth in PD medium (Fig. 2C). This may be because the most abundant proteins in HS from growth in CD medium represented a greater proportion of the total protein than they did in HS from growth in PD medium. Although the majority of the 20 most abundant proteins in the CD and PD HS samples were detected in both samples, the 20 most abundant proteins in each HS sample had only four proteins in common. The number of proteins detected in CD and PD CW samples was approximately equal (Fig. 2D), with many of the proteins found in both samples. Of note, the abundant proteins major allergen Asp f2 (expressed during zinc-limiting conditions at higher pH[37]), catalase B, and L-amino acid oxidase LaoA were not found in CW from growth in PD medium, and the abundant protein pheromone processing carboxypeptidase Sxa2 was not found in CW from growth in CD medium.

Proteomic comparison of A. fumigatus *to* C. posadasii

We detected 314 proteins via quantitative MS^E mass spectrometry in our *C. posadasii* extracts (Table S8), which was compared to the HS proteome *A. fumigatus*. The percent sequence identity of homologous regions of the 20 most abundant proteins in each fraction is shown in Figure 3. Five of the most abundant 20 *A. fumigatus* HS proteins had homologues in the top 20 *C. posadasii* HS proteins. Of note, the *A. fumigatus* protein Asp f3 (Pmp20), which has been successfully used to vaccinate mice against aspergillosis,[14] had a homolog in *C. posadasii* protein Pmp1 with 69% sequence identity. Pmp1 also protected mice against coccidioidomycosis when used as a vaccine.[15]

Interspecies protein homologies

Twenty cell wall proteins of interest were analyzed using NCBIs BLAST to determine potential homology with proteins from other medically relevant fungi, including *C. albicans, Cryptococcus neoformans, S. cerevisiae*, *Acremonium alcalophilum, Mucor circinelloides, Rhizopus oryzae, Penicillium marneffei, Fusarium oxysporum,* and *C. posadasii*, as well as homology with human and mouse

Table 1. Interspecies homology of select *A. fumigatus* cell wall proteins[a]

Protein	Accession[b]	kDa	Hs	Mm	Ca	Cn	Sc	Aa	Mc	Ro	Pm	Fo	Cp
Sxa2	XP_749486.1	59.8	29/56	36/27	28/87	60/100	27/81	29/76	28/85	25/84	64/100	40/93	47/90
All2	P79017.2	32.8	0	0	42/99	0	30/65	53/74	0	0	0	49/87	50/98
Crf1	Q8J0P4.2	40.3	0	33/26	48/66	32/56	49/62	48/67	38/43	34/42	67/65	59/67	49/66
Bys1	XP_748143.1	16.0	0	0	0	0	0	51/86	0	0	42/97	30/85	58/87
Ecm33	Q4WNS8.1	41.5	0	0	30/86	0	28/85	32/82	30/75	28/80	49/100	32/55	46/92
EglC	Q4WG16.1	44.7	0	0	27/61	0	30/56	57/65	26/54	25/62	65/70	62/65	28/61
GpiX[c]	XP_751929.1	39.6	0	0	0	0	0	0	0	0	55/100	0	49/45
Alp2	P87184.1	52.6	32/81	33/68	60/84	51/83	54/90	66/90	53/86	52/89	76/100	66/96	73/99
Gel4	P0C956.1	58.9	0	0	54/83	41/85	53/85	47/80	45/81	44/78	52/92	59/44	52/98
AbfB	Q4WL66.1	52.6	0	0	0	0	0	0	0	27/29	82/100	77/98	0
PHOa	Q8X176.1	49.1	0	0	0	0	0	0	43/72	45/66	66/87	52/78	0
CbhA	Q4WNA2.1	48.1	0	0	0	0	0	51/100	0	0	64/99	63/100	0
Al15	O60022.1	16.0	0	0	0	0	0	57/71	0	0	41/94	52/76	46/97
CpdS	XP_731524.1	57.1	29/70	28/70	29/69	41/85	30/68	26/95	31/76	28/66	69/91	45/85	52/85
CatB	Q92405.1	79.9	43/51	40/61	41/53	45/86	40/49	59/99	45/98	47/92	70/100	61/100	49/67
LaoA	XP_748830.1	78.6	30/26	25/72	0	0	0	35/98	35/96	0	0	72/80	25/46
Suc2p	XP_749260.1	57.3	0	0	0	49/97	49/63	0	0	0	0	48/97	0
Pep1	P41748.2	38.0	30/80	30/81	28/79	30/89	31/79	39/77	35/93	39/89	48/100	52/97	41/83
ChiA1	XP_747968.1	88.6	0	0	37/38	0	0	0	32/34	38/33	56/35	40/27	56/38
Crf2	Q4WI46.1	46.7	0	0	43/98	41/76	47/78	51/73	38/44	39/41	56/97	54/73	56/93

[a]Each cell lists % identity/% length of alignment against the most significant protein match. [b]XP accessions from NCBI REFSEQ. Others are from UniProtKB. [c]GpiX refers to an uncharacterized GPI-anchored cell wall protein. Hs, *Homo sapiens*; Mm, *Mus musculus*; Ca, *Candida albicans*; Cn, *Cryptococcus neofomans*; Sc, *Saccharomyces cerevisiae*; Aa, *Acremonium alcalophilum*; Mc, *Mucor circinelloides*; Ro, *Rhizopus oryzae*; Pm, *Penicillium marneffei*; Fo, *Fusarium oxysporum*; Cp, *Coccidiodes posadasii*

proteins (Table 1). Proteins with both % identity and percent coverage less than 25% were listed as 0, representing nonhomologous proteins. Many proteins were found to share high degrees of homology (>50% identity and >70% coverage) with proteins from other fungi, while retaining low homology (<35% identity and <30% coverage) with human or mouse proteins. Such proteins are potential broad-spectrum antifungal vaccine candidates. Crf1, a highly abundant *A. fumigatus* CW protein, has already shown cross-protection against *A. fumigatus* and *C. albicans* in mouse models of infection, demonstrating the feasibility of a cross-protective vaccine.[17]

Discussion

Although several non-cell wall proteins existed in our *A. fumigatus* CW fractions (of which NAD-dependent malate dehydrogenase, ubiquitin, spermidine synthase, cytochrome b5, and a hypothetical protein were the most abundant), cell wall proteins did appear to be highly enriched in these fractions compared to other proteins. Over one-half of the most abundant proteins in this fraction have predicted extracellular localization, which includes cell wall proteins. Several proteins with predicted extracellular localization were also detected in CF, but such proteins were usually also detected in HS as well, and many of these proteins have known cell wall localization. Furthermore, many abundant CF and HS proteins were not detected in CW fractions. This implies that any noncell wall protein contaminants in our CW fractions are only a particular subfraction of other *A. fumigatus* proteins. Thus, we may hypothesize that certain abundant cell wall proteins may ultimately be released into the extracellular environment, either to serve a specific function or to simply be lost during cell wall remodeling. Finally, several proteins lacking extracellular localization prediction from WoLF PSORT may in fact still be cell wall proteins with unknown export and translocation pathways. A study of the conidial cell wall proteome of *A. fumigatus* revealed similar proteins lacking classical signal peptides.[38] The proteins discovered in conidia have very little overlap with the cell wall proteins we encountered in hyphae, implying that the cell wall proteome undergoes major changes between the conidia and hyphae stages of the fungal lifecycle. This change in the cell wall proteome may result

in an antifungal vaccine against one morphologic form being ineffective against another form (i.e., a vaccine against conidial cell wall might not induce protection against the hyphal cell wall). It may therefore be desirable to develop vaccines using antigens expressed in both early and later stages of the fungal lifecycle. Our protein identifications from HS fractions were very similar to previous studies.[39,40] One study on the secreted proteome of *A. fumigatus* discovered many proteins detected in our CF fraction, but also several proteins with intracellular localization.[41]

We found the cell wall proteome of *A. fumigatus* to be rich in proteins related to carbohydrate digestion and cell wall structure formation and maintenance. Indeed, the 20 most abundant proteins from growth in CD and PD media CW fractions contained many proteins in this category, including Crf1, Bys1, EglC, Ecm33, CbhA, AbfB, Gel4, and Gel1. Several other less abundant proteins of this category were detected in the CW fraction from growth in each medium type individually, as were several proteins related to protein digestion and processing. Although the cell wall proteome was very similar during growth in CD or PD medium, there were significant differences in expression of proteins related to peptide processing and digestion. Asp f2 (All2) and LaoA were found only in CW from growth in CD medium, while Sxa2 was found only from growth in PD CW fraction. PD contains tryptic digest of casein, and CD contains no peptides, which could account for this difference.

When determining optimal proteins for vaccine candidates against fungal infection, it is essential to ensure that such a protein is expressed at high levels during pulmonary infection, because lung infections are most relevant and severe. Thus, proteins that are expressed under growth conditions simulating both a rich and a minimal supply of nutrients should be considered the best candidates for vaccine trials. Ideally, the *A. fumigatus* proteome could be assessed under conditions similar to those found during lung infection.

A high level of homology between proteins in different fungal species is another essential aspect of a cross-protective vaccine candidate. Our study comparing *A. fumigatus* and *C. posadasii* hyphal extracts indicates that several abundantly expressed proteins have high degrees of homology between these species. The same argument may apply to fungal cell wall proteins, and our preliminary data on the cell wall proteome of *A. terreus* agrees with this notion (data not shown). If these proteins have identical or highly similar and protective epitopes, they could serve as cross-protective vaccines. The most promising candidates would have relatively low homology to human and mouse proteins, and also high homology to proteins from *C. albicans*, second only to *A. fumigatus* as a cause of lethal invasive fungal infections. Therefore, our best potential cross-protective vaccine candidates include Crf1, Ecm33, Alp2, Gel4, and Crf2.

Animal vaccination experiments are currently planned or underway using recombinant Sxa2, Asp f2, Crf1, Bys1, Ecm33, EglC, GpiX, Alp2, Gel4, PHOa, and Crf2. Also planned is a broader mass spectrometry study to compare the entire proteome of many different species of pathogenic fungi, including filamentous fungi and yeast.

Acknowledgments

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Conflicts of interest

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Most abundant 20 proteins in hyphae sonicate of *A. fumigatus* grown in Czapek Dox medium.

Table S2. Most abundant 20 proteins in culture filtrate of *A. fumigatus* grown in Czapek Dox medium.

Table S3. Most abundant 20 proteins in cell wall of *A. fumigatus* grown in Czapek Dox medium.

Table S4. Most abundant 20 proteins in hyphae sonicate of *A. fumigatus* grown in Potato Dextrose medium.

Table S5. Most abundant 20 proteins in cell wall of *A. fumigatus* grown in Potato Dextrose medium.

Table S6. List of proteins in hyphae sonicate (HS), culture filtrate (CF), and cell wall (CW) extracts of *A. fumigatus* grown in Czapek Dox medium. PLGS (Protein Lynx Global Server) score is a measure of the quality of the mass spectrometry–based protein identification.

Table S7. List of proteins in hyphae sonicate (HS) and cell wall (CW) extracts of *A. fumigatus* grown in Potato Dextrose medium. PLGS (Protein Lynx Global Server) score is a measure of the quality of a mass spectrometry–based protein identification.

Table S8. List of proteins in hyphae extract of *C. posadasii*.

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