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Study of the essentiality of the *Aspergillus fumigatus triA* gene, encoding RNA triphosphatase, using the heterokaryon rescue technique and the conditional gene expression driven by the *alcA* and *niiA* promoters

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ABSTRACT

The identification of essential genes represents a critical step in the discovery of novel therapeutic targets in *Aspergillus fumigatus*. Structural analyses of the *Saccharomyces cerevisiae* RNA triphosphatase pointed out this enzyme as an attractive therapeutic target for fungal infections. In addition, demonstration of the essentiality of the *S. cerevisiae* RNA triphosphatase encoding gene enhanced the value of this potential therapeutic target. Nevertheless, consideration of a fungal RNA triphosphatase as an ideal therapeutic target needs confirmation of the essentiality of the respective gene in a fungal pathogen. In this work, we analyzed the essentiality of the *A. fumigatus triA* gene, encoding RNA triphosphatase, by conditional gene expression and heterokaryon deletion. Using the conditional gene expression driven by the *alcA* promoter (*alcA*^{P}), we found that TriA depletion causes morphological abnormalities that result in a very strong growth inhibition. Nevertheless, since a strict terminal phenotype was not observed, the essentiality of the *triA* gene could not be ensured. Accordingly, the essentiality of this gene was analyzed by the heterokaryon rescue technique. Results obtained unequivocally demonstrated the essentiality of the *A. fumigatus triA* gene, indicating the suitability of the RNA triphosphatase as an ideal therapeutic target to treat *A. fumigatus* infections. Besides, a second conditional gene expression system, based on the *niiA* promoter (*niiA*^{P}), was utilized in this work. Although the *niiA*^{P}-mediated repression of *triA* was less severe than that driven by the *alcA*^{P}, a strong growth inhibition was also found in *niiA*^{P}-*triA* strains. Finally, *E*-tests performed to determine whether *triA* down-regulated cells became more sensitive to antifungals suggest a synergic effect between amphotericin B and another antifungal inhibiting the *A. fumigatus* RNA triphosphatase activity.

1. Introduction

Aspergillus fumigatus is the most important pathogen of the genus *Aspergillus*. Among other diseases, this species causes invasive aspergillosis, a common life-threatening infection in immunocompromised patients (for reviews see Brakhage et al., 1999; Denning, 1998; Latgé, 1999, 2001; Pfaller and Diekema, 2004). The estimated annual incidence of invasive aspergillosis is 12–34 per million population (Wilson et al., 2002; Pfaller et al., 2006).

Despite the progress made in the diagnosis of aspergillosis and the recent introduction in therapy of a novel family of antifungals, the echinocandins, the prognosis of patients suffering invasive aspergillosis has not improved. Accordingly, when the most effective treatment is applied the mortality rate still exceeds 60–65% (Denning, 1996, 2000; Perea and Patterson, 2002; Marr et al., 2002, 2004; Steinbach et al., 2003; Pfaller et al., 2006). The main reason for these alarming figures is the low efficiency of the antifungal therapies available to treat invasive aspergillosis. Consequently, there is a real necessity of novel and effective antifungal agents to be added to the few antimycotics clinically used (i.e.: amphotericin B, voriconazole, caspofungin) to treat this disease.

The identification of essential genes in a microbial pathogen, such as *A. fumigatus*, represents a crucial step in the discovery of new therapeutic targets that will eventually lead to the development of novel antifungal drugs. Previous studies in model yeast *Saccharomyces cerevisiae* showing, among others, the essentiality of the *cet1* gene, encoding RNA triphosphatase, pointed out the suitability of this enzyme as a potential therapeutic target in pathogenic fungi (Tsukamoto et al., 1997; Ho et al., 1998; Lima et al., 1999).

RNA triphosphatase catalyses the first step of cap formation, a structure present in the 5′ terminus end of mRNA of eukaryotic

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cells and their viruses. The 5′ cap structure is essential and is required for efficient pre-mRNA splicing, export, stability and translation initiation (Shuman, 2000; Gu and Lima, 2005). RNA triphosphatase hydrolyses the 5′ triphosphate end of the nascent transcript to a diphosphate end. Then, the diphosphate end is capped with GMP by RNA guanylyltransferase. Finally, the 5′ guanine base is methylated by RNA methyltransferase at the guanine N7 position (Lima et al., 1999; Shuman, 2000).

Although the three capping reactions are universal in eukaryotes, there is a surprising difference in the structure and catalytic mechanism of the RNA triphosphatase component between lower and higher eukaryotes. Metazoans and plants have a two-component capping system consisting of a bifunctional triphosphatase–guanylyltransferase polypeptide and a separate methyltransferase enzyme. In contrast, fungi contain a three component system consisting of separate triphosphatase, guanylyltransferase and methyltransferase enzymes (Shuman, 2000).

Primary structures of mammalian RNA triphosphatases considerably differ from their fungal counterparts. In addition, the catalytic mechanism of RNA triphosphatases in fungi and mammals are completely different (Ho et al., 1998; Lima et al., 1999; Pei et al., 1999; Changela et al., 2001). In fact, while mammalian RNA triphosphatases belong to the metal-independent family, fungi contain RNA triphosphatases that belong to the divalent-cation-dependent group. Taking into account such biological differences, fungal triphosphatases have been considered as promising targets for antifungal drug discovery (Lima et al., 1999; Pei et al., 2001). Nevertheless, all these premises, especially the essentiality of the gene encoding RNA triphosphatase, have to be proven for a specific fungal pathogen, such as *A. fumigatus*. In this way, it is important to quote a report indicating the dispensability for growth of the RNA triphosphatase encoding gene from the human pathogenic yeast *Candida albicans* (Dunyak et al., 2002), which ruled out the appropriateness of this theoretically ideal molecular target in this species.

In this work, we firstly confirmed that the *A. fumigatus triA* gene, the ortholog of the *S. cerevisiae cet1* gene, encodes a typical fungal RNA triphosphatase. Then, we used complementary genetic approaches to ascertain the essentiality of the *A. fumigatus triA* gene. Using the heterokaryon rescue technique (Osmani et al., 2006) we demonstrated the essentiality of the *A. fumigatus triA* gene and thus, the usefulness of the RNA triphosphatase as a valuable therapeutic target to treat invasive aspergillosis. Besides, we analyzed the phenotype of *A. fumigatus* strains in which TriA depletion was driven by two different regulatable promoters, the *A. nidulans alcA*^{P} and the *A. fumigatus niiA*^{P}. Comparison of the phenotype of the Δ*triA* spores with that of strains in which *triA* down-regulation was controlled by the *alcA*^{P} or the *niiA*^{P}, showed valuable information on the feasibility of both conditional expression systems for essential gene identification and future applications of gene down-regulation in *A. fumigatus*.

2. Materials and methods

2.1. Strains and plasmids

Aspergillus fumigatus 237 (Smith et al., 1994; Olivas et al., 2008) is the wild-type isolate used in this work. The *A. fumigatus sC29* mutant (De Lucas et al., 2001), unable to use sulfate as the sole sulfur source, was utilized in transformation experiments using the *A. nidulans sC* gene as the fungal selective marker. The *A. fumigatus* KU80ΔpyrG (Δ*akuB*^{KU80}; *pyrG*$^{-}$) mutant strain (da Silva Ferreira et al., 2006), was used in transformations that employed the *Neurospora crassa pyr-4* gene as the fungal selective marker.

Plasmid pBluescriptSK^{+} (Sambrook et al., 1989) was utilized for cloning the *A. fumigatus triA* gene that encodes the mRNA capping nucleoside-triphosphatase. Vector pGEMTeasy (Promega) was employed to subclone PCR-amplified fragments that were subsequently cloned into appropriate vectors (see below). Plasmid pAL3 (Waring et al., 1989), that contains the *Neurospora crassa pyr-4* gene and the promoter of the *A. nidulans alcA* gene (*alcA*^{P}), was used in this study to construct the promoter replacement vectors pALSCRTF and pNIIASCRTF. Plasmid pKTB1 which contains the *Neurospora crassa pyr-4* marker (Van den Brulle et al., 1999) and plasmid pSCDNUDC (Romero et al., 2003), containing the *A. nidulans sC* gene, were also used in this study. All plasmid were propagated in *Escherichia coli* DH5α (BRL, USA). Standard molecular biology techniques were performed according to methods described by Sambrook et al. (1989).

2.2. Aspergillus fumigatus media

A. fumigatus was generally grown in *Aspergillus* complete medium (CM) (Armitt et al., 1976) and in *Aspergillus* minimal medium (MM) (Armitt et al., 1976; De Lucas et al., 1997) with 1% (w/v) glucose as sole carbon source. This MM contains 70 mM $NaNO_3$ and 0.05% (w/v) $MgSO_4$ as the sole nitrogen and sulfur source, respectively. Another minimal medium, named Ammonium Minimal Medium (AmMM), similar to MM but containing 10 mM ammonium tartrate as sole nitrogen source was used to repress the *A. fumigatus niiA* promoter (*niiA*^{P}). In addition, 3% glucose MM and YEPD medium, also containing 3% glucose (Romero et al., 2003), were utilized to repress the *A. nidulans alcA* promoter (*alcA*^{P}). Finally, 0.1 M glycerol MM was employed to select *sC*$^{+}$ transformants containing the *alcA*^{P}-*triA* fusion gene, while 1% glucose MM was used for selection of *sC*$^{+}$ strains with the *niiA*^{P}-*triA* fusion gene. Uridine (2.44 mg/ml) and 20 mM uracil were used as nutritional requirements for *A. fumigatus* KU80ΔpyrG mutant strain. L-methionine (10 mM) was added to the medium when the *A. fumigatus sC29* strain was cultivated. Transformation of *A. fumigatus* was done according to established procedures (De Lucas et al., 2001).

2.3. Construction of the triA gene replacement vector

The nucleotide sequence of the *triA* gene, encoding RNA triphosphatase, plus 2 kb from its upstream and downstream regions was obtained by a TBLASTN search at the *A. fumigatus* genome project database (http://www.sanger.ac.uk/Projects/A_fumigatus/) using the *A. fumigatus* mRNA capping nucleoside-triphosphatase protein sequence (XP_751975) (Nierman et al., 2005) as a query. This genomic sequence was utilized to design primers LSSTRI and RTRIKpn (containing a *Kpn*I site) (Table 1). Both primers were employed for PCR amplification of a 3.9-kb genomic region containing the *A. fumigatus triA* coding region (2522-bp) plus around 700-bp of its upstream and downstream sequences. The PCR-amplified product was cloned into pGEMTeasy to give pGEMRTF. A 3.7-kb *Kpn*I–*Sac*I genomic fragment was excised from this vector and cloned into pBluescript SK^{+} previously digested with the same enzymes to give plasmid pBlueRTF.

Deletion of the *A. fumigatus triA* gene was carried out as follows. An inverse PCR reaction was performed using the vector pBlueRTF and primers INVTRISS and INVTRICla (containing a *Cla*I site) (Table 1). The 5.1-kb resultant fragment, lacking most of the *triA* ORF (from nucleotide +539 to +2279 from the ATG start codon), was phosphorylated and self-ligated to give pDRTF. Digestion of this plasmid with *Not*I and *Cla*I permitted to clone a fungal selection marker between the *triA* promoter and terminator regions. Two different markers were used to construct the *triA* gene deletion cassettes used in this work: the *Aspergillus nidulans sC* marker and the *Neurospora crassa pyr-4* gene. The *A. nidulans sC* marker