VeA Regulates Conidiation, Gliotoxin Production and Protease Activity in the Opportunistic Human Pathogen *Aspergillus fumigatus*

Running Title: Role of VeA in *Aspergillus fumigatus*

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Abstract

Invasive aspergillosis by *Aspergillus fumigatus* is a leading cause of infection-related mortality in immunocompromised patients. In this study we show that veA, a major conserved regulatory gene that is unique to fungi, is necessary for normal morphogenesis in this medically relevant fungus. Although deletion of veA results in a strain with reduced conidiation, over-expression of this gene further reduced conidial production, indicating that veA has a major role as regulator of development in *A. fumigatus* and that normal conidiation is only sustained in the presence of VeA wild-type levels. Furthermore our studies revealed that veA is a positive regulator in the production of gliotoxin, a secondary metabolite known to be a virulent factor in *A. fumigatus*. Deletion of veA resulted in a reduction of gliotoxin production with respect to the wild type control. This reduction in toxin coincided with a decrease in gliZ and gliP expression, necessary for gliotoxin biosynthesis. Interestingly, veA also influences protease activity in this organism. Specifically, deletion of veA resulted in a reduction of protease activity; this is the first report of a veA homolog with a role in controlling fungal hydrolytic activity. Although veA affects several cellular processes in *A. fumigatus*, pathogenicity studies in a neutropenic mouse infection model indicated that veA is dispensable for virulence.
The ubiquitous fungus *Aspergillus fumigatus* is one of the most common human fungal pathogens, particularly in immunodepressed patients. This group includes those with hematological malignancies, those individuals with genetic immunodeficiencies, individuals infected with HIV, and cancer patients undergoing chemotherapy (24, 41, 53, 61, 67, 95). This population group is increasing (50) due to the HIV pandemic, the higher number of transplants, and immunosuppressive and myeloablative therapies for autoimmune and neoplastic diseases (31, 24, 50, 79). Mortality rates caused by *A. fumigatus* infections in immunodepressed patients range from 40% to 90% (i.e. 44, 50, 60, 77, 79).

The main point of entry leading to infection by *A. fumigatus* is the respiratory tract. *Aspergillus fumigatus* infection can cause aspergilloma, and invasive aspergillosis (IA) among immunodepressed individuals. *Aspergillus fumigatus* asexual spores (conidia) are small in size compared to those of other Aspergillus species, being only 2.5-3.0 μm in diameter. This allows them to reach the lung alveoli (74, 76). In individuals with healthy immune systems, spores that are not removed by mucociliary clearance, are eliminated by epithelial cells or alveolar macrophages, by phagocytosis and killing of conidia, triggering a proinflammatory response that recruits neutrophiles that eliminate hyphae. Failure in these defenses leads to IA. Due to the fact that conidia are the main inoculum in *A. fumigatus* infections, it is important to elucidate the genetic mechanism governing the production of asexual spores in this fungus. A homolog of the VeA fungal global regulator was found in *A. fumigatus* (6, 45, 85), and was reported to affect conidiation in a nitrogen source-dependent manner (44).

VeA homologs have been described to regulate asexual and sexual development in numerous fungal species (i.e. 26, 38, 39, 52, 100). VeA function has been particularly characterized in the model fungus *Aspergillus nidulans*, where it was found that VeA forms a nuclear protein complex. Mechanistically, VeA is transported to the nucleus by the α-importin KapA (4, 85.). In the nucleus, VeA interacts with the chromatin-remodeling LaeA protein (6), the red phytochrome-like protein FphA, that
interacts with the blue light-response elements LreA and LreB (68), and VelB, another velvet family protein (6, 7, 58).

In addition to its role in regulating development VeA has been demonstrated to control secondary metabolism in several fungal genera (reviewed by 17). The first study demonstrating the association of VeA with regulation of secondary metabolism was carried by our group in 2003 (38), where we showed that VeA was necessary for the production for the mycotoxin sterigmatocystin. VeA controls the expression of the transcription factor gene aflR (38), necessary for sterigmatocystin gene cluster activation (103). VeA is found in numerous fungal species, particularly in Ascomycetes (57). Orthologs of VeA in other fungi have also been demonstrated to regulate secondary metabolism, for example aflatoxin in A. flavus and A. parasiticus (16, 26), fumonisin and fusarins in Fusarium verticillioides (56) and F. fujikori, where it was also found to regulate gibberellins and bikaverin (96), trichothecenes in Fusarium graminearum (54), dothistromin in Dothistroma septosporum (19), penicillin in A. nidulans (38), cephalosporin C in Acremonium chrysogerum (25), polyketide ML-236B (substrate in pravastatin production) in Penicillium citrinum (5), and T-toxin in Cochliobolus heterostrophus (99) among others.

Whether VeA affects secondary metabolism in A. fumigatus remains to be determined. Secondary metabolites are part of the chemical arsenal necessary for niche specialization (15) including host-fungus interactions. Aspergillus fumigatus synthesizes several toxic secondary metabolites. Some of these compounds act as immunosuppressants, which may influence pathogenesis processes. Among them, the most characterized is gliotoxin. Some studies have described that gliotoxin is important for virulence while others have showed that it is unimportant (reviewed by 23, 47). Gliotoxin has been described as having immunosuppressive properties (i.e. 3, 12, 20, 29, 55, 62, 66, 83, 86, 92, 94, 101, 102). This compounds has also been shown to inhibit phagocytosis in macrophage and to induce apoptosis. Gliotoxin has been detected in A. fumigatus infected animals and humans at concentrations at least as high as those required to detect an effect in vitro (i.e. 28, 72).
Other virulence factors also allow *A. fumigatus* to become an opportunistic pathogen in the case of immunocompromised patients. Examples of these virulence factors include conidial cell components (10, 14, 33, 34, 48, 91). Additionally, Kolattukudy et al., (1993)(42) reported that certain fungal hydrolytic activities could also influence pathogenicity. For example, mutants defective in elastolytic serine protease showed a decreased in virulence (42). Also, the *A. fumigatus* strain lacking *hacA*, encoding a transcriptional regulator of the unfolded protein response (73), showed a reduced capacity for protease secretion and reduction in virulence. Whether VeA affects hydrolytic activity in *A. fumigatus* has not previously been investigated.

The effect of VeA on virulence has been mainly characterized in plant pathogenic fungi. For example, we found that the *F. vercillioides veA* null mutant fails to produce disease in seedlings grown from seeds infected with the veA deletion mutant (57). Deletion of veA also reduced plant pathogenicity in *Aspergillus flavus* (27), *A. parasiticus* (16) *Fusarium verticillioides* (57), *F. fujikuroi* (96), *F. graminearum* (54), and *Cochliobolus heterostrophus* (99) among others. Importantly, a recent study has shown for the first time that VeA also influences pathogenicity in animals by the opportunistic pathogenic fungus *Histoplasma capsulatum* (49).

In the present study we demonstrate that veA not only controls morphogenesis by regulating conidiation, but it also controls gliotoxin gene expression and concomitant gliotoxin biosynthesis in *A. fumigatus*. Importantly, we report for the first time a relationship between hydrolytic enzymes, specifically protease activity, and the VeA regulator in fungi. Additionally, we evaluated the possible role of veA in pathogenity in a murine model.

**MATERIALS AND METHODS**

**Strains and culture conditions.** *Aspergillus fumigatus* strains used in this study are listed in Table
1. All strains were grown on Czapek-Dox medium (Difco), unless otherwise indicated, plus the appropriate supplements for the corresponding auxotrophic markers (36). Solid medium was prepared by adding 15 g/liter agar. Strains were stored as 30% glycerol stocks at -80°C.

**Generation of the deletion, complementation and over-expression strains.** The deletion cassette was generated by fusion Polymerase Chain Reaction (PCR) as outlined in Szewczyk et al., (2006)(89). A 1.2 kb 5’ UTR fragment was first amplified from *A. fumigatus* genomic DNA with primers 5’F-Afu577 and 5’R-Afu578 (Table 2). A 1.2 kb 3’UTR fragment was also amplified from genomic DNA with primers 3’F-Afu579 and 3’R-Afu580 (Table 2). The intermediate DNA fragment containing the *A. fumigatus pyrG* was amplified from plasmid p1439 (84) using primers Afu5PyrG581 and Afu3PyrG582 (Table 2). *pyrG* was utilized as transformation marker, achieving a complete gene replacement of veA in CEA17, a commonly used pyrG auxotroph derived from the wild-type pathogenic isolate CBS144-89 (40, 80, 32).

A complementation strain was also generated by transformation of the veA deletion mutant with the veA wild-type allele. The complementation veA strain was constructed by PCR amplifying a 6 kb genomic DNA fragment included the coding region of the *A. fumigatus veA* gene, 2.7 kb 5’UTR and 1.5 kb 3’UTR, with primers veAcompFXbaI394 and veAcompRXbaI478. This PCR product was then digested with *XbaI* and cloned into pBC-Hygro plasmid containing the hygromycin B resistance marker (hyg)(FGSC) previously digested with *XbaI*, resulting in pSD20. A 10.3 kb fragment was then amplified from pSD20 using primers pBC-HygroF769 and pBC-HygroR770, which was then used to transform the veA deletion strain TSD1.15.

The *A. fumigatus veA* over-expression strain (OEveA) was constructed by transforming CEA17 with plasmid pSD21, resulting in the TSD2.8 strain. The over-expression plasmid was constructed as follows: First, *A. fumigatus veA* was amplified by PCR from genomic DNA using primers veAF398_AscI and veAR399_NotI (Table 2). This fragment was digested with AscI and NotI and ligated to plasmid
pTMH44.2 (gift from N.P. Keller) previously digested with AscI and NotI. pTMH44.2 contains the
gpdA(p) and trpC(t) from A. nidulans. A. fumigatus pyrG was PCR amplified from p1439 with primers
PyrGF-Xba1-1439 and pyrGR-Xba1-1439 (Table 2) and cloned into XbaI of pTMH44.2 resulting in the
final transformation plasmid pSD21.

The A. fumigatus gliZ over-expression strain (OE gliZ) was constructed by transforming CEA17
with plasmid pSD37.1, resulting in the TSD26.1 strain. pSD37.1 was constructed as follows: First, A.
fumigatus veA was amplified by PCR from genomic DNA using primers gliZF_AscI1040 and
gliZR_Not11041 (Table 2). This fragment was then digested with Ascl and NotI and ligated to plasmid
pSD21 previously digested with Ascl and NotI.

All A. fumigatus polyethylene glycol-mediated transformations were performed as previously
described (89). Transformants were first screened by PCR using primers veA5F428 and linkerR642 for
deletion, and veAF398_AscI and veAR399_NotI for complementation. Selected transformants were
further confirmed by Southern analysis (75). OEveA strain was confirmed using PCR with primers
gpdAF592 and veAR399_NotI, and OEgliZ strain was confirmed using PCR with primers gpdAF592 and
gliZR_Not11041 (Table 2).

**Morphological analysis.** For assessment of colony growth A. fumigatus strains were point
inoculated on Czapek-Dox medium and colony diameter was measured after 5 days. Then, 16 mm
diameter cores were harvested 1cm from the center of the plates. Cores were homogenized in water and
conidia were counted using a hemacytometer. The experiments were done in the dark and in the light.

**Gliotoxin analysis.** Analysis of gliotoxin production was conducted in a time-course experiment.
Plates containing 25 mL of liquid Czapek-Dox medium were inoculated with approximately 10^7 conidia
per plate. Stationary cultures were incubated at 37°C. Supernatant from 72 h and 120 h old cultures was
filtered through sterile Miracloth™ (Calbiochem). Seventeen mL of culture supernatants from the 72h
and 120h time points were extracted with chloroform. The extracts were then allowed to dry and
resuspended in 1 mL of methanol. The methanol soluble extracts were then filtered with a 0.22µm filter, dried again and resuspended in 500µl of methanol. Gliotoxin analysis was performed as described by Cramer et al., (2006)(22) with some modifications. Briefly, 20 µl from each methanol solution were analyzed in a Waters 1525 HPLC system equipped with a binary pump and a Waters 717 autosampler. HPLC separation was performed at 37°C on a Phenomenex C18 4.6 X 25 mm, 5 micron analytical column connected to a column guard. UV detection was at 264 nm (Waters 2487 Dual λ Absorbance Detector). Compounds were eluted using an isocratic mobile phase of water:acetonitrile:trifluoroacetic acid (65:34.9:0.1) with a flow rate of 1 mL/min. Gliotoxin was detected at a wavelength of 264 nm. Peak areas of the analyzed samples were compared to those from standard gliotoxin (Sigma-Aldrich, MO, USA).

**Gene expression analysis.** Total RNA was isolated from mycelia harvested from 48h and 72h liquid stationary cultures using TRIzol (Invitrogen, USA) following manufacturer’s recommendations. Five micrograms of total RNA was treated with DNAseI RQI (Promega) to remove DNA contamination. One microgram of DNAse treated RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). Expression of gliZ and gliP was analyzed by quantitative real time PCR (qRT-PCR). The reactions were performed with an Mx3000p thermocycler (Agilent Technologies) using SYBR Green Jumpstart Taq Ready mix (Sigma, MO, USA). The primers used in qRT-PCR are listed in Table 2. Similarly, expression of brlA was analyzed in 72 h liquid stationary cultures by qRT-PCR. The primers used in qRT-PCR are also listed in Table 2.

**Protease activity.** The wild type, deletion veA, complementation and over-expression strains were point inoculated on Czapek–Dox medium containing 5% skim milk powder (Difco), in triplicates, and incubated for four days. Degradation halos indicated protease activity. This experiment was also repeated using glucose minimum medium (37) supplemented with skim milk.

An azocasein assay was also performed as previously described by Reichard et al., (1997) (71) with some modifications. Azocasein (Sigma MO USA) at a concentration of 5mg/mL was dissolved in a
solution containing 50mM Tris buffer (pH 7.5), 0.2M NaCl, 5mM CaCl2, 0.05% Brij 35 and 0.01% 

sodium azide. The entire content of 3 day old culture plates was blended with 25 mL of distilled water 
and collected in a 50mL Falcon tube. The tubes were centrifuged at 3800 rpm for 5 minutes at 4°C. One 

mL of supernatant was centrifuged again at 10,000 rpm for 3 minutes at 4°C. One hundred microliters of 
the supernatant was mixed with 400µl of azocasein solution. The samples were incubated at 37°C in a 
water bath for 90 minutes. Twenty percent trichloroacetic acid was added to stop the reactions and 
samples were left at room temperatures for 30 minutes, followed by centrifugation at 8000g for 3 minutes. Three hundred microliters of supernatant was then mixed with 300µl of 1M NaOH. The absorbance of the 
released azo group was measured in 96 well flat bottom plates (BD Falcon) using a plate reader (Biotek) 
at 436nm in duplicates. Distilled water mixed with azocasein was used as blank.

In a separate experiment, the wild type, deletion veA, complementation and over-expression 
strains were incubated on Czapek-Dox medium substituting sodium nitrate with 0.4% BSA or 0.1% 
peptone as nitrogen sources, as described previously (9), and incubated at 37°C for 3 days to observe 
possible differences in the ability to grow utilizing a non-hydrolyzed nitrogen source.

**Fluorescence microscopy.** *Aspergillus fumigatus* CEA17 strain (Table 1) was transformed with 
the veA::gfp::pyrG4::fum fusion PCR products as described by Szewczyk et al. (2006)(89). Primers used for 
fusion PCR are listed in Table 2. Plasmid p1439 (83) was used as template for the PCR amplification of 
the intermediate fragment. Integration was confirmed by PCR and Southern blot analysis (data not 
shown). Conidia from the selected transformant TSD21.1 (Table 1) were inoculated as described 
previously (85). Briefly, conidia were inoculated on the surface of cover slips immersed in Watch 
minimal medium (65). The cultures were incubated for 16 hours at 37°C in the dark or in the light. 
Samples were observed with a Nikon Eclipse E-600 microscope with a 60x oil-immersion objective, 
Nomarski optics and fluorochromes for GFP (ex 470, em 525) detection. Micrographs were taken using a 
Hamamatsu ORCA-ER high-sensitivity monochrome digital CCD camera with MicroSuite5 image
capture and optimization software. DIC and DAPI images, to indicate nuclear localization, were taken with an exposure of 200 ms and GFP with 1 s.

**Osmotic and oxidative stress assays.** To test sensitivity to osmotic stress, Czapek-Dox medium was supplemented with 0.6M KCl, 1.2M sorbitol or 1M sucrose. Strains were then point-inoculated on these media. After 3 days of incubation at 37°C, colony diameter was measured.

Sensitivity of *A. fumigatus* strains to oxidative stress was determined as previously described by Sugareva et al. (2006)(87) with some modification. Spores (5 x10⁶ spores/mL) were top-agar inoculated with 5 mL of Czapek-Dox medium (0.5% Agar) on 25 mL plates of solid Czapek-Dox medium (1.5% Agar). After solidification, 7 mm cores were made at the center of each plate and 100 μl of 2mM water soluble menadione (Sigma-Aldrich, MO, USA) was added. The plates were incubated at 37°C for 30h, and growth inhibition halos were measured. The experiment was performed with three replicates.

Menadione sensitivity was also tested by adding various concentrations of menadione to the medium (0μM, 5μM, 7.5μM, 10μM and 15μM) after autoclaving, and measuring the colony diameter after 48 hours. The experiment was performed with four replicates.

**Effect of pH on growth.** To test a possible veA-dependent effect of different pH on *A. fumigatus* growth, Czapek-Dox medium was adjusted to various pH levels (3, 4, 5, 6, 7, 8, 9 and 10) and point inoculated with wild type, deletion veA, complementation and over-expression strains. Colony diameter was measured after 48 h of incubation.

**Cell wall defect tests.** To identify possible alterations in cell wall due to alterations caused by the deletion or over-expression of veA, the strains were inoculated on Czapek-Dox medium containing 0.05% or 0.1% SDS. With the same goal, a similar experiment was also carried out adding 5μg/mL, 10μg/mL, 20μg/mL, 25μg/mL, 50μg/mL or 100 μg/mL of Congo red to Czapek-Dox medium (69, 73, 93). The cultures were incubated at 37°C in the dark. After 72h colony diameter was observed. Cell wall integrity was also tested by adding 32μg/ml and 64μg/ml of the antifungal compound nikkomycin Z, as described...
in (84).

Pathogenicity tests. Female Six-week-old, outbred Swiss ICR mice (Harlan Sprague Dawley), weighing 24 to 27 g were immunosuppressed (neutropenic) by intraperitoneal injection of cyclophosphamide (150 mg/kg of body weight) on days –4, –1, and 3 with a single dose of cortisone acetate (200 mg/kg), given subcutaneously. The mice were anesthetized via isoflourance inhalation in a bell jar on day 0. The mice were infected by intranasal inoculation, mimicking the natural route of infection. Inocula were prepared from four strains: isogenic wild-type, ΔveA, complementation strain and OEveA strain grown on GMM plates. Conidia were harvested by flooding fungal colonies with 0.85% NaCl with Tween 80, enumerated with a hemocytometer, and adjusted to a final concentration of 6 log<sub>10</sub> CFU/mL (to ensure inoculum viability, counts and the viability of the inocula were verified by triplicate serial plating on GMM plates). Sedated mice (10 mice/fungal strain) were infected by nasal instillation of 50 µl of the inoculum (day 1) and monitored three times daily for 7 days post-infection. All surviving mice were sacrificed on day 7. The tissue fungal burden in whole-lung homogenate was quantified by qPCR. Briefly, in this qPCR procedure we assessed the fungal tissue burden of infected animals by use of a single copy (FKS) to determine the number of fungal cell nuclei present (13, 21, 36). The primers used for qPCR are listed in Table 2. Cumulative mortality curves were generated and the statistical analysis using the log rank test was utilized to perform pair-wise comparisons of survival levels among the strain groups.

Statistical Analysis. Statistical analysis was done, unless otherwise indicated, by analysis of variance (ANOVA) in conjunction with Tukey test using Statistical Package for Social Sciences (SPSS 19, IBM). The means were compared at p-value of 0.05.

RESULTS
veA affects colony pigmentation and conidiation in A. fumigatus. Studies in the model filamentous fungus A. nidulans have shown that veA regulates morphogenesis in a light-dependent manner, acting as a positive regulator of sexual development and a negative regulator of asexual development (100). Krapmman et al. (2005) (44) reported a reduction in conidiation on medium containing nitrate as nitrogen source in an A. fumigatus veA deletion mutant. The corresponding deduced amino acid sequence presents 55% identity with A. nidulans VeA, 67% with A. flavus VeA and 68% with A. parasiticus VeA, all experimentally characterized orthologs (Suppl Fig. 1, and 38, 26, 16). In our study we generated a deletion veA strain and also an OEvEa strain with a constitutively active promoter to further investigate the role of veA in A. fumigatus conidiation as well as fungal growth. The construction of these strains was carried out as detailed in the section Materials and Methods. Polyethylene glycol-mediated transformation of A. fumigatus CEA17 strain with the deletion DNA cassette yielded several transformants. These transformants were analyzed by PCR (data not shown) and Southern blot (Fig. 1A and B). Complementation strains were obtained by transforming the selected deletion strain (TSD1.15) with an A. fumigatus veA wild-type allele (Fig. 1B). OEvEa strains were also generated and confirmed by PCR (Fig. 1C and D). Expression analysis by qRT-PCR indicated absence of A. fumigatus veA expression in the ΔveA strain, recovery of veA expression after complementation with the veA wild-type allele, and higher accumulation of transcripts when veA was over-expressed. This was observed at both time points tested (48h and 72h after inoculation)(Fig. 1E).

The veA deletion mutants showed slightly smaller colonies on Czapek-Dox (Fig. 2A and B). These mutants also accumulated a dark pigment in the back of the colonies when grown on GMM (36) (Suppl. Fig. 2), which was not produced by the wild-type strain. Colony pigmentation is also observed in A. nidulans ΔveA strains growing on the same medium (data not shown). Complementation of the A. fumigatus veA strain with the wild-type allele repressed the production of this pigment (Suppl. Fig. 2).

Deletion of veA resulted in a 50% reduction in conidiation rate with respect to the levels observed
in wild-type control cultures growing on Czapek-Dox (Fig. 2A and C). Interestingly, over-expression of veA lead to an even more pronounced decrease of conidiation (85% reduction) and abundant proliferation of aerial mycelium. The same effects were observed in additional veA deletion and OEveA mutants generated in this study (data not shown). Furthermore, expression of brlA, encoding a transcription factor that regulates conidiation (2), was also decreased in the deletion and over-expression strains compared to the expression levels in the control strains (Fig. 2D). Although A. fumigatus OEveA strain is deficient in conidiation, it only presented small variations in growth rate with respect to the control strain; slightly smaller but denser colonies when grown on solid Czapek-Dox medium (Fig. 2A and C) and slightly greater biomass production when growing in liquid stationary cultures (Suppl. Fig. 3) compared to the wild-type strain under the same experimental conditions.

Although in A. nidulans it has been established that veA regulation of morphogenesis is light-dependent (8, 17, 100), in A. fumigatus the effect of veA on conidiation was similar in either dark (Fig. 2A and C) or light cultures (Suppl. Fig. 4), suggesting that, differently from the model fungus A. nidulans, light is not a relevant factor in the veA-mediated regulation of conidiation in A. fumigatus.

Gliotoxin production and expression of gliotoxin gene cluster genes is veA-dependent. Aspergillus fumigatus produces gliotoxin, a secondary metabolite that in some cases has been shown to be associated with pathogenicity, and that has been detected in mice lungs during infection (12). Previously it has been demonstrated that veA orthologs regulate secondary metabolism in other fungal species (i.e. 17, 19, 26, 38, 56). For this reason we investigated whether veA also controls the biosynthesis of gliotoxin in A. fumigatus. We compared the production of gliotoxin in A. fumigatus wild type, ΔveA, complementation and OEveA strains at 3 days and 5 days after inoculation. Our HPLC analysis indicated that in the absence of veA, gliotoxin production was reduced 5-fold with respect to the wild type, and reached only 25% of wild-type levels after 5 days of incubation (Fig. 3). Interestingly, OEveA produced only trace amounts of gliotoxin at both time points.
Previous studies have shown that \textit{gliP}, encoding a nonribosomal peptide-synthase (NRPS) \cite{22, 46, 81, 88}, and \textit{gliZ}, encoding a putative Zn2Cys2 binuclear transcription factor \cite{12}, both located in the gliotoxin gene cluster, are necessary for gliotoxin biosynthesis. We examined whether the effect of \textit{veA} on gliotoxin production was the result of a possible role of \textit{veA} in regulating the expression levels of \textit{gliZ} and \textit{gliP} (Fig. 4A and B respectively). In this study gene expression was evaluated by qRT-PCR at 48h and 72h after inoculation. Our results indicated that \textit{gliP} expression levels were only approximately 10% in \textit{ΔveA} strain with respect to the wild-type strain at both 48h and 72h after inoculation (Fig. 4B). Parallely, over-expression of \textit{veA} also resulted in a decrease of \textit{gliP} expression compared to the wild type expression levels. The decrease in \textit{gliP} transcript accumulation correlated with the decrease in gliotoxin production observed in these strains (Fig. 3). The expression of the transcription factor-encoding gene \textit{gliZ} was also reduced in the \textit{ΔveA} to only 10% with respect to wild-type levels (Fig. 4A). Surprisingly, over-expression of \textit{veA} showed an unexpected increase in the expression of \textit{gliZ} compared to wild-type levels.

An additional experiment including the wild type, OE\textit{veA}, and an OE\textit{gliZ} strain (Table 1) was carried out under the same experimental conditions. Again, the OE\textit{veA} strain produced less gliotoxin than the controls, \textit{gliP} was expressed only 10% or less with respect to the wild-type levels while \textit{gliZ} expression levels increased in comparison to the wild-type control. The OE\textit{gliZ} strain produced higher levels of gliotoxin, coinciding with elevated expression levels of both \textit{gliP} and \textit{gliZ} with respect to the wild type (Fig. 5).

\textit{veA} is required for normal protease activity. Hydrolytic enzymes are an important factor that allows \textit{A. fumigatus} to survive in the host environment \cite{73}. Previous studies showed that a protease deficient mutants are unable to grow in mouse lung in vivo. While some proteases have shown no role in virulence \cite{i.e. 9, 35 reviewed in 1, 50, 51}, others have been shown to be associated with this process \cite{i.e. 43, 73}. For this reason, in our study we examined the possible role of \textit{veA} in controlling protease activity.
in *A. fumigatus* and observed that this hydrolytic activity is *veA*-dependent. This is relevant since this is the first finding of a connection between VeA and hydrolytic activity in fungi.

In solid cultures containing casein (5% skim milk), degradation halos were observed in the wild-type and *veA* complemented cultures (Fig. 6A). However, the degradation halos in the Δ*veA* cultures were reduced in comparison to those observed in the control cultures. The azocasein quantitative analysis detected only 15% protease activity in the Δ*veA* cultures with respect to the wild-type strain (Fig. 6B). The OEveA strain also showed a 40% reduction in protease activity (Fig. 6B). Thus, normal expression of *veA* is necessary to maintain wild-type levels of protease activity in *A. fumigatus*.

Additionally, in a separate experiment the wild type, Δ*veA*, complemented strain and OEveA strains were point-inoculated on Czapek–Dox solid medium in which sodium nitrate was substituted with 0.4% bovine serum albumin or 0.1% peptone as nitrogen source, and incubated for 3 days. The growth of these strains was not as robust in Czapek–Dox-BSA as that observed on Czapek–Dox-peptone medium. This effect was especially notable in Δ*veA* colonies (Suppl. Fig. 5).

**Deletion or over-expression of *veA* does not increase sensitivity to pH, oxidative stress, osmotic stress, or presence of SDS or Congo red in *A. fumigatus*.** Silencing of the *H. capsulatum* veA homolog, *VEA1*, resulted in strains that were more sensitive to acidic conditions (49), challenging their ability to grow at low pH. To test whether *veA* affects the capacity of *A. fumigatus* to grow at different pH we inoculated the wild type, Δ*veA*, complemented strain and OEveA strains on Czapek-Dox (pH ranging from 3-10) and incubated for 3 days. Our results indicated that changes in pH did not affect the growth of these strains (Suppl. Fig. 6).

Deletion of another *veA* ortholog in *Cochliobolus heterostrophus* showed a greater sensitivity to oxidative stress in this mutant (99). The ability to respond to the presence of reactive oxygen species (ROS) might be beneficial for the growth of *A. fumigatus* in the lung. While deletion of the conidial specific catalase *catA* results in an increase in sensitivity to H$_2$O$_2$, no differences in conidial killing were
observed in vivo in murine alveolar macrophages cultures (63). On the other hand, mycelial catalases $cat1$ and $cat2$ play a role in protecting the fungus in vivo; the mutants were sensitive to the oxidative burst of polymorphonuclear leukocytes (PMNL’s) (63). In our study we examined whether $veA$ is involved in the oxidative stress response in $A. fumigatus$. We challenged the wild type, $\Delta veA$, complemented strain and $OEveA$ strain cultures on Czapek–Dox supplemented with different concentrations of menadione as detailed in Materials and Methods section. However, the addition of this compound to the culture medium equally affected the ability of all the strains to grow in the same manner (Suppl. Fig. 7) inhibiting growth at 20 mM menadione concentration.

The possible role of $veA$ in osmotic stress response was examined by addition of either sucrose (1M), sorbitol (1.2M) or KCl (0.6M) to the medium. Also, the possible effect of $veA$ on the integrity of the fungal cell wall was tested by addition of SDS, Congo red, or nikkomycin Z to Czapek-Dox, as described in the Materials and Methods section. In these three experiments the effect on growth caused by the supplementation of these compounds was $veA$-independent in $A. fumigatus$ (data not shown and Suppl. Fig. 8).

**Subcellular localization of Aspergillus fumigatus VeA.** Previous studies in the model filamentous fungus $A. nidulans$ have demonstrated that the VeA protein is transported to the nucleus, where it forms a nuclear complex with other proteins (4, 6, 85). With the purpose of elucidating the subcellular localization of VeA in $A. fumigatus$, we generated a strain containing VeA fused to GFP. Observations of this strain using fluorescence microscopy indicated that $A. fumigatus$ VeA localizes in both cytoplasm and nuclei, preferentially accumulating in nuclei, as revealed when comparing with images of micrographs with DAPI staining indicating nuclear location (Fig. 7). Similar results were obtained in light cultures (Suppl Fig. 9), indicating that, differently from $A. nidulans$ VeA, $A. fumigatus$ VeA subcellular localization was light-independent.
Aspergillus fumigatus veA does not affect pathogenicity in the neutrophenic mouse model.

veA is necessary for virulence in different plant pathogenic fungi (i.e. 27, 54, 57, 96). Furthermore a recent characterization of the H. capsulatum veA ortholog by Smulian’s group in collaboration with our group showed for the first time that a veA ortholog is necessary for normal virulence in animals (49). In the present study we investigated if veA was also a virulence factor in A. fumigatus in a neutrophenic mouse model experiment. Seven days after infection with wild type, ΔveA, complemented strain or OEvA strains no significant differences in survival rate were detected among the strains.

Fungal burden in mouse lungs of dead or sacrificed animals seven days after infection was measured by qPCR, and normalized against values obtained from mice sacrificed just after infection. The data showed no significant difference in lung colonization among the A. fumigatus strains tested.

DISCUSSION

Aspergillus fumigatus is a primary causative agent of human infections. IA affects immunocompromised patients resulting in a devastating disease with high mortality rates. Even with the recent advances in medical technology, A. fumigatus infections are on the rise. This organism disseminates by producing abundant air-borne conidia that can easily reach the lung alveoli due to their small size leading to infection in immunosupressed hosts (74, 76). In this study we demonstrated that the global regulator veA is important for normal production of conidia. VeA has been shown to control development in other fungal species (i.e. 6, 25, 26, 38, 39, 52). The role of veA as a negative regulator of asexual development has been particularly well characterized in the model fungus A. nidulans, where it was demonstrated that the light-dependent subcellular localization of the VeA protein controls the asexual/sexual developmental balance (4, 85). The deletion of veA in A. nidulans also affects the brlA α/β
transcript ratio involved in the activation of conidiation (38). The reduction of conidiation in the *A. fumigatus* deletion *veA* strain observed in our studies is in agreement with previous work showing a nitrate-dependent reduction (44). However, differing from this report, our experiments indicated that *veA* over-expression resulted in an even more notable negative effect on conidiation. It is possible that this difference could be due to the use of different culture media. A reduction of *brlA* expression in the deletion and over-expression strains parallels the differences in conidiation observed in Czapek-Dox cultures. Similar to our findings, *A. nidulans* over-expression of the *veA* ortholog also leads to a decrease in conidiation (39). Our study revealed that both, abnormally high levels as well as absence of the VeA regulator in *A. fumigatus* are detrimental for proper asexual development in this fungus. It is possible that certain balanced stoichiometry with respect to other regulatory factors, perhaps with other proteins of the VeA complex (6, 7, 58) in the nucleus (Fig.7), might be necessary for normal conidiation in this organism.

Differently from *A. nidulans*, where VeA control of morphogenesis is light-dependent, in *A. fumigatus* this regulatory process was light-independent, observing similar results in either dark or light cultures. With respect to sexual development, the *A. fumigatus* genome contains homologs of mating type genes (70, 98), as well as other genes such as *nsdD* (9), *nsdC* or *steA* (data not shown), demonstrated to control sexual development in *A. nidulans*. Sexual development in *A. fumigatus* has been elusive until 2009, when O’Gorman et al. (2009)(59) described fruiting bodies in this fungus. In our study *A. fumigatus* CEA10 did not reproduce sexually, hence future research might be needed to demonstrate the possible role of VeA in *A. fumigatus* sexual development. It is known that *A. fumigatus veA* restores cleistothecial formation in the *A. nidulans veA* deletion mutant (44). This, together with the fact that *veA* controls sexual development in other fungal species (i.e. 39, 49, 52, 99) suggests a role of *veA* in *A. fumigatus* sexual development.
Although veA regulates morphogenesis in *A. fumigatus*, only a small effect on growth was observed. Variation in veA expression did not affect the fungus response to pH or high levels of ROS, while veA orthologs in other fungal species are necessary for the genetic response to adapt and grow under these conditions (49, 99). In *A. fumigatus*, veA is not required for osmotic stress response either. This suggests that *A. fumigatus* might have a robust capacity to adapt to different environmental stresses using alternative veA-independent regulatory mechanisms that allows this fungus to survive. Cell wall integrity was not affected by veA in *A. fumigatus*, whereas in *F. verticillioides* deletion of the veA ortholog FvVE1 leads to defects in the cell wall that can be compensated with osmotic stabilizers (52). Similar results were also observed in *Acremonium chrysogenum* veA ortholog studies (25).

Numerous studies have shown that VeA is also a master regulator of secondary metabolism in many fungal species (i.e. 5, 16, 19, 25, 26, 38, 54, 56, 96, 99). In *A. fumigatus* however, the involvement of VeA in secondary metabolism has not been investigated until now. Previous research revealed a role of fungal secondary metabolites as virulence factors during *A. fumigatus* lung colonization in immunosuppressed mice (81, 88). The most well known among these compounds is gliotoxin (i.e. 3, 12, 20, 28, 29, 55, 62, 66, 72, 83, 86, 88, 92, 94, 101, 102). The gene cluster responsible for the synthesis of gliotoxin has been found (30). It is comprised of 22 genes and located in chromosome VI. Independent groups have disrupted the nonribosomal peptide synthase encoding gene gliP within this cluster (22, 46, 88). These strains fail to produce gliotoxin. Similar results were obtained when *gliZ*, encoding a putative Zn2Cys2 binuclear transcription factor, also within this gene cluster, was deleted (12), suggesting that *gliZ* acts as specific positive regulator of the gliotoxin gene cluster. Location of genes encoding specific transcription factors in fungal secondary metabolic gene clusters is a common occurrence. One well-known example is *aflR*, encoding a transcription factor that activates the aflatoxin and sterigmatocystin gene clusters in *A. flavus* and *A. parasiticus*, and *A. nidulans* respectively (i.e. 18, 64, 97, 103). In these and other fungi, the transcription factor within the clusters has been often found to be controlled by VeA.
(17), functionally connected to LaeA (6, 8). Our experiments indicated that VeA is required for wild-type levels of gliotoxin, and that, similarly to the observed effect on conidiation, the absence or over-expression of veA has a negative effect on the production of this mycotoxin. Expression levels of gliP, used as an indicator of cluster activation, were down-regulated in both deletion veA and over-expression veA strains. In our study we also observed that VeA influences the expression of gliZ. The A. fumigatus strain lacking veA presented a reduction in gliZ expression with respect to the wild type, suggesting that abnormally low levels of gliZ in ΔveA are detrimental to the proper expression levels of the gliotoxin structural genes in the cluster, and therefore negatively affecting the production of this mycotoxin. Surprisingly, expression levels of gliZ were greater in the over-expression veA strain, in spite of the observed low levels of gliP expression and reduced gliotoxin accumulation. When gliZ was over-expressed in a veA wild-type background, high levels of both gliP and concomitant gliotoxin accumulation were observed. It is possible that veA in A. fumigatus could influence the expression of structural genes in the gliotoxin clusters by other unknown mechanisms in addition to the effect on the expression of gliZ. Detrimental effects on the production of certain secondary metabolites by both deletion and over-expression mutants of veA homologs have been previously reported. For example, in A. nidulans both deletion or over-expression of veA leads to a reduction in penicillin production (38, 82).

Other virulent factors that could influence A. fumigatus infection are hydrolytic enzymes (42). The action of some hydrolases facilitates tissue penetration in the infected A549 lung epithelial cell lines (41). Several proteases have been characterized in A. fumigatus with different effects on virulence (9, 35, 43). Deficiencies of elastolytic serine protease (42) or the protease secretion-deficient hacA, mutant (73) presented a reduction in pathogenicity. As mentioned, whether VeA affects hydrolytic activity in A. fumigatus has not previously been investigated. Our study revealed that Aspergillus fumigatus ΔveA and OEveA strains present a notable reduction in protease activity, particularly the ΔveA strain, with only 10% activity with respect to activity observed in the wild type. These results indicate an additional role of veA
that has not been reported before in fungi, regulating hydrolytic activity, in this case protease activity in

*A. fumigatus*.

Due to the fact that both deletion and over-expression of veA are detrimental for gliotoxin production and also for protease activity we predicted a decrease in pathogenicity in these mutants. Furthermore, absence of LaeA, shown to interact with VeA in *A. nidulans*, resulted in a decrease in pathogenicity in neutropenic mice (11). Surprisingly, in our study neither ΔveA nor OEveA strains showed a difference in virulence with respect to the control strains. Fungal burden data showed the same ability to colonize the lungs in the ΔveA or OEveA strains as compared to the control strains. It is possible that LaeA might control a separate set of virulence factors relevant in the neutropenic immune-suppressed environment, for example secondary metabolites other than gliotoxin, control by both regulators (Fig. 3 and 11), or different characteristic of the spore surface (11). Although veA did not show a relevant role in pathogenicity in *A. fumigatus*, the veA ortholog in *H. capsulatum* was required for normal virulence in mice (49), suggesting that the role of veA in virulence might be different in other pathogenic fungi that affect animals and humans. It is also possible that although veA was not necessary for pathogenicity in neutropenic mice, this regulator could be important during *A. fumigatus* infection in non-neutropenic immunosuppressed hosts. Future studies will contribute to define the relationship of VeA in infection in other immunosuppressed environments.

In conclusion, we have shown that *A. fumigatus* veA is a regulator of conidiation and also revealed veA as a positive regulator of gliotoxin, affecting expression of genes in the gliotoxin gene cluster. We have shown that in *A. fumigatus* protease activity is regulated by veA; a novel finding connecting this master regulator with fungal hydrolytic activity. Based on the conservation of VeA in other filamentous fungi, particularly in Ascomycetes, it is likely that VeA orthologs could affect hydrolytic activity in other fungal species. Another important observation is that alterations in veA expression (either deletion or over-expression of veA) did not, however, result in changes in pathogenicity under the experimental...
conditions tests, while deletion of the veA ortholog in other species, such as *H. capsulatum* (49), lead to a reduction of virulence. This suggests that the possible use of VeA as a target in the design of a general strategy against a broad range of fungal infection might not be feasible, and that although VeA still retains its high potential for this purpose, the role of VeA in pathogenicity must be studied on a case-by-case basis in different fungal species.

**ACKNOWLEDGEMENTS**

This work was funded by NIH R03AI079496. We wish to thank Robert Cramer for helpful discussion, and we also thank Barbara Ball for technical support.

**Table 1.** Strains used in this study.

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**Table 2.** Primers used in this study. Restriction sites are underlined.
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*Integrated DNA Technologies, Coralville, IA

**REFERENCES**


Espeso. 2009. Importin alpha is an essential nuclear import carrier adaptor required for proper sexual and asexual development and secondary metabolism in *Aspergillus nidulans*. Fungal Genetics and Biology **46**:506-515.


the fungal cell wall. Journal of Medical and Veterinary Mycology 35:189-196.


with hydrocortisone. Eukaryotic Cell 6:1562-1569.


94. Waring, P. 1990. DNA fragmentation induced in macrophages by gliotoxin does not require protein-synthesis and is preceded by raised inositol triphosphate levels. Journal of Biological Chemistry 265:14476-14480.


Figure legends
Fig. 1. DNA and RNA analyses for the verification of the deletion and over-expression *A. fumigatus* mutants. (A) Schematic representation showing *EcoRI* sites in the *A. fumigatus* wild-type veA locus, and the deletion veA construct generated to replace veA by the *A. fumigatus pyrG* gene (*pyrG<sup>A. fum</sup>*) utilized as transformation marker gene. The fragment used as probe template for Southern blot analysis is also shown. Size of the DNA fragments predicted for the Southern blot analysis using this probe is also shown for both the wild type and deletion veA mutant. (B) Southern blot analysis. The veA gene replacement construct was transformed in CEA17. *EcoRI*-digested genomic DNA of CEA10 wild type (WT), ΔveA transformant (TSD1.15) and complementation transformant (TSD3.5) was hybridized with the probe shown in (A). Additional deletion transformants also showed the same band pattern (data not shown). The extra band in the analysis of the complementation strain indicates integration of the wild-type veA allele in the TSD1.15 genomic DNA. Additional complementation transformants were also obtained. Integration of the full complementation cassette in these strains was also confirmed by PCR (data not shown). (C) Diagram showing the veA over-expression DNA cassette (from pSFD21 plasmid) used in this study. Vertical lines indicate the annealing sites for the primers gpdAF592 and veAR399_NotI (Table 2) used in the diagnostic PCR of four veA over-expression transformants shown in (D), with a predicted PCR product of 1917 bp. (E) Expression analysis of veA by qRT-PCR using primers veA_qRTPCR_F814 and veA_qRT PCR_R815 (Table 2).

Fig 2. Role of veA in *A. fumigatus* colony growth and conidiation. A) *Aspergillus fumigatus* wild type (WT), veA, complementation (com) and OEveA (OE) strains were point-inoculated on Czapek-Dox medium and incubated for 5 days in the dark. B) Colony diameter measurements. C) Quantification of conidial production. Cores (16mm diameter) were taken 1cm from the center of the plates and homogenized in water. Spores were counted using a hemocytometer. D) Relative expression levels of
brlA after 72 hours in liquid stationary cultures. *Aspergillus fumigatus* 18s was used as internal reference gene. Error bars represent standard error.

Fig. 3. *veA* controls gliotoxin production. *Aspergillus fumigatus* wild type (WT), Δ*veA*, complementation (com) and OEv*veA* (OE) strains were grown in liquid stationary cultures in Czapek-Dox medium. Samples were collected for toxin analysis 72 h and 120 h after inoculation, and extracted with chloroform as detailed in Material and Methods section. Gliotoxin production in these cultures was analyzed by HPLC. The experiment was done with three replicates. Error bars represent standard error. Different letters indicate samples that are significantly different (*p* ≤ 0.05).

Fig. 4. Expression of *gliZ* and *gliP* is regulated by *veA*. Transcriptional pattern of *gliZ* (A) and *gliP* (B) was evaluated by qRT-PCR. Total RNA was isolated at 48 and 72 h from liquid stationary Czapek-Dox cultures incubated in the dark at 37°C. Primer pairs *gliZ_qRTPCR_F* and *gliZ_qRTPCR_R*, and *gliP_qRTPCR_R* and *gliP_qRTPCR_R* (Table 2) were used to measure expression of *gliZ* and *gliP*, respectively. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method described by Schmittgen and Livak (2008)(77). Expression of the *A. fumigatus* 18S gene was used as internal reference. Values were normalized to that of the wild type at 48 h considered as 1. The error bars indicate the ranges for three replicates.

Fig. 5. *veA* controls gliotoxin production through additional mechanisms besides influencing *gliZ* expression. (A) HPLC analysis of gliotoxin from *Aspergillus fumigatus* wild type (WT), OEv*veA* and OEv*gliZ* after 72 and 120 h of incubation in liquid stationary cultures. (B) and (C) Transcriptional pattern of *gliZ* and *gliP* respectively. Total RNA was isolated at 48 and 72 h after inoculation. Primer pairs *gliZ_qRTPCR_F* and *gliZ_qRTPCR_R*, and *gliP_qRTPCR_R* and *gliP_qRTPCR_R* (Table 2)
were used to measure expression of gliZ and gliP, respectively. The relative expression levels were calculated using the 2^{-ΔCt} method described by Schmittgen and Livak (2008) (77). Expression of the *A. fumigatus* 18S gene was used as internal reference. Values were normalized to that of the wild type at 48 h considered as 1. The error bars indicate the ranges for three replicates.

**Fig. 6.** levels of veA expression are necessary for normal protease activity. (A) *Aspergillus fumigatus* wild type (WT), ΔveA, complementation (com) and OEveA (OE) strains were point-inoculated on Czapek-Dox medium containing 5% skim milk. The plates were grown at 37°C in the dark. Degradation halos indicate protease activity. (B) Quantification of proteolytic activity measured by azocasein assay. The experiment included four replicates and was repeated twice with similar results. Error bars represent standard error. Different letters indicate statistically significant differences (p≤0.05).

**Fig. 7.** *Aspergillus fumigatus* VeA preferentially localizes in nuclei. (A) Representation of the strategy to fuse GFP to VeA. The tagged construct was introduced at the veA locus by a double-recombination event. (B) Micrographs showing the subcellular localization of the VeA::GFP fusion described in (A) in *A. fumigatus*. From left to right, Normaski images, DAPI images, and green fluorescence images are included. DAPI images were used as nuclear localization internal control. Scale bars represent 10µm. Arrows indicate nuclei.

**Fig. 8.** veA is dispensable in *Aspergillus fumigatus* virulence. (A). Neutropenic CD-1 mice were infected intranasally with conidia from *Aspergillus fumigatus* wild type (WT), ΔveA, complementation (com) and OEveA (OE) strains. Survival rates are shown. (B) Fungal burden in mouse lungs measured by qPCR. DNA isolated from whole mouse lungs was used. The experiment included 10 mice per group.
Fig. 1. DNA and RNA analyses for the verification of the deletion and over-expression A. fumigatus mutants. (A) Schematic representation showing EcoRI sites in the A. fumigatus wild-type veA locus, and the deletion veA construct generated to replace veA by the A. fumigatus pyrG gene (pyrG<sup>A, fum</sup>) utilized as transformation marker gene. The fragment used as probe template for Southern blot analysis is also shown. Size of the DNA fragments predicted for the Southern blot analysis using this probe is also shown for both the wild type and deletion veA mutant. (B) Southern blot analysis. The veA gene replacement construct was transformed in CEA17. EcoRI-digested genomic DNA of CEA10 wild type (WT), ΔveA transformant (TSD1.15) and complementation transformant (TSD3.5) was hybridized with the probe shown in (A). Additional deletion transformants also showed the same band pattern (data not shown). The extra band in the analysis of the complementation strain indicates integration of the wild-type veA allele in the TSD1.15 genomic DNA. Additional complementation transformants were also obtained. Integration of the full complementation cassette in these strains was also confirmed by PCR (data not shown). (C) Diagram showing the veA over-expression DNA cassette (from pSD21 plasmid) used in this study. Vertical lines indicate the annealing sites for the primers gpdAF592 and veAR399_NotI (Table 2) used in the diagnostic PCR of four veA over-expression transformants shown in (D), with a predicted PCR product of 1917 bp. (E) Expression analysis of veA by qRT-PCR using primers veA_qRTPCR_F814 and veA_qRTPCR_R815 (Table 2).
Fig 2. Role of veA in *A. fumigatus* colony growth and conidiation. A) *Aspergillus fumigatus* wild type (WT), veA, complementation (com) and OEv eA (OE) strains were point-inoculated on Czapek-Dox medium and incubated for 5 days in the dark. B) Colony diameter measurements. C) Quantification of conidial production. Cores (16mm diameter) were taken 1cm from the center of the plates and homogenized in water. Spores were counted using a hemocytometer. D) Relative expression levels of *brlA* after 72 hours in liquid stationary cultures. *Aspergillus fumigatus* 18s was used as internal reference gene. Error bars represent standard error.
Fig. 3. veA controls gliotoxin production. *Aspergillus fumigatus* wild type (WT), ΔveA, complementation (com) and OEveA (OE) strains were grown in liquid stationary cultures in Czapek-Dox medium. Samples were collected for toxin analysis 72 h and 120 h after inoculation, and extracted with chloroform as detailed in Material and Methods section. Gliotoxin production in these cultures was analyzed by HPLC. The experiment was done with three replicates. Error bars represent standard error. Different letters indicate samples that are significantly different (p<0.05).
Fig. 4. Expression of gliZ and gliP is regulated by veA. Transcriptional pattern of gliZ (A) and gliP (B) was evaluated by qRT-PCR. Total RNA was isolated at 48 and 72 h from liquid stationary Czapek-Dos cultures incubated in the dark at 37°C. Primer pairs gliZ_qRTPCR_F838 and gliZ_qRTPCR_R839, and gliP_qRTPCR_R841 and gliP_qRTPCR_R841 (Table 2) were used to measure expression of gliZ and gliP, respectively. The relative expression levels were calculated using the 2^(-ΔΔCt) method described by Schmittgen and Livak (2008) (75). Expression of the A. fumigatus 18S gene was used as internal reference. Values were normalized to that of the wild type at 48 h considered as 1. The error bars indicate the ranges for three replicates.
Fig. 5. veA controls gliotoxin production through additional mechanisms besides influencing gliZ expression. (A) HPLC analysis of gliotoxin from *Aspergillus fumigatus* wild type (WT), OEveA and OEgliZ after 72 and 120 h of incubation in liquid stationary cultures. (B) and (C) Transcriptional pattern of *gliZ* and *gliP* respectively. Total RNA was isolated at 48 and 72 h after inoculation. Primer pairs *gliZ* qRTPCR_F838 and *gliZ* qRTPCR_R839, and *gliP* qRTPCR_R841 and *gliP* qRTPCR_R841 (Table 2) were used to measure expression of *gliZ* and *gliP*, respectively. The relative expression levels were calculated using the $2^{-\Delta\Delta C_{t}}$ method described by Schmittgen and Livak (2008) (75). Expression of the *A. fumigatus* 18S gene was used as internal reference. Values were normalized to that of the wild type at 48 h considered as 1. The error bars indicate the ranges for three replicates.
Fig. 6. Wild-type levels of veA expression are necessary for normal protease activity. (A) *Aspergillus fumigatus* wild type (WT), ΔveA, complementation (com) and OEveA (OE) strains were point-inoculated on Czapek-Dox medium containing 5% skim milk. The plates were grown at 37°C in the dark. Degradation halos indicate protease activity. (B) Quantification of proteolytic activity measured by azocasein assay. The experiment included four replicates and was repeated twice with similar results. Error bars represent standard error. Different letters indicate statistically significant differences (p≤0.05).
Fig. 7. *Aspergillus fumigatus* VeA preferentially localizes in nuclei. (A) Representation of the strategy to fuse GFP to VeA. The tagged construct was introduced at the veA locus by a double-recombination event. (B) Micrographs showing the subcellular localization of the VeA::GFP fusion described in (A) in *A. fumigatus*. From left to right, Nomarski images, DAPI images, and green fluorescence images are included. DAPI images were used as nuclear localization internal control. Scale bars represent 10μm. Arrows indicate nuclei.
Fig 8. *veA* is dispensable in *Aspergillus fumigatus* virulence. (A). Neutropenic CD-1 mice were infected intranasally with conidia from *Aspergillus fumigatus* wild type (WT), Δ*veA*, complementation (com) and OEVeA (OE) strains. Survival rates are shown. (B) Fungal burden in mouse lungs measured by qPCR. DNA isolated from whole mouse lungs was used. The experiment included 10 mice per group.