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Development of a Ligand-Directed Approach To Study the Pathogenesis of Invasive Aspergillosis

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Invasive aspergillosis is a leading cause of infectious death in immunosuppressed patients. Here, we adapted a phage display library-based selection to screen and identify binding peptides to the surface of *Aspergillus fumigatus* conidia and hyphae. We identified a peptide (sequence CGGRLGPFC) that reliably binds to the surface of *Aspergillus fumigatus* hyphae. Binding was not *Aspergillus* strain specific, as it was also observed in hyphae of other *Aspergillus* clinical isolates. Furthermore, CGGRLGPFC-displaying phage targets *Aspergillus fumigatus* hyphae on formalin-fixed paraffin-embedded histopathology sections of lung tissue recovered from mice with invasive pulmonary aspergillosis. This approach may yield reagents such as peptidomimetics for novel diagnostic and therapeutic interventions in invasive aspergillosis.

Invasive aspergillosis, mainly caused by *Aspergillus fumigatus*, is the most common opportunistic mycosis in immunosuppressed patients with leukemia, bone marrow and solid-organ transplant recipients and a frequent cause of morbidity and mortality (17, 20). As the pathogenesis of invasive aspergillosis involves inhalation of airborne conidia in susceptible hosts, pneumonia is the most common clinical manifestation (17, 20). As in other filamentous molds, there are two developmental programs of growth of *Aspergillus* species: conidia and hyphae (4, 20). *Aspergillus* conidia are continuously inhaled from the environment and, in normal hosts, most are efficiently phagocytosed by resident lung macrophages (4, 20). Some conidia escape phagocytosis and germinate to hyphae, the invasive form of *Aspergillus* species. Hyphae are then destroyed by neutrophils in immunocompetent hosts (4, 20).

The high mortality of invasive aspergillosis reflects the severe net state of immunosuppression of affected patients, delayed diagnosis, and the suboptimal in vivo efficacy of antifungal agents against *Aspergillus* species (17, 20). Hence, the combination of early detection along with targeted delivery of antifungal agents to the site of infection early on, when the tissue fungal burden is relatively low, could be of critical importance in improving the outcome of invasive aspergillosis.

Biopanning and Rapid Analysis of Selective Interactive Ligands (BRASIL) is an approach for the screening and identification of cell-surface-binding peptides from phage display libraries (10). Phage display technology has been used to identify vascular receptors in tumors and to characterize protein interacting sites in the context of mammalian cells (2, 13, 14, 27). However, this methodology has not as yet been applied to the study of infections such as invasive aspergillosis. We hypothesized that the surfaces of conidia and hyphae of *Aspergillus fumigatus* may contain binding sites that can be accessible and targeted by binding peptides.

In the present study, we first identify peptide ligands that bind in vitro to the surface of conidia and hyphae of *Aspergillus fumigatus*. Then, we show that a cyclic peptide motif (sequence CGGRLGPFC) binds to hyphae of several clinical mold isolates in vitro and to *Aspergillus fumigatus* hyphal elements present in lung tissue recovered from a murine model of invasive pulmonary aspergillosis. These data suggest that a ligand-directed system to targeting lesions in invasive aspergillosis can be developed by a combinatorial selection approach.

MATERIALS AND METHODS

Fungal isolates. We tested the *Aspergillus fumigatus* clinical isolate 293 (AF293) used in the *Aspergillus* sequencing project (http://www.sanger.ac.uk/Projects/A_fumigatus/) (7). We also tested a panel of mold isolates (three *Aspergillus fumigatus* isolates [AF66, AF76, and AF101], two *Aspergillus terreus* isolates [AT11 and AT48], two *Aspergillus flavus* isolates [AF117 and AF122], two *Aspergillus niger* isolates [AN42 and AN75], and two *Rhizopus* isolates [Z160 and Z161]) recovered from cancer patients presenting with invasive mycoses at The University of Texas M. D. Anderson Cancer Center.

To obtain conidia, AF293 was plated on yeast agar glucose plates (0.5% yeast extract, 1.0% dextrose, 0.2% vitamin mixture, 0.1% trace elements, 1.5% agar, 1% $MgSO_4$) at 37°C; conidia were harvested 3 days later, counted by using hemocytometry, and suspended in phosphate-buffered saline (PBS). To obtain hyphae, AF293 conidia (suspensions of 10^4 conidia/ml) were incubated in liquid yeast agar glucose medium at 37°C for 16 to 20 h, which allowed the transformation of >95% of conidia to hyphae with lengths varying from 50 to 70 μm as determined by using an inverted microscope. Following centrifugation, AF293 hyphae were suspended in PBS.

Biopanning and rapid analysis of selective interactive ligands (BRASIL). We screened a phage display library for AF293 conidium and hypha binding ligands by the BRASIL method (1, 10). The phage library that was used in the present study contained the insert CX_7C (C, cysteine; X, any amino acid residue). It was

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derived from a large-scale preparation of a phage random peptide library and was designed to display a constrained cyclic loop within the pIII capsid protein (28, 33). The preparation was optimized to create the highest possible insert diversity; thus, the diversity of displayed peptides in the library was approximately 10^8 to 10^9 (1). All phage clones of the library are evaluated in vitro to rule out a selective growth advantage (rather than binding capability) that may explain the enrichment observed during the rounds of panning. This is obviously a routine control in all experiments with phage display screenings. As such, there is no growth advantage for any of the clones in this library.

Conidia suspended in PBS (10^9 conidia/ml) or hyphae (a 100-μl aliquot from the concentrated hyphal PBS solution derived from centrifugation) were incubated with 10^9 transducing units (TU) of the phage library (10). The conidium- or hypha-phage coincubation was performed in the presence of PBS containing 1% bovine serum albumin on ice to prevent or minimize nonspecific internalization. Following a 16-h coincubation, the suspension was transferred to the top of a nonmiscible organic lower phase with an intermediate specific density and centrifuged (10). In pilot experiments, we first determined the optimal phage separation conditions with a dibutyl phtalate:cyclohexane (Sigma Chemical Co., St. Louis, MO) ratio of 6:1. Also, hyphae required greater centrifugation forces (100,000 × *g* versus 10,000 × *g* for conidia) for longer times (20 min versus 10 min for conidia). Following centrifugation, conidia or hyphae entered the lower organic phase and pelleted at the bottom of the tube, carrying with them only specifically bound phage. The pellet was then transferred to another clean tube as previously described (10).

Phage bound to conidia or hyphae were rescued by infection of log-phase *Escherichia coli* K91kan bacteria (10). Briefly, following coincubation of *Escherichia coli* K91kan with conidium- or hypha-bound phage for 30 min, the infected bacteria were initially incubated in Luria-Bertani (LB) liquid medium containing kanamycin for selection of *Escherichia coli* K91kan bacteria, tetracycline (low concentration, 0.2 μg/ml) for the genes responsible for tetracycline resistance in the host bacteria to be induced (phage contains the tetracycline resistance gene) (1, 29, 33), and voriconazole (4 μg/ml). Voriconazole, an antifungal agent with good in vitro and in vivo activity against *Aspergillus* species (26), was used to suppress subsequent conidial and hyphal growth. Following that, bacteria were incubated overnight at 37°C in LB liquid medium containing kanamycin, tetracycline (high concentration; 40 μg/ml, for the selection of the phage-infected *Escherichia coli* K91kan bacteria), and voriconazole. The next day, amplified phage was separated from *Escherichia coli* K91kan bacteria by polyethylene glycol–NaCl precipitation and used in subsequent selection rounds (titer, 10^9 TU) to enrich for the phage binding specifically to conidia or hyphae (10). After four rounds of selection for AF293 conidia and five rounds of selection for AF293 hyphae, we sequenced the DNA corresponding to peptide inserts of randomly picked phage clones for AF293 conidia and hyphae. Peptide sequences were then analyzed according to enrichment and by Clustal W sequence alignment. Selected motifs were used to search nonredundant protein databanks (National Center for Biotechnology Information [NCBI] BLAST [http://www.ncbi.nlm.nih.gov/BLAST]).

To evaluate phage binding, we used the fd-tet insertless phage as a negative control (1, 10). In parallel experiments, we coincubated conidia or hyphae with selected peptide-displaying or control phage and quantified phage bound to conidia or hyphae relative to fd-tet. We tested (in triplicate) various experimental conditions in independent experiments (i.e., phage inputs of 10^6 to 10^{10} TU, dibutyl phtalate: cyclohexane ratios of 6:1 versus 9:1, and incubation periods of 4 h versus 16 h).

Murine model of invasive pulmonary aspergillosis. The binding of CGGRL GPFC-displaying phage (relative to fd-tet binding) on AF293 hyphae was assessed on formalin-fixed, paraffin-embedded histopathology sections of lung tissue from mice with invasive pulmonary aspergillosis. This specific peptide-displaying phage was used because it was the one enriched in the screening and for which homology searches suggested that it corresponded to molecules of the extracellular matrix present in the lung parenchyma. Female Swiss Webster mice weighting 20 to 25 g each (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were immunosuppressed with intraperitoneal cyclophosphamide (Sigma) (150 mg/kg of body weight; 200 to 250 μl of a 15-mg/ml sterile saline solution) and infected intranasally with AF293 conidia (inoculum, 35 μl of a 10^9 conidia/ml solution or ~35×10^6 conidia per mouse) (21). A hyperacute pneumonia ensues, and mice typically succumb to their infection within 4 days (21). We euthanized the mice 4 days postinfection by CO_2 narcosis and cervical dislocation, removed their lungs, fixed the lungs with 10% formaldehyde, and embedded them in paraffin wax.

Phage overlay assays and immunohistochemistry studies. Lung tissue sections (each, 5 μm) were deparaffinized in xylene and rehydrated in a graded alcohol series. Endogenous peroxidase was blocked by PBS containing 3% hydrogen peroxide for 10 min at room temperature (RT). For epitope retrieval, tissue sections were heated in target retrieval solution (DakoCytomation, Carpinteria, CA) in a standard steamer for 30 min and then allowed to cool to RT for 15 min. After equilibration with Tris-buffered saline containing 0.05% Tween 20, the slides were incubated with protein-blocking solution (DakoCytomation) for 15 min to block nonspecific protein binding sites (32). Subsequently, 2×10^9 TU of the CGGRLGPFC-displaying or control phage were added and incubated for 2 h at RT. An anti-phage antibody (Sigma) (10 μg/ml; 200-μl volume of a 1:500 dilution in antibody diluent; DakoCytomation), or rabbit immunoglobulin G (IgG, negative control; DakoCytomation) was then added to the slides and incubated overnight at 4°C. After three washes with Tris-buffered saline containing 0.05% Tween 20, the peroxidase-conjugated anti-rabbit secondary antibody was added for 20 min at RT. Detection of immunoreaction was then achieved by using the LSAB+ system (DakoCytomation) containing streptavidin-biotin-peroxidase complex for 20 min incubation at RT (32). Color was developed with 3,3′-diaminobenzidine and hydrogen peroxide (DakoCytomation), and slides were subsequently counterstained with 100% hematoxylin, dehydrated, and mounted (32). At least two different sections from each mouse with macroscopic hemorrhagic lesions consistent with invasive pulmonary aspergillosis were tested per experiment. Each experiment was repeated independently two times on different days by similar methods.

Immunofluorescence studies. Steps until the addition of anti-phage antibody (or rabbit IgG negative control) were performed as described above for immunohistochemistry studies. After an overnight incubation at 4°C and three washes with PBS, the tissue sections were incubated with fluorescence-labeled secondary donkey anti-rabbit antibody (Molecular Probes, Eugene, OR) at a concentration of 1:200 for 1 h and then counterstained with DAPI (4′,6′-diamidino-2-phenylindole; Molecular Probes) for 1 min (19). Subsequently, tissue sections were washed with PBS and mounted with Vectorshield mounting medium (Vector Laboratories, Burlingame, CA) (19). In control experiments, we tested for potential cross-reactivity of anti-phage or the secondary anti-rabbit antibody with *Aspergillus* hyphae. At least two different histopathology sections from each mouse with invasive pulmonary aspergillosis were tested per experiment. Each experiment was repeated two times independently on different days.

Statistical analysis. Differences in the number of bound phages recovered from various binding experiments were analyzed by using the Mann-Whitney two-tailed *t* test (GraphPad Prism 3 software program; GraphPad Software, Inc., San Diego, California). *P* values of ≤0.05 were considered statistically significant.

RESULTS

Selection of peptide motifs that bind to the surface of *Aspergillus fumigatus* conidia and hyphae. Analysis of the peptides recovered from phage selection for *Aspergillus* conidia or hyphae according to enrichment and by Clustal W sequence alignment revealed that several amino acid residues were shared among multiple motifs (Table 1). Searches for each of these peptides in NCBI BLAST revealed that some corresponded or matched to motifs contained in human proteins (Tables 2 and 3).

Specifically, a peptide motif (sequence CWGHSRDEC) with high similarity to collagen VI was evaluated further as a potential ligand on the surface of *Aspergillus fumigatus* AF293 conidia. This most commonly recovered CWGHSRDEC motif showed enrichment during successive rounds of panning (i.e., from 9.7% in the third round of selection to 28.3% in the fourth round of selection) and was found to reside on the surface of the von Willebrand domain I of collagen VI (http://www.ncbi.nlm.nih.gov/structure/). The second-most-common conidium-associated recovered CLLSATPSC motif also showed enrichment during successive rounds of panning (i.e., from 3.3% in the third round of selection to 21.7% in the fourth round of selection). In addition, other molecules of the extracellular matrix (e.g., proteoglycans) and immune system effector molecules (e.g., complement components) were also identified as potential ligands on the surface of AF293 conidia (Table 2). More diversity inpeptide motifs was observed in the results for

TABLE 1. Analysis of the peptides recovered from phage selection for *Aspergillus* conidia or hyphae according to enrichment and by Clustal W sequence alignment[a]

Motif	Panning	Alignment
G[HR][RS]R[DL]E	*Aspergillus* conidia	WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		WGHSRDE
		SGGRRLE
		SGGRRLE
		SGGRRLE
		SGGRRLE
A[T]PS	*Aspergillus* conidia	LLSATPS
		LLSATPS
		LLSATPS
		LLSATPS
		LLSATPS
		LLSATPS
		LLSATPS
		LLSATPS
		LLSATPS
		LLSATPS
GG-R[GR]L[DE]	*Aspergillus* conidia	GGVRGLD
		GGVRGLD
		GGVRGLD
		GGVRGLD
		GGVRGLD
		GGVRGLD
		SGG-RRLE
		SGG-RRLE
		SGG-RRLE
		SGG-RRLE
		TGG-RVLS
GARA[DS][AG][As]	*Aspergillus* conidia	GARASGS
		GARASGS
		GARASGS
		GARASGS
		GARASGS
		ARADAAT
		ARADAAT
GSS	*Aspergillus* conidia	ATRDGSS
		ATRDGSS
		ATRDGSS
		ATRDGSS
		SGSSIDQ
		GSSSGNF
Others	*Aspergillus* conidia	DANRVGG
		GAIRSGL
		GWQGFGS
		SGEVGRG
		GGWGPGS
		AGSGLSN
		AGGQKSI
		TGGRVLS
		VGVRPDS
		GWHPGQE
		XXHAGQA
		EGLRRSA
		EYLGLAR
No apparent pattern (RS motif)	*Aspergillus* conidia	RESVRDR
		NEYSRPG
		XAGPXRT
		TVDSARS
		-DGRGMA
		XTRSGHX
		LEWTGLD
		ATGTHGP
G[AST]XVS	*Aspergillus* hyphae	GPIVSFG
		AGTGVS-G
		GGSKVSA
		RNGSHVS
		GAAVSIL
		GAVVSVD
[GD][RS]LG	*Aspergillus* hyphae	GGRLGPF
		GGRLGPF
		GDRLPHF
		RSIGRLG
		PAEGSLG
LhRSp	*Aspergillus* hyphae	VLLRSSG
		FLLRSND
		RLWRSTG
		VPLSRST
GXRS	*Aspergillus* hyphae	IGGRSTL
		ARIGSRS
		GVRSSSA
R[GST]XXWS	*Aspergillus* hyphae	RGFAWSP
		RSERWSG
		RTSGWSE
ASV	*Aspergillus* hyphae	DMLGASV
		ASVHTLD
		ASVARII
AAG	*Aspergillus* hyphae	AAGIGGD
		DMAAGMA
		WGVAAGG
G[DQ]XSXV	*Aspergillus* hyphae	GDASGVV
		GDGSWVG
		GGQHSEV
LXGR	*Aspergillus* hyphae	LLGRISR
		LLLGRFA
		EIALRGR
LXS[NSK]S	*Aspergillus* hyphae	DLTSNSR
		ALKSSSN
		RPLGSKS
Others	*Aspergillus* hyphae	VGTDYVG
		VLNDLVA
		RMFNSVA
		WMFGSGA
		NPGYWGN
		YYPGYDA
		GALGDXX
		QALGQLD
		IGRAPQM
		GRFMQLL
		GLDTRGG
		GAGHAGG
		IHEYGVQ
		TRYEVGV
		GRAFTSI
		RYLRAVT
		PIG--LGLV
		GPHKGLV
		LGVSLIA
		YFGVSRS
		PFVAYDP
		GSPVAYS
		KSSTTAS
		STTRENH
No apparent patterns (RS motif)	*Aspergillus* hyphae	RWSEKMR
		RIWSDYR
		LLYSNGR
		LSRDRVR
		RWTLAGC
		TGRRHSV
		XKXRELX
		WRQSTEG
		FHPLRDG
		FDWQGRV
		GQDTGSL
		QPREFEL
		XCDSSVS
		YNSLVTH

[a] Peptide sequences preferentially isolated from the surface of *Aspergillus* conidia and hyphae were aligned to obtain motifs shared between different peptides (Clustal W program; European Molecular Biology Laboratory). The software registers sequence identities and similarities among multiple peptide sequences and aligns the sequences by placing peptides with the most similarity or identity next to one another. Similarity between peptides at the level of amino acid sequence is presented by boldface type.

TABLE 2. Candidate proteins mimicked by selected peptide motifs after selection for AF293 conidia[a]

Extended motif	Human protein containing the motif	Protein description	Accession no.	Alignment	Homology score[b]
WGHSRDE	Collagen VI	ECM protein	NP_001839	WG SRD	25.8
DLGRVGG				DLG VG	25.0
ISKQGGA				IS QG A	23.1
GAIRSGL				AI+ GL	22.3
GRGVEGS				RGV+G+	22.3
TAADARA				TAAD	21.6
GSSSGNF				GS+S G NF	20.8
GWQGFGS				G+QG GS	20.8
RESVRDR				+++RDR	18.9
QDISSGS				++ SSG+	18.5
QDISSGS	Phosphacan	Proteoglycan	AAC52383	D SSGS	24.6
VGVRPDS				VGV DS	23.9
RALGLYE				RAL L+E	22.3
WGHSRDE				HSR+E	22.3
LLSATPS				S+TPS	21.9
NSLGSGA				SL SG+	21.9
SGSARAG				S S+ AG	21.9
LLSATPS	Versican	Proteoglycan	AAC24358	LLS TPS	29.6
ATRDGSS				ATR GS+	26.2
TAADARA				AA+AR	22.3
GRDSPSA				+ LGSG	21.9
NSLGSGA				SPSA	21.6
SSGDRTA				SGDR	21.6
DANRVGG				NRVG	21.6
LLSATPS	Syndecan	Proteoglycan	NP_037158	LL+ATP+	26.6
SSGDRTA				+SG+ TA	22.7
GGVRGLD				GG+ GL	20.8
LLSATPS	Heparan sulfate receptor	Proteoglycan receptor	AAA37087	LL+ATP+	26.6
TAADARA				T+ DA+A	21.9
GGVRGLD				GG+ GL	20.8
LLSATPS	DSD-I proteoglycan	Proteoglycan	CAB41976	S+TPS	21.9
GRDSPSA				GRDS	21.6
GRGVEGS				VEGS	21.6
GWQGFGS				GFGS	21.6
GARASGS	T-cell receptor β-chain	Cellular immune response	AAD29501	ARASG	25.8
SSGDRTA				SGD TA	21.2
RALGLYE				RA G+ E	20.4
GARASGS	Cytokeratin XIV, XVIII	Keratin-like molecule	XP_094275	GAR SGS	
GARASGS	Tumor necrosis factor ligand superfamily IX	Membrane receptor	NP_003802	GARAS GS	29.6
ELRRGGS				ELRR G GS	24.3
NEYSRPG				YS PG	20.8
GARASGS	Nebulin-related anchoring protein	Anchoring protein	AAL99185	ARASG	25.8
RESVRDR				+SVRD	22.3
ELRRGGS	Lymphocyte α-kinase	Protein kinase	NP_079420	ELRRGG	30.0
GSSSGNF				GSS G+	21.9
NSLGSGA				NSLG+	21.9
QDISSGS				SSGS	21.6
VGVRPDS				GVR D	20.8
GAIRSGL	1, 4-alpha-glucan branching enzyme	Biosynthetic enzyme	JX0243	GA+ SG	22.3
DLGRVGG				GRVG	21.6
TVDSARS				VD A S	21.6
GGWGPGS				GGW GS	20.8
GPRSYEN				PR YE	20.8
SSGDRTA	Cartilage aggregating proteoglycan	Proteoglycan	CAA42701	SSGDRTA	30.0
SLVRGGT				SLVR	21.6

Continued on facing page

TABLE 2—*Continued*

Extended motif	Human protein containing the motif	Protein description	Accession no.	Alignment	Homology score[b]
SSGDRTA	IgG heavy-chain variable region	Humoral immune response	CAC29392	SSGDRT	30.0
GRGVEGS				G+G+E	18.5
GSSSGNF				+SSG+	18.1
RESVRDR				+SV+ R	18.5
GGWGPGS	Properdin precursor (factor P)	Complement component	Q64181	G+G WG	21.6
SGFGQWG				G+G WG	21.6
TAADARA				T D RA	20.8
GGWGPGS	Thrombospondin I	ECM protein	NP_035710	GGWGP S	29.3
GDGRGMA				GDGRG A	28.9
GRGVEGS				GRGVE	25.8
AGGQKSI				A GQ KSI	24.6
QDISSGS				QD+SS	22.3
RESVRDR				R+ V DR	21.6
SGGRRLE				GRRL	21.6
TVDSARS				TVDS	21.6
GGWGPGS	Chemokine ligand I	Membrane receptor	NP_002987	WGPGS	25.8
EGLRRSA				EGLR RS	24.6
GARASGS				ASGS	21.6
GRGVEGS				GV GS	21.2
LLSATPS				L TPS	21.2
RALGLYE	C_9 molecule	Complement component	AAA51889	GLYE	21.6
ELRRGGS				L RGG+	20.8
SLVRGGT	C_8-β molecule	Complement component	BAC41370	LVRGGT	30.0
ELRRGGS				L RGG+	20.8
GRGVEGS				G GV +GS	20.8
LLSATPS	Major histocompatibility complex class I heavy chain	Major histocompatibility complex	AAG02528	LL TPS	25.4
TAADARA				TAA+ +A	22.3
ISKQGGA				I+KQ GG	20.0
IGRAPQM	Immunity-associated protein	GTP immune-associated nucleotide	NP_787056	RAPQM	

[a] For similarity searches, peptides recovered from panning were analyzed with BLAST (National Center for Biotechnology Information [NCBI]) searches for similarity to known human proteins in the NCBI database. Motif regions of 100% identity between peptides selected from panning and the candidate protein are shown. Conserved amino acid substitutions are indicated by plus signs. ECM, extracellular matrix.

[b] A complete seven-amino-acid match gave a homology score of 34, and a complete six-amino-acid match gave a homology score of 30. The score matrix was based on modified BLOSUM62 to minimize the problem that some rare amino acids in the BLOSUM62/PAM30 matrix are heavily weighted, which tends to overpower the perfect matches of four- to five-amino-acid stenches.

potential ligands binding to the surface of AF293 hyphae (Table 3). Again, a variety of molecules of the extracellular matrix including collagen types I to VII, IX to XI, and XV; thrombospondins I to V, laminin V; basilin (an elastin-like protein); agrin (a component of the lung and kidney basal membrane); intercellular adhesion molecule (ICAM) V; and receptors of effector immune system cells such as T lymphocytes and macrophages were identified as potential *Aspergillus* hyphal ligands (Table 3). As shown here, phage selection for *Aspergillus* conidia and hyphae yielded peptides that mimic several human proteins known to be present in the lungs (6).

Validation of phage binding to conidia and hyphae of *Aspergillus* and other molds. We then characterized the binding of CWGHSRDEC-displaying and CGGRLGPFC-displaying phage, the phage clones most frequently recovered from AF293 conidia (Table 2) and hyphae (Table 3) and analyzed the degree of their binding to AF293 conidia and hyphae relative to control phage by the BRASIL method. Satisfyingly, we found that binding of the CWGHSRDEC-displaying and CGGRLGPFC-displaying phage recovered from AF293 conidia and hyphae was significantly higher than control in all experimental conditions tested ($P < 0.01$) (Fig. 1A and B).

We next tested whether this binding would also be seen in other patient-derived mold isolates. Again, CWGHSRDEC-displaying phage recovered from conidia of different *Aspergillus* and *Rhizopus* isolates was significantly higher than that of a control ($P < 0.01$), with the exception of conidia of *Aspergillus flavus* A122, where no significant binding of the CWGHSRD EC-displaying phage was observed relative to control phage (Fig. 1C). Similarly, the CGGRLGPFC-displaying phage recovered from hyphae of different *Aspergillus* and *Rhizopus* isolates was significantly higher than control ($P < 0.001$), with the exception of hyphae of *Aspergillus flavus* A117, where no significant binding of the CGGRLGPFC-displaying phage was observed relative to control phage (Fig. 1D). The reason(s) for the lack of binding in either A117 or A122 isolates remain(s) an open question; future studies focusing on larger number of

TABLE 3. Candidate proteins mimicked by selected peptide motifs after selection for AF293 hyphae[a]

Extended motif	Human protein containing the motif	Protein description	Accession no.	Alignment	Homology score[b]
GGRLGPF	Collagen I–VII, IX–XI	ECM proteins	NP_031763	GRLGP	25.8
GSVGTGA				G VG TGA	25.4
AILSVGL				ILSV L	24.3
VGVEYRT				VG EYRT	24.3
GGAHGAG				GGA AG	24.3
GDGSWVG				G GS VG	23.5
IIRAVSA	Collagen XIV–XVII, XIX	ECM proteins	AAB19038	II+ VS	22.3
GGRLGPF				G LGP+	21.9
GLSGEAP				GL GE	21.6
GSVGTGA	Collagen XXII, XXIV	ECM proteins	NP_690850	GSV GTG	25.4
GPIVSFG				GPI +FG	25.0
GGRLGPF				G+LGP	21.9
GGRLGPF	Thrombospondin I–V	ECM proteins	NP_035710	GGRLG F	28.9
ADYGPYY				AD GP Y	24.6
GGAAVGW				G AVG+	21.9
RGRLAIE				RG L A+E	21.9
GVRSSSA				G +SSS	21.6
NGWYGPN				NG +GP	21.6
GDGSWVG				DGSW	21.6
GGRLGPF	Nicein	Laminin-like protein	NP_061486	GGRLG F	28.9
RGRLAIE				RG L A+E	21.9
GQDTGSL				GQD S	21.2
GGAHGAG				GGA G G	20.8
QPREFEL				R+F++	15.4
GGAHGAG	Laminin V	ECM protein	AAM03454	GG AH GAG	22.3
GRFMQLL				+FMQ L	21.2
GLSGEAP				LS EA	20.8
GGSKVSA				GG SA	20.0
GGRLGPF	Laminin gamma-III chain	ECM protein	AAD29851	RLGPF	25.8
QPREFEL				REF+L	22.3
GGAHGAG				GG AH GAG	22.3
AMGAAMD				A+G AA D	21.6
RGRLAIE				G LA+E	21.6
RPLGSKS				R LGS+	21.6
LSRDRVR				SR +RVR	19.2
GSVGTGA	Macrophage scavenger receptor-like I	Cellular immune response	NP_057324	GS GTG	25.4
GGRLGPF				GGR LGP	22.7
DLQGLAQ				+LQGL	21.9
HNERTTS				RTTS	21.6
RSIGRLG				S+G LG	21.6
GGRLGPF	T-cell receptor β-chain	Cellular immune response	AAC69961	GRLGP	25.8
LLLGRFA				LLLG A	24.3
RSIGRLG				RS GRLG	23.9
VSAGLMD				+ AGL+D	22.3
PIGLGLV				+G GLV	21.9
DLTHVSA	Basilin	ECM protein	AAM53532/ NP_660140/ Q8K482	DLTHVS	30.0
AILSVGL				A+LSV	22.7
DLQGLAQ				DLQ L +	21.6
GQDTGSL				G+ GSL	21.6
GTSRWLR	Agrin	Basic membrane component	NP_067617	T+RWLR	26.2
RYLRAVT				YL AVT	25.8
VPLSRST				+PLS ST	25.4
GLSGEAP				LS GEAP	25.0
VVGSADG				V SADG	25.0
VLLRSSG				V+LRS+	22.3

Continued on facing page

TABLE 3—*Continued*

Extended motif	Human protein containing the motif	Protein description	Accession no.	Alignment	Homology score[b]
XCDSSVS	NEPH2 immunoglobulin domain	Protein-protein interaction molecule	XP_235986	CDSSVS	30.0
GAAVSIL				GA V+ L	21.9
GRAFTSI				G +FT+I	21.9
NGWYGPN				NG+Y N	21.6
FHPLRDG				LRDG	21.6
XCDSSVS	Cartilage homeoprotein I	ECM protein	Q91574	CDS+VS	26.2
VVGSADG				GSA+G	21.9
GLSGEAP				LSG A	21.6
GVRSSSA				RSSS	21.6
IGGRSTL				+GG TL	21.6
NSSSKLA				SSSK	21.6
GGSKVSA				GGS V++	21.6
HNERTTS				H+ RT S	21.6
VVGSADG	NEPH1 immunoglobulin domain	Protein-protein interaction molecule	AAK00529	VVGSAD	30.0
DVSVVAG				D +VVAG	25.4
VLLRSSG				VLL SG	25.0
GRAFTSI				GR FT	21.6
HNERTTS				ERT S	21.6
RGRLAIE				RGRL	21.6
ISTFARG	T-cell receptor α-chain	Cellular immune response	CAB92465	ISTF RG	30.0
VGVEYRT	ICAM V	Intercellular adhesion molecule	AAH26338	VGVEYR	30.0
GTSRWLR				GT R LR	25.0
GGAHGAG				GGA GA	25.0
PIGLGLV				P GLGL	24.6
AILSVGL				A+L++GL	23.5
VGVEYRT	Telencephalin	Intercellular adhesion molecule	NP_032345	VGVEYR	30.0
GTSRWLR				GT R LR	25.0
PIGLGLV				P GLGL	24.6
GGAAVGW				GGAA G	24.6
AILSVGL				A+L++GL	23.5
LLGRISR				LLG ++R	22.3
NPGYWGN	Immunoglobulin alpha VH4	Humoral immune response	AAG21420	PGYWG	25.8
NPGYWGN	Immunoglobulin heavy chain, VH3 family	Humoral immune response	AAQ05568	PGYWG	25.8
IGGRSTL				GR TL	21.6
NPGYWGN	Iron transporter	Molecule transporter	NP_011072	NPGYW	25.8
YFGVSRS				Y+G S+S	23.5
VSAGLMD				+SAGL	22.7
HNERTTS				E+TTS	21.9

[a] For similarity searches, peptides recovered from panning were analyzed with BLAST (NCBI) searches for similarity to known human proteins in the NCBI database. Motif regions of 100% identity between peptides selected from panning and the candidate protein are shown. Conserved amino acid substitutions are indicated by plus signs. ECM, extracellular matrix.

[b] A complete seven-amino-acid match gave homology score of 34, a complete six-amino-acid match gave homology score of 30, and the score matrix was based on modified BLOSUM62 to minimize the problem that some rare amino acids in the BLOSUM62/PAM30 matrix are heavily weighted, which tends to overpower the perfect matches of four- to five-amino-acid stenches.

isolates of each *Aspergillus* species should address the possibility of *Aspergillus* species-specific differences in phage binding.

These results show that the two most commonly recovered peptide-displaying phage from AF293 conidia and hyphae also bind to the surface of conidia and hyphae of a variety of other clinical mold isolates recovered from cancer patients, suggesting that this binding is not likely strain specific.

A selected binding peptide targets *Aspergillus fumigatus* hyphal elements on lung tissue recovered from mice with invasive pulmonary aspergillosis. Having characterized the in vitro binding of CGGRLGPFC-displaying phage to hyphae of various mold isolates, we then assessed whether this phage clone also binds to *Aspergillus* hyphae recovered from mice with invasive pulmonary aspergillosis. We focused on studying the binding of hypha-associated peptides to mouse lung tissue with invasive pulmonary aspergillosis because *Aspergillus* hyphae are the predominant fungal forms that are seen during invasive growth in tissues, although *Aspergillus* conidia and conidiophores can occasionally be seen (4, 20). To that end, we performed immunohistochemistry and immunofluorescence studies of immunosuppressed mice infected with *Aspergillus fumigatus*.

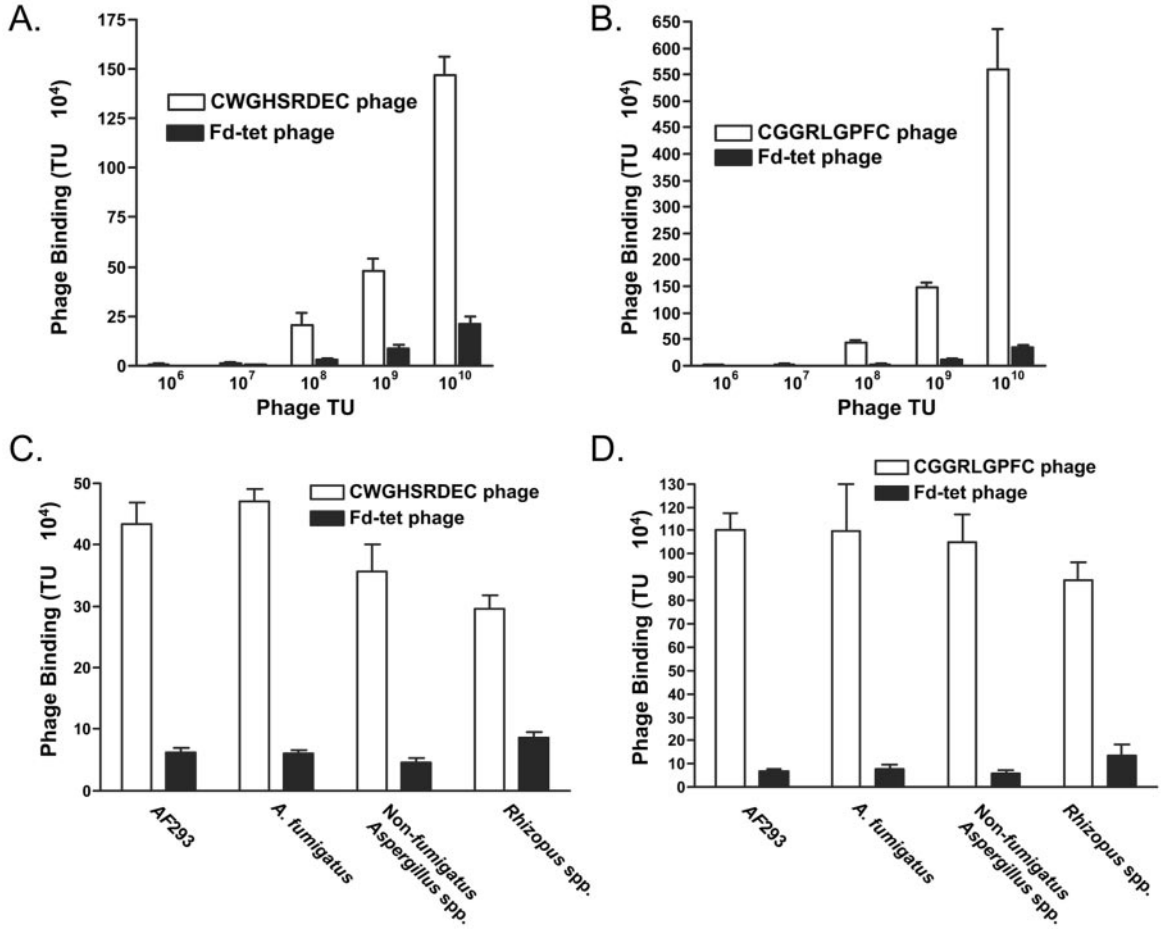

FIG. 1. Validation of phage binding to conidia and hyphae of *Aspergillus* and *Rhizopus* clinical isolates. (A) Binding of the CWGHSRDEC-displaying (relative to control) phage to conidia of AF293 using different TU of the phage in independent experiments (10^6 to 10^{10} TU). (B) Binding of the CGGRLGPFC-displaying (relative to control) phage to hyphae of AF293 using different TU of the phage in independent experiments (10^6 to 10^{10} TU). (C) Binding of the CWGHSRDEC-displaying (relative to control) phage (titer, 10^9 TU) to conidia of patient-derived *Aspergillus* and *Rhizopus* isolates. (D) Binding of the CGGRLGPFC-displaying (relative to control) phage (titer, 10^9 TU) to hyphae of patient-derived *Aspergillus* and *Rhizopus* isolates. Values are averages from three independent experiments after one round of biopanning; bars represent standard deviations. (No significant binding of the CWGHSRDEC-displaying phage was observed relative to control phage to conidia of the *Aspergillus flavus* isolate AF122 [data are not shown in Fig. 1C)] and no significant binding of the CGGRLGPFC-displaying phage was observed relative to control phage to hyphae of the *Aspergillus flavus* isolate *AF117* [data are not shown in Fig. 1D]).

Consistently, CGGRLGPFC-displaying phage binding to *Aspergillus* hyphae was strong and present in all tested sections of lung tissue in the mice with invasive pulmonary aspergillosis (Fig. 2C and F). In contrast, control phage binding to AF293 hyphae was either absent or barely detectable relative to CGGR LGPFC-displaying phage binding in the lungs (Fig. 2B and E). IgG did not immunoreact with *Aspergillus* hyphae that was previously coincubated with CGGRPGPFC-displaying phage (Fig. 2A and D).

By using immunofluorescence, CGGRLGPFC-displaying phage bound specifically to AF293 hyphae on lung tissue sections from mice with invasive pulmonary aspergillosis (Fig. 3). The possibility that this hypha-specific signal was due to anti-phage or anti-rabbit secondary antibody cross-reactivity with *Aspergillus* hyphae was excluded by the addition of anti-phage antibody without previously adding CGGRLGPFC-displaying phage and by the addition of anti-rabbit secondary antibody without previously adding anti-phage antibody, respectively.

Thus, the results of immunostaining that demonstrate specific and reproducible binding to the mouse lung tissue with invasive pulmonary aspergillosis are consistent with our in vitro findings that CGGRRGPFC-displaying phage binds to the surface of *Aspergillus* hyphae.

DISCUSSION

In the present study, we used a combinatorial peptide approach for selecting cell surface motifs from phage display libraries for an infectious disease application. As a proof of concept, we used the BRASIL method to screen for enriched peptides on the surface of conidia and hyphae of the medically significant fungus *Aspergillus fumigatus*. To isolate shared and different motifs between both developmental programs of growth of *Aspergillus fumigatus*, we performed parallel screens of a phage display library with both conidia and hyphae.

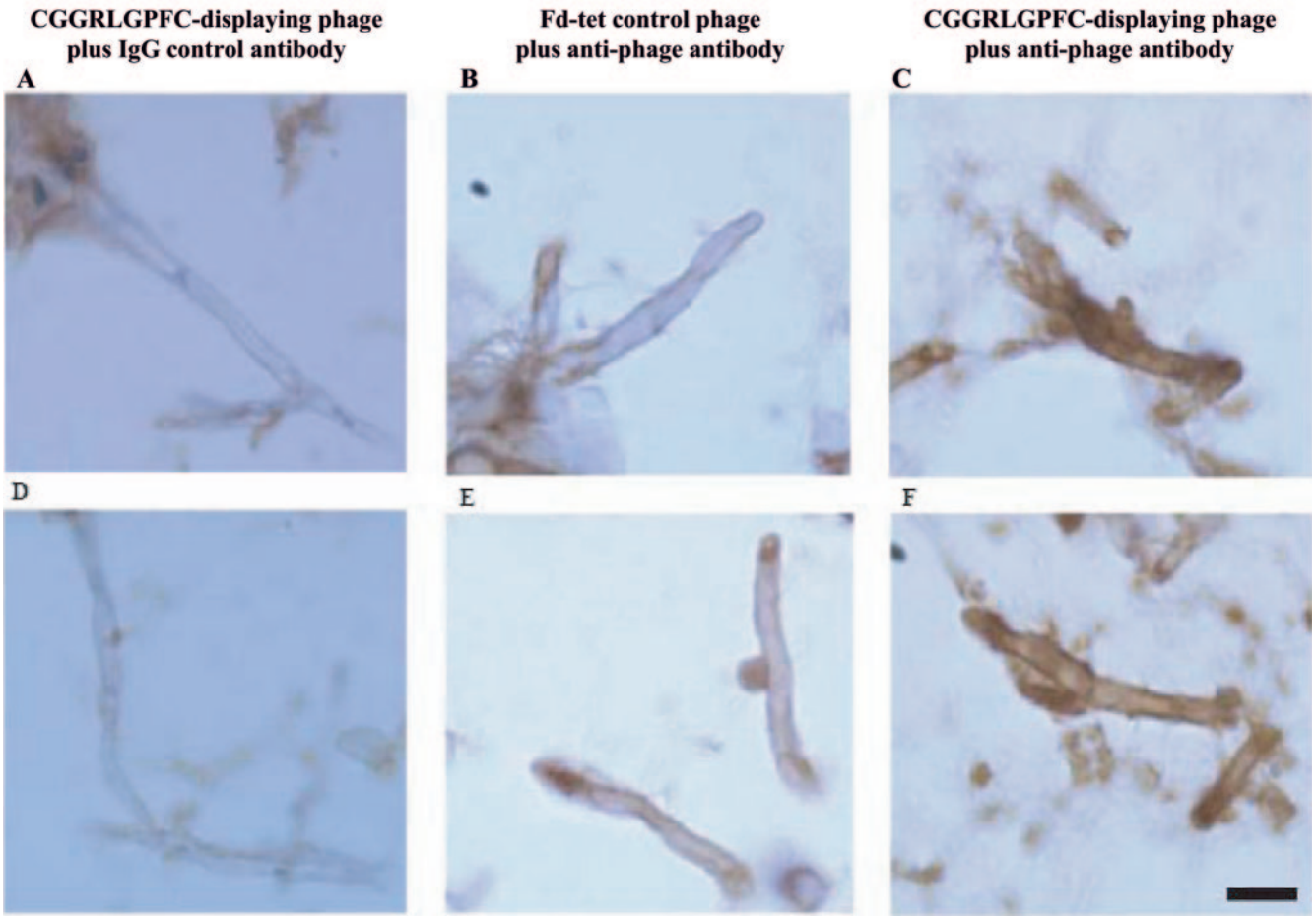

FIG. 2. Immunohistochemistry analysis following phage overlay assays on representative lung tissue sections from mice with invasive pulmonary aspergillosis. (A and D) Results following addition of CGGRLGPFC-displaying phage and rabbit IgG. (B and E) Results following addition of control phage and anti-phage antibody. (C and F) Results following addition of CGGRLGPFC-displaying phage and anti-phage antibody. Note the stronger signal on *Aspergillus* hyphae than the signal generated by the addition of control phage. Bar, ~15 to 20 μm.

We identified several peptide motifs after phage selection for *Aspergillus* conidia and hyphae that mimic human proteins of the extracellular matrix such as various collagen subtypes and proteoglycans. These proteins, which are quite abundant in the human airways and lung parenchyma (6), likely play a significant role in the initial attachment of *Aspergillus* conidia to airway and lung tissue molecules and in the subsequent invasion of *Aspergillus* hyphae into the lungs. Of interest is also the identification of hypha binding peptide motifs that mimic ligands associated with effector immune cells such as macrophage and T-lymphocyte receptors as potential ligands on the *Aspergillus* hyphal surface, since macrophages and T lymphocytes are critical components of the human immune response against invading *Aspergillus* (4, 20). Nevertheless, the true identity of the proteins mimicked but those targeting peptides remains an open question that should be the focus of future studies.

The fact that we did not find sequences from the initial rounds of selection that were identical between *Aspergillus* conidia and hyphae is probably explained by the fact that we sequenced the DNA corresponding to peptide inserts of approximately 50 to 70 randomly picked phage clones for *Aspergillus* conidia and hyphae. Thus, if we had sequenced more phage clones, it is very likely that we would have found similar sequences from the selection of *Aspergillus* conidia and hyphae. This assumption is based on other experiments that demonstrated that representative conidium-associated displaying phage (e.g., CWGHSRDEC and CLLSATPSC) also bound (even though less avidly) to *Aspergillus* hyphae (data not shown). Similarly, representative hypha-associated displaying phage (e.g., CGGRLGPFC and CIIRAVSAC) also bound (although less avidly) to *Aspergillus* conidia (data not shown). We believe that this phenomenon may reflect common or ubiquitous binding sites on the surface of *Aspergillus* conidia and hyphae and is likely explained by their common origin, since *Aspergillus* hyphae germinate from conidia.

The BRASIL method has been widely utilized for the selection of specific ligands because of the intrinsic ability of this methodology to minimize background phage binding (10). Moreover, the system is well suited for preclearing of the library before one selects peptides with the desired specificity. This is an important step, because it decreases the chances of isolating nonspecific binding phage, as these are more than likely to get precleared before the specific selection starts.

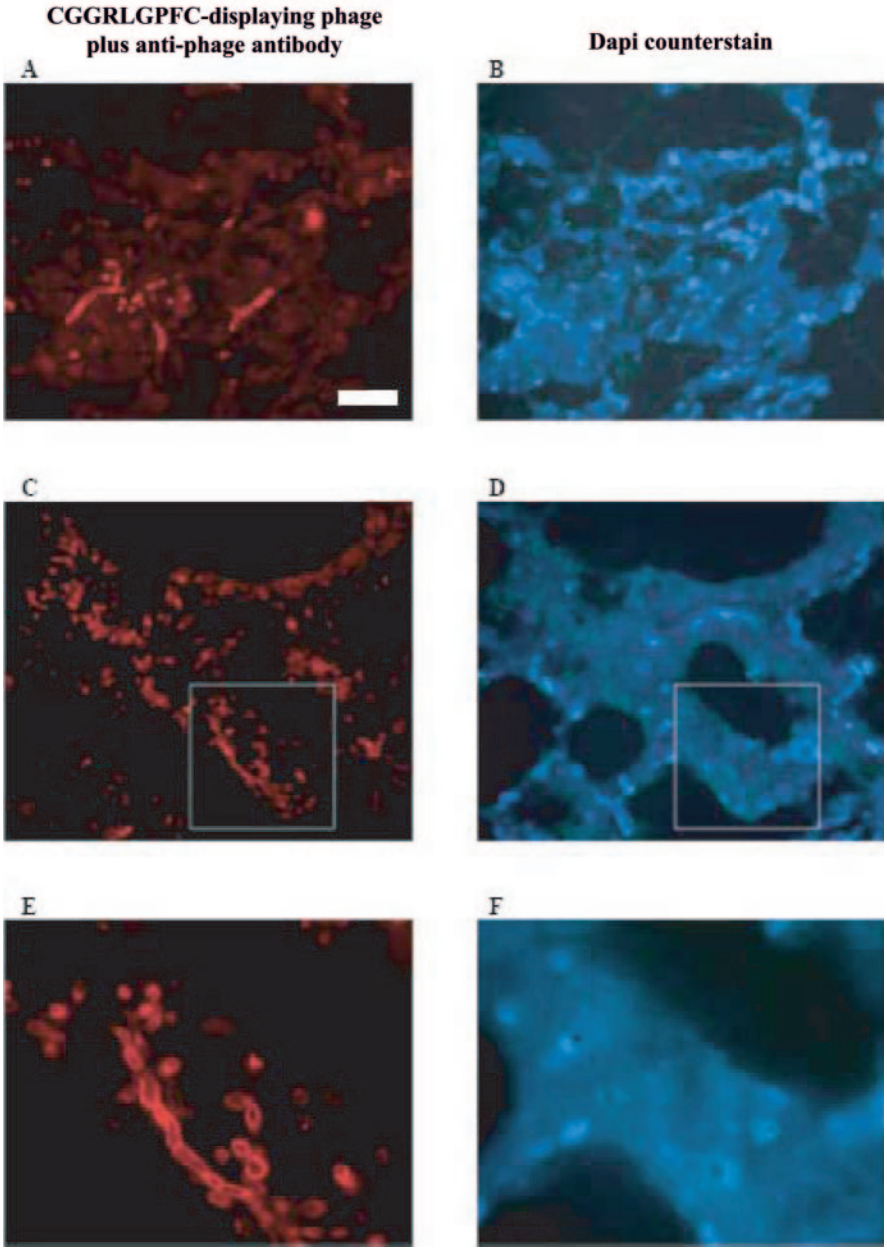

FIG. 3. Immunofluorescence studies of representative lung tissue sections from mice with invasive pulmonary aspergillosis. (A, C, and E) Hypha-specific signal achieved following addition of CGGRLGPFC-displaying phage and anti-phage antibody. (B, D, and F) Respective DAPI counterstaining. Panels E and F represent a higher magnification (×730) of panels C and D, respectively (×292). Bar, ~25 to 30 μm.

It is worth mentioning also that successful phage display screenings do not always result in substantial enrichment from round to round. This is a positive indication that is often utilized as a parameter for prioritizing peptide leads (10). Furthermore, differences in transducing unit counts are a strong indication of specificity and they do not necessarily (or usually) reach log-scale differences to be considered highly selective. Moreover, the BRASIL technology often results in the detection of binding events that are quite robust, given the inherent stringency of the assay.

However, it is the extensive validation at the level of each individual phage that determines the level of certainty of how selective and specific a given ligand is and in recognizing a protein target. Validation at the individual phage level is required, and in our case, it stood the test under well-controlled experimental conditions. The alignment analysis of the peptides suggested that the binding sites in question might well be conformationally defined, since the exact linear amino acid primary sequence of any given peptides was not enriched during the screening. However, we did find residues of the peptides with homology scattered throughout the isolated sequences. Of note, this is a relatively common observation in phage display random peptide screenings (3, 9, 23, 34).

Detection of early invasion by *Aspergillus* hyphae is the focus of early diagnostic and therapeutic strategies for invasive aspergillosis (17, 20). Therefore, we examined whether the CGGRLG PFC-displaying phage clone, the most commonly recovered peptide-displaying phage from AF293 hyphae, also binds to the surface of hyphae of other patient-derived *Aspergillus* isolates in vitro. In fact, we found that the CGGRLGPFC-displaying phage bound to 8 (89%) of 9 *Aspergillus* isolates tested, suggesting that CGGRLGPFC binding is not strain specific. Also of interest is that CGGRLGPFC-displaying phage bound to hyphae of both *Rhizopus* isolates tested; *Rhizopus* is a rare, yet emerging, opportunistic mold that shares common pathogenic and clinical features with *Aspergillus* (18, 20).

To extend this in vitro observation that CGGRPGPFC-displaying phage binds consistently to *Aspergillus* hyphae also in vivo, we sought to determine whether the CGGRLGPFC-displaying phage binds to *Aspergillus* hyphal elements on lung tissue sections from mice with invasive pulmonary aspergillosis. By using immunohistochemistry analysis, we found that CGG RLGPFC-displaying phage clone binds reproducibly to hyphae in contrast to control phage. Similarly, by using immunofluorescence we found a hypha-specific signal of the CGGRLG PFC-displaying phage in all histopathology sections tested from mice with invasive pulmonary aspergillosis.

Our findings have potential therapeutic implications. Early invasion by *Aspergillus* hyphae in the lung parenchyma is frequently missed by even the most sensitive available radiographic methods, such as high-resolution computed tomography of the lungs, which typically has a threshold of detection of lesions of $\geq$1 mm (12). Thus, identification of hypha-specific peptides may lead to new immunolabeling radiographic approaches for detection of early invasion of *Aspergillus* hyphae in the lungs.

Finally, antifungal drug efficacy against *Aspergillus* species in vivo is suboptimal, and this is partially explained because *Aspergillus* is an angiotropic mold that invades blood vessels, resulting in tissue infarcts and low tissue perfusion (31). By detecting *Aspergillus* lesions early before the formation of such grossly hypoperfused lesions and, ideally, by coupling an antifungal with hypha-specific peptides, it might be possible to enhance the activity of antifungal agents in vivo by increasing local and early delivery to the site of infection. Targeted delivery of cytotoxic drugs (2), proapoptotic peptides (8, 16), cytokines (5), metalloprotease inhibitors (15), genes (11, 25), or liposomes (30) to tumor vasculature receptors has resulted in marked therapeutic efficacy in tumor-bearing mice and is the subject of current research. Our study adds invasive aspergillosis as an example of a prototypic infectious disease that can be approached in a similar way.

We believe that a peptidomimetic approach based on peptides holds more promise as a delivery tool than antibodies for several reasons. As opposed to antibodies, which can be difficult and expensive to produce on a large scale, small molecules such as peptidomimetics offer great stability (24). Moreover, they are suitable for the simple design of targeted compounds by Merrifield synthesis, without the need for chemical conjugation (24). Also, although peptides often show lower-affinity binding constants than antibodies, their tissue penetration profile may compensate for it (24). Finally, one must be careful when extrapolating data derived from comparing antibodies to ligand peptide-based binding, particularly in the context of antibody versus peptide phage display selection. It may be true that inhibitory peptides isolated by phage display are unlikely to serve as agents per se but (i) they may form the basis for rational peptidomimetic development and (ii) they may serve as tools for finding targets against which a good antibody may also be a suitable choice for translational applications.

Direct comparison of peptides and antibodies targeting vascular receptors has been performed for both CD13-targeting ligands (28) and aminopeptidase A-targeting ligands (22). In both instances, peptides or antibodies were able to home to tumors by binding to these vascular addresses and to functionally block proangiogenic activity of these proteins in the context of tumor vasculature. It is possible that lower-affinity ligands might preferentially accumulate at sites in which the target is highly expressed. As opposed to higher-affinity ligands, such as antibodies, peptides bind to target receptors in a manner that is directly dependent on receptor expression levels. However, these two approaches are nonmutually exclusive and may actually be complementary: as discussed above, peptides may be useful for identifying targets for antibody-based therapy and also for rationally developing peptidomimetic drugs. Hence, future work should compare and contrast different classes of *Aspergillus* ligands. Finally, selection of an antibody phage display library (rather than a peptide library) by using the BRASIL method may be possible in future studies. If so, selection of targeting antibodies may be feasible.

In conclusion, we show that a peptide-displaying phage binds to the invasive hyphal form of *Aspergillus fumigatus* both in vitro and to lung tissue recovered from mice with invasive pulmonary aspergillosis. Ligand-directed targeting of this opportunistic mycosis may lead to new imaging and therapeutic interventions in invasive aspergillosis. Finally, these results may become useful in studying host immune responses against *Aspergillus* by focusing on fundamental differences in ligand binding sites between effector immune cells of the innate immunity (e.g., resting lung macrophages) and those of the adaptive immunity (e.g., lymphocytes).

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REFERENCES

1. **Arap, W., M. G. Kolonin, M. Trepel, J. Lahdenranta, M. Cardó-Vila, R. J. Giordano, P. J. Mintz, P. U. Ardelt, V. J. Yao, C. I. Vidal, L. Chen, A. Flamm, H. Valtanen, L. M. Weavind, M. E. Hicks, R. E. Pollock, G. H. Botz, C. D. Bucana, E. Koivunen, D. Cahill, P. Troncoso, K. A. Baggerly, R. D. Pentz, K. A. Do, C. J. Logothetis, and R. Pasqualini.** 2002. Steps toward mapping the human vasculature by phage display. Nat. Med. **8:**121–127.
2. **Arap, W., R. Pasqualini, and E. Ruoslahti.** 1998. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science **279:** 377–380.
3. **Buhl, L., P. B. Szecsi, G. G. Gisselo, and C. Schafer-Nielsen.** 2002. Surface immunoglobulin on B lymphocytes as a potential target for specific peptide ligands in chronic lymphocytic leukaemia. Br. J. Haematol. **116:**549–554.
4. **Clemons, K. V., V. L. Calich, E. Burger, S. G. Filler, M. Grazziutti, J. Murphy, E. Roilides, A. Campa, M. R. Dias, J. E. Edwards, Jr., Y. Fu, G. Fernandes-Bordignon, A. Ibrahim, H. Katsifa, C. G. Lamaignere, L. H. Meloni-Bruneri, J. Rex, C. A. Savary, and C. Xidieh.** 2000. Pathogenesis I: interactions of host cells and fungi. Med. Mycol. **38:**99–111.

5. **Curnis, F., A. Sacchi, L. Borgna, F. Magni, A. Gasparri, and A. Corti.** 2000. Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13). Nat. Biotechnol. **18:**1185–1190.
6. **Davidson, J. M.** 1990. Biochemistry and turnover of lung interstitium. Eur. Respir. J. **3:**1048–1063.
7. **Denning, D. W., M. J. Anderson, G. Turner, J. P. Latge, and J. W. Bennett.** 2002. Sequencing the *Aspergillus fumigatus* genome. Lancet Infect. Dis. **2:**251–253.
8. **Ellerby, H. M., W. Arap, L. M. Ellerby, R. Kain, R. Andrusiak, G. D. Rio, S. Krajewski, C. R. Lombardo, R. Rao, E. Ruoslahti, D. E. Bredesen, and R. Pasqualini.** 1999. Anti-cancer activity of targeted pro-apoptotic peptides. Nat. Med. **5:**1032–1038.
9. **Ganglberger, E., K. Grunberger, B. Sponer, C. Radauer, H. Breiteneder, G. Boltz-Nitulescu, O. Scheiner, and E. Jensen-Jarolim.** 2000. Allergen mimotopes for 3-dimensional epitope search and induction of antibodies inhibiting human IgE. FASEB J. **14:**2177–2184.
10. **Giordano, R., M. Cardó-Vila, J. Lahdenranta, R. Pasqualini, and W. Arap.** 2001. Biopanning and rapid analysis of selective interactive ligands. Nat. Med. **7:**1249–1253.
11. **Grifman, M., M. Trepel, P. Speece, L. B. Gilbert, W. Arap, R. Pasqualini, and M. D. Weitzman.** 2001. Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsids. Mol. Ther. **3:**964–975.
12. **Kakinuma, R., H. Ohmatsu, M. Kaneko, K. Eguchi, T. Naruke, K. Nagai, Y. Nishiwaki, A. Suzuki, and N. Moriyama.** 1999. Detection failures in spiral CT screening for lung cancer: analysis of CT findings. Radiology **212:**61–66.
13. **Koivunen, E., B. H. Restel, D. Rajotte, J. Lahdenranta, M. Hagedorn, W. Arap, and R. Pasqualini.** 1999. Integrin-binding peptides derived from phage display libraries. Methods Mol. Biol. **129:**3–17.
14. **Koivunen, E., W. Arap, D. Rajotte, J. Lahdenranta, and R. Pasqualini.** 1999. Identification of receptor ligands with phage display peptide libraries. J. Nucl. Med. **40:**883–888.
15. **Koivunen, E., W. Arap, H. Valtanen, A. Rainisalo, O. P. Medina, P. Heikkila, C. Kantor, C. G. Gahmberg, T. Salo, Y. T. Konttinen, T. Sorsa, E. Ruoslahti, and R. Pasqualini.** 1999. Tumor targeting with a selective gelatinase inhibitor. Nat. Biotechnol. **17:**768–774.
16. **Kolonin, M. G., P. K. Saha, L. Chan, R. Pasqualini, and W. Arap.** 2004. Reversal of obesity by targeted ablation of adipose tissue. Nat. Med. **10:**625–632.
17. **Kontoyiannis, D. P., and G. P. Bodey.** 2002. Invasive aspergillosis in 2002: an update. Eur. J. Clin. Microbiol. Infect. Dis. **21:**161–172.
18. **Kontoyiannis, D. P., M. S. Lionakis, R. E. Lewis, G. Chamilos, M. Healy, P. Perego, A. Safdar, H. Kantarjian, R. Champlin, T. J. Walsh, and I. I. Raad.** 2005. Zygomycosis in the era of *Aspergillus*-active therapy in a tertiary care cancer center: a matched case-control observational study of 27 recent patients. J. Infect. Dis. **191:**1350–1360.
19. **Lai, R., G. Z. Rassidakis, L. J. Medeiros, V. Leventaki, M. Keating, and T. J. McDonnell.** 2003. Expression of STAT3 and its phosphorylated forms in mantle cell lymphoma cell lines and tumours. J. Pathol. **199:**84–89.
20. **Latge, J. P.** 1999. *Aspergillus fumigatus* and aspergillosis. Clin. Microbiol. Rev. **12:**310–350.
21. **Lewis, R. E., R. A. Prince, J. Chi, and D. P. Kontoyiannis.** 2002. Itraconazole preexposure attenuates the efficacy of subsequent amphotericin B therapy in a murine model of acute invasive pulmonary aspergillosis. Antimicrob. Agents Chemother. **46:**3208–3214.
22. **Marchio, S., J. Lahdenranta, R. O. Schlingemann, D. Valdembri, P. Wesseling, M. A. Arap, A. Hajitou, M. G. Ozawa, M. Trepel, R. J. Giordano, D. M. Nanus, H. B. Dijkman, E. Oosterwijk, R. L. Sidman, M. D. Cooper, F. Bussolino, R. Pasqualini, and W. Arap.** 2004. Aminopeptidase A is a functional target in angiogenic blood vessels. Cancer Cell **5:**151–162.
23. **Mintz, P. J., J. Kim, K. A. Do, X. Wang, R. G. Zinner, M. Cristofanilli, M. A. Arap, W. K. Hong, P. Troncoso, C. J. Logothetis, R. Pasqualini, and W. Arap.** 2003. Fingerprinting the circulating repertoire of antibodies from cancer patients. Nat. Biotechnol. **21:**57–63.
24. **Mori, T.** 2004. Cancer-specific ligands identified from screening of peptide-display libraries. Curr. Pharm. Des. **10:**2335–2343.
25. **Muller, O. J., F. Kaul, M. D. Weitzman, R. Pasqualini, W. Arap, J. A. Kleinschmidt, and M. Trepel.** 2003. Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors. Nat. Biotechnol. **21:**1040–1046.
26. **Murphy, M., E. M. Bernard, T. Ishimaru, and D. Armstrong.** 1997. Activity of voriconazole (UK-109,496) against clinical isolates of *Aspergillus* species and its effectiveness in an experimental model of invasive pulmonary aspergillosis. Antimicrob. Agents Chemother. **41:**696–698.
27. **Pasqualini, R., and W. Arap.** 2002. Profiling the molecular diversity of blood vessels. Cold Spring Harb. Symp. Quant. Biol. **67:**223–225.
28. **Pasqualini, R., E. Koivunen, R. Kain, J. Lahdenranta, M. Sakamoto, A. Stryhn, R. A. Ashmun, L. H. Shapiro, W. Arap, and E. Ruoslahti.** 2000. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res. **60:**722–727.
29. **Pasqualini, R., W. Arap, D. Rajotte, and E. Ruoslahti.** 2001. In vivo selection of phage-display libraries, p. 22.1–22.24. *In* C. F. Barbas III, D. R. Burton, J. K. Scott, and G. J. Silverman (ed.), Phage display: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Pastorino, F., C. Brignole, D. Marimpietri, M. Cilli, C. Gambini, D. Ribatti, R. Longhi, T. M. Allen, A. Corti, and M. Ponzoni.** 2003. Vascular damage and anti-angiogenic effects of tumor vessel-targeted liposomal chemotherapy. Cancer Res. **63:**7400–7409.
31. **Paterson, P. J., S. Seaton, H. G. Prentice, and C. C. Kibbler.** 2003. Treatment failure in invasive aspergillosis: susceptibility of deep tissue isolates following treatment with amphotericin B. J. Antimicrob. Chemother. **52:**873–876.
32. **Rassidakis, G. Z., L. J. Medeiros, S. Viviani, V. Bonfante, G. P. Nadali, T. P. Vassilakopoulos, O. Mesina, M. Herling, M. K. Angelopoulou, R. Giardini, M. Chilosi, C. Kittas, P. McLaughlin, M. A. Rodriguez, J. Romaguera, G. Bonadonna, A. M. Gianni, G. Pizzolo, G. A. Pangalis, F. Cabanillas, and A. H. Sarris.** 2002. CD20 expression in Hodgkin and Reed-Sternberg cells of classical Hodgkin's disease: associations with presenting features and clinical outcome. J. Clin. Oncol. **20:**1278–1287.
33. **Smith, G. P., and J. K. Scott.** 1993. Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol. **217:**228–257.
34. **Suphioglu, C., G. Schappi, J. Kenrick, D. Levy, J. M. Davies, and R. E. O'Hehir.** 2001. A novel grass pollen allergen mimotope identified by phage display peptide library inhibits allergen-human IgE antibody interaction. FEBS Lett. **502:**46–52.

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