RESEARCH LETTER

NCE102 homologue in Aspergillus fumigatus is required for normal sporulation, not hyphal growth or pathogenesis

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Abstract

In Saccharomyces cerevisiae, Nce102 encodes a 173 amino acid transmembrane protein, which acts as a key player in eisosome assembly and plasma membrane organization. Here, we describe the characterization of Nce102 homologue in the human pathogen, Aspergillus fumigatus. Our results demonstrated that AfuNce102 is continuously expressed during fungal growth. In addition, microscopic examination of an AfuNce102-GFP-expressing transformant confirmed the localization of the fusion protein to the endoplasmic reticulum with higher density fluorescence at the tip of the mycelium. During conidiogenesis, the protein was localized to the conidiophores and the conidia. Abnormal conidiation of AfuNce102 deletion mutant suggests a potential role for AfuNce102 in sporulation process.

Introduction

A nonclassical export pathway has been proposed in yeast as an alternative route for the secretion of proteins lacking signal sequence (Cleves et al., 1996; Nombela et al., 2006). Based on a screen for gene products involved in this nonclassical export pathway, three genes, Nce101, Nce102, and Nce103, have been identified as being involved in protein secretion (Cleves et al., 1996). In Saccharomyces cerevisiae, Nce102 encodes a 173 amino acid peptide containing four transmembrane domains. Early functional studies on Nce102 demonstrated that the deletion of this gene can severely disrupt the nonclassical secretion of heterologous mammalian galectin-1. This observation has led to a hypothesis that Nce102-related nonclassical export pathway may be involved in the transport of virulence factors to the cell surface of pathogenic microorganisms (Nombela et al., 2006). The yeast deletion mutant of Nce102 was also found to be more sensitive to diethylmaleate toxicity, suggesting a possible role for Nce102 in protection of the cell against oxidative stress (Desmyter et al., 2007).

Recently, a genome-wide screen in yeast has identified the Nce102 as a key player in plasma membrane organization (Frohlich et al., 2009). In yeast, the plasma membrane is highly organized and laterally divided into two overlapping compartments, membrane compartment of Can1 (MCC) and membrane compartment of Pma1 (MCP). Careful examination of these membrane compartments demonstrated that Nce102 is associated with MCC and the deletion of Nce102 gene affected the integrity of this compartment. Nce102 co-localizes with main cytosolic components of eisosomes, Pil1, and Lsp, and the deletion of Nce102 resulted in an altered number and distribution of eisosomes (Walther et al., 2006). These data suggest that Nce102 is required for normal eisosome and MCC formation.

In a recent study, the eisosomal protein homologues (PilA, PilB, and SurG) in Aspergillus nidulans have been characterized (Vangelatos et al., 2010). Detailed analysis of pilA, pilB, or surG deletion strains have confirmed the nonessential role of these proteins in fungal growth. Furthermore, the endocytosis process was not affected in these mutants, demonstrating a possible functional divergence of eisosomal proteins in Aspergilli. A similar study on
Eisosomal proteins of filamentous fungus *Ashbya gossypii* confirmed the important role of *pil1* homologue in polar growth and the nonessential role of *Nce102* homologue in growth and eisosomal stability (Seger et al., 2011). In the present study, we have characterized *Nce102* homologue, *AfuNce102*, in the human pathogen, *Aspergillus fumigatus*. Our results indicate that this gene is not essential for hyphal growth or pathogenesis, but it is required for normal sporulation. Localization studies using an enhanced green fluorescent protein (EGFP)-tagged *AfuNce102* construct demonstrated that *Afu-Nce102* is primarily localized to endoplasmic reticulum with more intensity at the hyphal tip. During the conidiosporeogenesis, the protein localized to conidiophores and conidia.

**Materials and methods**

**Strains, plasmids, and culture conditions**

*Aspergillus fumigatus* strain AF293 and its *PyrG* derivative were used for the isolation of the *Nce102* gene homologue and in gene disruption experiments. *Escherichia coli* Top10 (Invitrogen) cells were used in DNA recombinant procedures. pGEM-T Easy cloning system (Promega) was used for cloning of PCR products. Plasmid pGEM-GlaA-EGFP, comprised the *Aspergillus niger* gglA promoter, EGFP sequence, and gglA termination signal, was used for preparation of NCE-GFP-tagged construct. Plasmid pAN7.1 containing the hygromycin resistance gene as a fungal selection marker was used in co-transformation experiments of NCE-GFP-tagged construct (Punt et al., 1987).

Fungal strains were grown and kept on SAB agar or SAB agar medium supplemented with uridine and uracil (UU). Modified Vogel’s medium (Vogel, 1956) was used in isolation of fungal transformants. For phenotypic analysis of strains, radial growth rates were determined by cultivation of fungal spores (10⁴ spores) on center of SAB and modified Vogel’s agar plates at 30, 37, and 42 °C followed by serial measurement of colonies’ diameter for 5 days.

**DNA and RNA manipulations**

Fungal DNA was prepared as previously described (Moller et al., 1992). RNA samples were purified using a commercial kit (Qiagen, RNA easy® Mini kit). Molecular methods including ligation of DNA fragments, transformation of *E. coli*, and restriction mapping were performed according to Sambrook & Russell (2001). For RT-PCR reactions, cDNA was synthesized using ReverTraAidTM (Fermentas). In all cDNA synthesis reactions, 1 μg of total RNA (adjusted with Nanodrop 1000) was used. All PCRs were performed as 30 cycles of 95 °C for 1 min, 58 °C for 30 s, and 72 °C for 30 s. The *A. fumigatus* actin fragment (500 bp) was amplified as a loading control during all RT-PCRs.

**Construction of Nce102 deletion cassette and Nce-egfp fusion cassette**

Constructs were prepared to facilitate homologous recombination using Nce102 flanking regions surrounding a *pyrG* marker (Fig. 1a). A 4-kb fragment containing the entire Nce102 coding region with upstream and downstream flanking regions was cloned into the pGEM-Teasy vector. From this vector, a 1.8-kb 3’ flanking region

Fig. 1. (a) Schematic representation of disruption cassette construction. The native *AfuNce102* gene will be replaced by disruption cassette through homologous recombination. The positions of primers used in this study are shown. (b) Schematic representation of constructed plasmids used in gene deletion experiment (pNCEKO) and GFP tagging of *AfuNce102* (pNCE-GFP).
of the gene was amplified using primers NCE_KO3 and NCE_KO4 containing EcoRI and SalI sites, respectively (Table S1, Supporting information). This fragment was subsequently cloned into EcoRI/SalI site of pGEM-Teasy vector, yielding pNCE_ko1 plasmid. Likewise, primers NCE_KO5 and NCE_KO6 containing NotI and EcoRI sites were used to generate an approximately 1.8-kb 5′ flanking region of the gene, which was then cloned into NotI/EcoRI site of pNCE-kol1. To prepare the final construct, pNCE_KO, the A. fumigatus pyrG gene with its own promoter and terminator was cut from a previously prepared pMOD-pyrG plasmid using EcoRI and cloned into EcoRI site of pNCE_ko1 (Fig. 1b).

To generate the NCE-EGFP fusion construct, the full-length AfuNce102 cDNA was prepared by RT-PCR using primers NCE_F1 and NCE_R1 containing BglII and HindIII restriction sites, respectively (Table S1). This fragment was subsequently cloned into a BglII/HindIII digest of pGEM-EGP plasmid resulting in the pNCE-EGFP plasmid (Fig. 1b).

For the complementation study, a 3.5-kb PCR product containing AfuNce102 and its 5' and 3' flanking regions was amplified using primers NCE-F2 and NCE_KO2 (Table S1). The resulting fragment along with plasmid pAN7.1 was used in a co-transformation reaction to transform the AfuNce102 deletion strain.

Fluorescence microscopy
Mycelia were visualized using a Jenus fluorescence microscope. Digital images were acquired by an INFINITY lite digital camera (Lumenera, Canada) and were prepared using Adobe Photoshop cs version 8.0.

Conidia of NCE-EGFP-expressing strain were inoculated in maltodextrin medium (1%) on coverslips and incubated at 37 °C for 16 h. The EGFP fluorescence was directly observed using a standard FITC filter. For ER staining, ER-TrackerTM Red dye (Invitrogen) was used at a final concentration of 1 μM in PBS. The strain was grown on a coverslip covered with dye solution for 30 min at 37 °C and washed briefly in PBS before being observed under the microscope equipped with a Rhodamine filter. To stain the nuclei, the mycelia were grown on coverslips as previously described and covered with a DAPI solution (Sigma) for 30 min at room temperature. After washing in PBS, the stained mycelia were visualized using a standard DAPI filter.

Susceptibility testing
The MICs for a range of anti-fungal and chemical agents were determined in 96-well microtiter plates containing RPMI 1640 medium enriched by 2% glucose. A total of 10^4 spores per well were inoculated, and twofold serial dilutions across the concentration range of each test compound (0–200 μg mL⁻¹) were prepared. MICs were measured after 24 h for AF293 and AfuNce102 KO mutant.

Pathogenicity in murine model
A murine model for systemic aspergillosis was used as described before (Romano et al., 2006). Briefly, female BALB/C mice were immunosuppressed by intraperitoneal injection of cyclophosphamide (200 mg kg⁻¹). Freshly harvested conidia from parental strain, AF293, and AfuNce102 deletion strain (2.5 × 10⁵) were intravenously injected, and survival was monitored daily for up to 4 weeks in each group (n = 10). Statistical analysis of data was carried out by SPSS software version 16 (SPSS Inc., Chicago). P value of < 0.05 was considered significant in this analysis.

Animal studies were performed according to the instructions published by the ethic committee of Pasteur Institute of Iran.

Results
Identification of A. fumigatus Nce102 homologue
The S. cerevisiae Nce102 sequence (GeneID: 856272) was used to identify homologues in the A. fumigatus genome using BlastP. The top-scoring match (Afu2g01590) was chosen for further analysis. This ORF has been annotated as NCE102 in Broad Institute database (http://www.broadinstitute.org/annotation/genome/aspergillus_group), which was named as AfuNce102. AfuNce102 contains 656 base pairs with two introns at positions 43–120 and 388–437. This gene encodes a 175 amino acid protein containing four transmembrane domains. The prediction of transmembrane regions was performed using TMpred tool. The four transmembrane regions were predicted to be located at amino acids 15–33, 44–64, 72–93, and 125–148. Signal peptide predication was performed using SignalP3.0 server and identified the first 34 amino acids as a putative signal peptide with a predicted cleavage site located between amino acid 34 and 35.

The AfuNce102 aligned with Nce102 homologues from other aspergilli including Aspergillus flavus, A. nidulans, A. niger, and Aspergillus clavatus with a high identity percentage ranging from 72% to 83%.

RT-PCR analysis using primers NCE_RT1 and NCE_RT2 showed that AfuNce102 was expressed during germination and throughout the hyphal growth.
Disruption of Nce102 in A. fumigatus

A deletion cassette containing 1.8-kb 5‘ and 3‘ flanking region of nce102 surrounding the pyrG marker was prepared (Fig. 1a and b). The cassette was digested by NotI/XbaI, and the deletion fragment was used for transformation of A. fumigatus AF293 pyrG− strain. Primary PCR screening of transformants demonstrated that in one transformant out of 32, the gene has been deleted. RT-PCR analysis confirmed that in this mutant, AfuNce102 has been deleted. This transformant showed a cotton-like colony appearance and a clear delay in conidiation at 37 °C (Fig. 2a). In the wild-type strain, normal conidiophores were observed within the center of colony after 16 h of inoculation in minimal medium agar, and after 48 h, conidiophores were observed throughout the colony. In contrast to the wild type, the AfuNce102 deletion mutant showed a low frequency of conidiophores after 16 h of incubation (Fig. 2d). The size of conidiophores and the number of spores per conidiophore were reduced markedly, and instead, a large number of undifferentiated aerial hyphae were produced. However, after 2 days, conidiophores were visible at the colony margin with a low density in the colony center. The mutant was also not able to produce any conidia at room temperature in minimal medium (Fig. 2b).

Despite the conidiation abnormalities, the growth of mutant under a range of conditions such as variable carbon and nitrogen sources and differing incubation temperatures (30, 37, and 42 °C) were examined. The results showed no significant difference in growth under these conditions when compared with the wild type, indicating that the AfuNce102 is not involved in the growth of A. fumigatus under tested conditions. Germination studies of wild-type and deletant spores in SAB or MM liquid medium confirmed a similar pattern of germination time and the frequency of germinated spores (data not shown).

Conidiophore development can be triggered by various environmental signals, and the brlA gene acts as a key regulator in this process (Adams et al., 1988). To check if the brlA expression has been affected by AfuNce102 deletion, the transcription level of brlA was measured after 16 and 24 h incubation of both mutant and parent strains in minimal medium using semi-quantitative RT-PCR. The results indicated that the lack of AfuNce102 function did not influence the transcriptional level of brlA (data not shown).

It has been proposed that fluG gene as the most upstream component of FluG pathway is responsible for the synthesis of a low molecular weight extracellular factor that can activate the fungal sporulation program (Lee & Adams, 1994; Wieser & Adams, 1995). As the contiguous cultivation of fluG deletant and the wild-type strain have resulted in complementation of the fluG defect in the mutant, we tested the possible suppression of conidiation defect in AfuNce102 deletion mutant by growing the strain next to the wild type on minimal medium agar. The results demonstrated that the conidiation abnormality in AfuNce102 deletion mutant was not suppressed when it was grown next to the wild-type strain (data not shown).

MIC levels against a range of known antifungal drugs or chemical compounds were determined to test their effect on the AfuNce102 mutant. No difference in MIC between the wild type and the mutant was observed for itracnazole, hygromycin B, nystatin, and calcofluor white; however, the mutant showed an eightfold increase in sensitivity to the sphingolipid synthesis blocker, Myriocin t, compared with the parental strain (MIC values: 25 μg mL−1 for mutant and 200 μg mL−1 for parent strain).

Complementation of the AfuNce102 deletion mutant

The AfuNce102 deletion mutant was transformed with a 3.5-kb PCR product containing AfuNce102 and 5‘ and 3‘
flanking regions. As this fragment did not contain any fungal selection marker, pAN7.1 plasmid containing hygromycin resistance gene was used as the second plasmid in co-transformation reaction. A positive transformant was selected and tested on minimal medium. The expression of AfuNce102 driven by its own promoter resulted in normal sporulation and growth phenotype (data not shown).

**Localization of AfuNce102-GFP fusion protein**

To investigate the intracellular localization of AfuNce102, a C-terminal fusion construct, driven by the glaA inducible promoter, was prepared and transformed into the *A. fumigatus* AF293 parent strain. A positive transformant was isolated and grown in inducing medium containing maltodextrin 1% as the sole carbon source. This transformant was directly analyzed by fluorescent microscopy. In young mycelia, Nce102 tagged with EGFP was primarily detected in ER with a tip-high gradient (Fig. 3d). The fluorescence was also detectable at the septum (Fig. 3a and e). In old hyphae, the ER localization of EGFP-tagged protein was more clear, and the EGFP fluorescence was frequently observed in ring-like structures (Figs 3e and 4b). DAPI staining of mycelia demonstrated that these ring structures are nuclei (Fig. 4b and c). During the conidiophore formation, a faint and diffused fluorescence was detected in the vesicle, and later, a strong signal was observed in phialides and mature conidia (Fig. 5). A variable intensity of EGFP fluorescence was observed among phialides.

As the expression of AfuNce102 under the control of glaA promoter may result in a nonphysiological level of the tagged protein, we tested the growth phenotype of AfuNce102-GFP transformant in the inducing medium. The results showed that overexpression of AfuNce102-GFP did not affect the growth phenotype of the *A. fumigatus*, including the radial growth rate or sporulation (data not shown).

![Fig. 3. Pattern of AfuNCE102:GFP fluorescence in mycelium of Aspergillus fumigatus grown in the presence of 1% maltodextrin for 16 h at 37 °C. (a), (b), and (c) represent phase-contrast image, fluorescent image and overlay image, respectively. Arrows indicate septum location. (d) represents the reticulotubular distribution of AfuNCE102-GFP throughout the germling with a clear localization at the tip. Arrow head in (e) shows the ring structures (nuclei) surrounded by reticulotubular network (ER). Bars: 5 μm.](image1)

![Fig. 4. (a) ER-Tracker-stained germling of NCE-GFP transformant grown in the presence of Maltodextrin 1%. The fluorescent pattern of ER-Tracker (red) is similar to AfuNce102-GFP distribution. (b) The reticulum network surrounds nuclei in DAPI-stained germlings (c). Arrowheads in (b) and (c) indicate the positions of the nuclei. Bars: 5 μm.](image2)
Virulence of AfuNce102 deletion mutant

To test whether the deletion of AfuNce102 can affect the virulence of A. fumigatus in an animal model, the survival of infected, temporarily immunocompromised mice was monitored for 4 weeks. Figure 6 illustrates the survival curves during the experiment. In statistical analysis of survival percentages using Mann–Whitney test, a significant survival difference was observed between the group infected with wild type spores and the control group, which only received cyclophosphamide ($P = 0.029$). The difference of survival between the group infected by AfuNce102 deletant spores and the control group was also significant ($P = 0.04$). However, the difference of survival between two infected groups was not statistically significant ($P = 0.34$). These comparisons support the conclusion that the virulence of fungus has not been affected by AfuNce102 gene deletion.

Discussion

So far, several studies have documented the role of Nce102 in membrane organization, eisosome assembly, and endocytosis in yeast (Grossmann et al., 2008; Frohlich et al., 2009). Despite the conservation of the main eisosomal proteins among the ascomycetes, the functional role of these proteins may diverge (Vangelatos et al., 2010). For instance, while the deletion of Pil1 leads to clustering of the remaining eisosome components, aberrant plasma membrane invaginations and the reduction of the endocytic rate in yeast (Walther et al., 2006), the deletion of Pil1 homologue in A. oryzae, and A. nidulans had no effect on endocytosis (Higuchi et al., 2009; Vangelatos et al., 2010).
In view of the important role of Nce102 in eisosome assembly in yeast and the possible involvement in non-classical export of some virulence factors to the cell surface (Nombela et al., 2006), we carried out a gene knock out study to understand the role of Nce102 homologue in the growth and pathogenesis of *A. fumigatus*. We first identified the gene in fungal genome data base, cloned it, and generated a deletion mutant. The intracellular localization of AfuNce102 was also examined using EGFP-tagged AfuNce102.

*AfuNce102* deletion mutant showed a clear delay in conidiophore formation at 37 °C and severely affected sporulation at 25 °C. Asexual sporulation is a complex process that requires highly coordinated activity of upstream and central developmental pathways. For instance, FluG pathway contains several upstream developmental activators that can activate an overlapping regulatory pathway containing key conidiation regulators like brlA and wetA (Extebeste et al., 2010). In examination of *brlA* expression levels as the central regulator of conidiation, we did not detect any difference between the parental strain and the *AfuNce102* deletion mutant indicating that *AfuNce102* may not influence *brlA* expression in *A. fumigatus*. AfuNce102 does not seem to be related to an extracellular sporulation activating factor (s), which is thought to be a product of *fluG* gene (D’Souza et al., 2001). This was concluded as the conidiation defect of *AfuNce102* deletant was not suppressed when the mutant was grown in the vicinity of the wild type.

In addition to the main regulatory pathways, several reports have introduced other key players in sporulation process. For example, Soid-Raggi et al. (2006) have identified a transmembrane flavoprotein, Tmpa, which is necessary for conidiophore formation in *A. nidulans*, and Li et al. (2007) demonstrated the role of normal sphingolipid metabolism in asexual sporulation. Although the deletion of eisosomal proteins, Pil A, PilB, or SurG, in *A. nidulans* has not changed the growth phenotype, sporulation, or spore survival (Vangelatos et al., 2010), the deletion of Nce102 homologue in *A. fumigatus* caused abnormal sporulation. The most severe defect in conidiation was observed at 25 °C. This may indicate an additional function for AfuNCE102 in fungal development. It has been proposed that Nce102 can modulate plasma membrane organization through sphingolipid signaling in yeast. The overexpression of Nce102 in yeast can block the inhibitory effect of a sphingolipid synthesis blocker, myriocin, on eisosomes (Frohlich et al., 2009). Although the role of sphingolipid signaling in eisosome assembly was not investigated in the current study, the increased sensitivity of AfuNce102 deletion mutant to myriocin may point to the role of the protein in sphingolipid sensing as proposed before (Frohlich et al., 2009). The presence of sphingolipid based signal transduction pathway in *A. nidulans*, and its role in fungal development has previously been observed (Li et al., 2007).

In a localization study, AfuNCE102-EGFP fusion protein showed a reticulotubular distribution representing ER localization. This is similar to the cellular localization of NCE102 in yeast reported by Kumar et al. (2002) and the cytoplasmic distribution of another eisosomal transmembrane protein, SurG, in *A. nidulans* (Vangelatos et al., 2010). The localization of AfuNce102 to ER was more prominent in the basal region of elongated hyphae with frequent ring-like structures that represent the ER envelope around the nuclei. This may indicate the accumulation of AfuNce102 protein in older regions of hyphae over time. EGFP fluorescent was also observed along the septa. This could be due to the strategic positioning of ER as a supplying center of material for septum formation as suggested by Maruyama et al. (2006). Alternatively, AfuNce102-EGFP may be directly targeted to the septum or trapped in the septum during septum formation.

During conidiogenesis, AfuNce102 localized to conidiophores and mature conidia. This is consistent with the results presented by Vangelatos et al. (2010), which demonstrate the co-localization of eisosomal proteins during conidiogenesis. In *A. nidulans*, the eisosomal proteins, PilA, PilB, and SurG, are localized at the periphery of resting conidia, and it is expected that the transmembrane protein, AfuNce102, co-localizes with eisosomes as reported previously.

The virulence of AfuNce102 deletion mutant was comparable to that of the wild type. This suggests that AfuNce102 is not required for pathogenesis in the systemic infection model used in the present study.

In conclusion, we have shown that AfuNce102 is involved in sporulation process in *A. fumigatus*. Although the localization data presented in this study were derived from the expression of AfuNce102-EGFP under the control of a strong and nonphysiological promoter, the targeting of GFP fusion protein to the conidiophores and mature conidia along with an abnormal sporulation in deletion mutant may be relevant to the potential role in sporulation.

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**References**


Supporting Information

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

Table S1. Primers used in this study.

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