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Modulation of fungal sensitivity to staurosporine by targeting proteins identified by transcriptional profiling

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ABSTRACT

An analysis of the time-dependent genetic response to the death-inducer staurosporine was performed in *Neurospora crassa* by transcriptional profiling. Staurosporine induced two major genes encoding an ABC transporter and a protein with similarity to regulatory subunits of potassium channels. The transcriptional response is dependent on the activity of a novel transcription factor. Deletion mutants in differentially expressed genes displayed altered sensitivity to staurosporine, underscoring significant proteins involved in the response to the drug. A null-mutant of the ABC transporter (abc3) is extremely sensitive to staurosporine, accumulates more staurosporine than the wild type strain and is defective in energy-dependent export of the drug, indicating that the ABC3 protein is the first described staurosporine transporter. It was located in the plasma membrane by immunofluorescence microscopy. The combination of inhibitors of ABC transporters or of potassium channels with staurosporine leads to an enhanced activity against *N. crassa* and pathogenic fungi paving the way to the development of more potent and specific antifungals. Our results highlight the general use of transcriptional profiling for the identification of novel proteins involved in cell death and their potential use as drug targets.

1. Introduction

Programmed cell death (PCD) is a process of cell suicide essential for the development and survival of the organism. The process was firstly described in higher eukaryotes, but more recently found to occur in fungi as well (Madeo et al., 1997). Interference with PCD has had increasing relevance in public health issues due to the fact that its stimulation may prove to be fundamental in cancer treatments and in the control of infectious diseases (Ramsdale, 2008; Reed, 2006; Sharon et al., 2009). Thus, an understanding of the complex networks that ultimately lead to cell death is necessary. It is anticipated that modulation of PCD can be achieved by the identification and targeting of key components regulating the process.

PCD can be induced with drugs like staurosporine (STS), an alkaloid from microbial origin firstly described as antibacterial and antifungal (Omura et al., 1977). STS has been widely used as a protein kinase inhibitor or as a PCD-inducing agent (Gescher, 2000). The STS concentration needed to induce PCD (100 nM–10 μM) is much higher than the k_I for inhibition of protein kinases (~5 nM), indicating that STS interferes with other cellular functions (Jarvis et al., 1994). Although STS effects on apoptosis have been largely studied, nothing is known so far about its trafficking and transport. Several STS analogs are in advanced clinical trials as anticancer agents (Osman et al., 2010).

The filamentous fungus *Neurospora crassa* has been a valuable research model organism (Davis and Perkins, 2002), particularly following its genome sequencing (Galagan et al., 2003) and targeted disruption (Colot et al., 2006). It undergoes PCD following the addition of external pro-apoptotic drugs (Castro et al., 2008, 2010; Videira et al., 2009) or as a consequence of heterokaryon incompatibility (Glass and Dementhon, 2006). Specific mutants of respiratory chain complex I are particularly sensitive to STS (Castro et al., 2010). In this work, we performed a transcriptomic analysis of *N. crassa* exposed to STS in order to identify novel mechanisms associated with PCD. We further showed that targeting specifically identified proteins by chemical means allows modulation of *N. crassa* sensitivity to STS and this effect could be extended to the pathogenic fungi *Aspergillus fumigatus* and *Candida albicans*. The

Abbreviations: PCD, programmed cell death; STS, staurosporine.

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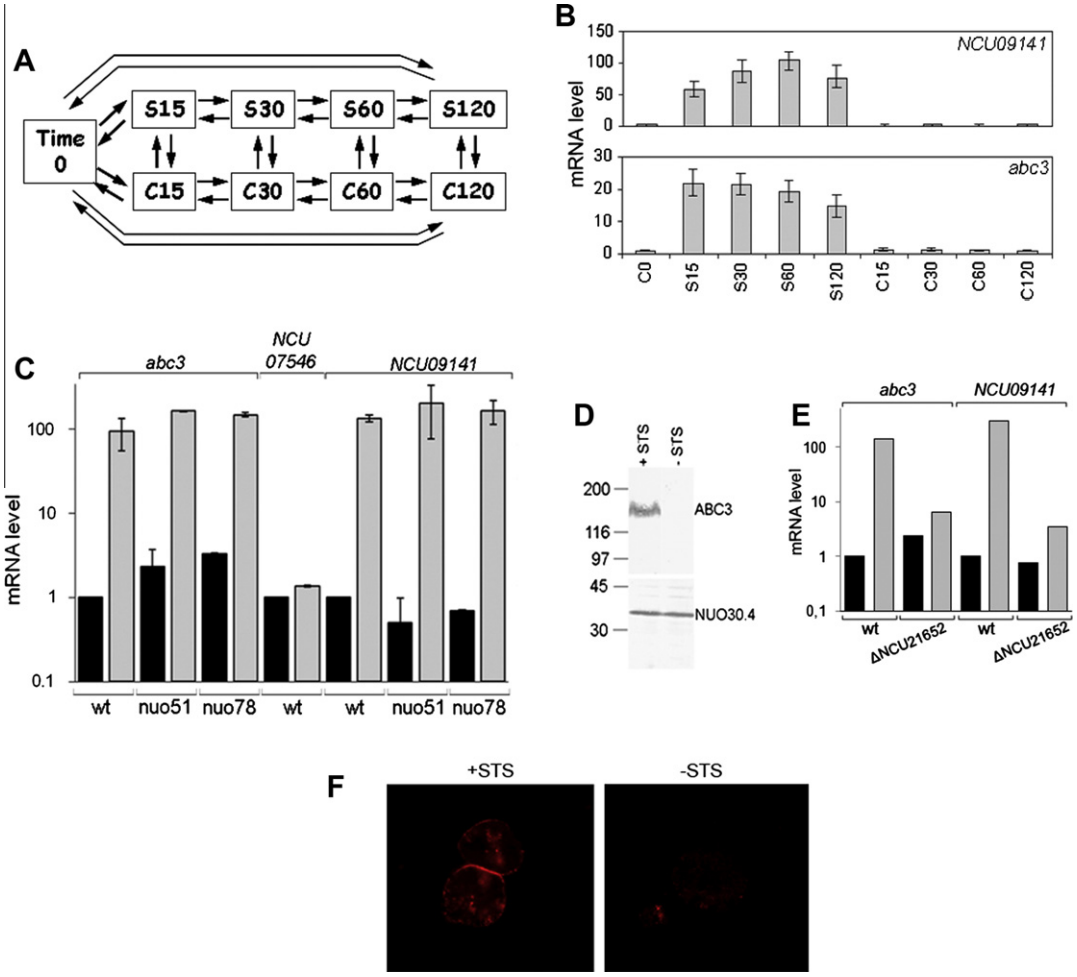

Fig. 1. Expression levels and protein localization after STS treatment. (A) Scheme of the microarray experiment. *N. crassa* conidia were germinated in minimal medium for 5 h (time 0) and then incubated in the absence (C) or in the presence of 12.5 μM STS (S). Samples were withdrawn at the indicated time points (min) and used for di-swap microarray hybridizations (arrows, with the arrowhead pointing the sample labeled with Cy5) and quantification of gene expression levels. (B) Time-course relative expression levels of the genes encoding NCU09141 (upper panel) and ABC3 (lower panel). (C) Quantification by RT-PCR of relative expression levels of the genes encoding ABC3, NCU07546 and NCU09141 performed in the wild type strain (wt) and the complex I mutants (nuo51 and nuo78) following 5-h germination and 30 min incubation in the absence (black bars) or presence of STS (gray bars). (D) Western blots of total protein extracts from 16 h-grown fungal cells exposed to 12.5 μM STS for 3 h or untreated-cells using antiserum against ABC3 and antiserum against the constitutive 30.4 kDa subunit of complex I, as control. Standard molecular weights (kDa) are indicated on the left. (E) Quantification by RT-PCR of relative expression levels of the genes encoding NCU09975 (*abc3*) and NCU09141 (shown above) performed in the wild type strain and the ΔNCU21652 mutant (shown below) following 5-h germination and 30 minutes incubation in the absence (black bars) or presence of STS (gray bars). (F) Localization of the ABC3 protein by immunofluorescence using confocal microscopy in the *N. crassa* slime mutant after 16 h of growth followed by 3 h in the presence (left) or absence (right) of 12.5 μM STS.

higher activity of STS when combined with specific protein inhibitors against pathogenic fungi has potential medical implications.

2. Material and methods

2.1. Strains, growth techniques and chemicals

Wild type *N. crassa* (FGSC 2489), the cell wall-less slime mutant (FGSC 4761), and several deletion strains generated by the Neurospora Genome Project (Dunlap et al., 2007), were obtained from the Fungal Genetics Stock Center (McCluskey, 2003). The mitochondrial complex I nuo mutants have been reviewed (Marques et al., 2005). Standard procedures were employed for growth and handling of *N. crassa*, *A. fumigatus* ATCC 46645 and *C. albicans* SC5314 strains (Castro et al., 2010; Davis and de Serres, 1970). *N. crassa* conidial germination was evaluated by optical microscopy. For spot assays, serial 3-fold dilutions of cellular suspensions from all fungi were spotted on agar plates (GFS for *N. crassa* (Davis and de Serres, 1970), Sabouraud for *A. fumigatus* and *C. albicans*) containing drugs, so that the last spot contained ~50 cells, and incubated at 26 °C (37 °C for *A. fumigatus*). We prepared stock solutions of 10.7 mM STS/DMSO (LC-Laboratories), 50 mM Verapamil/ethanol (Sigma–Aldrich), 1 M sodium orthovanadate (Sigma–Aldrich) and 100 mM 4-aminopyridine (Sigma–Aldrich).

2.2. Microarray experiments and data analysis

Closed-circuit designs were employed for microarray comparisons (Fig. 1A), because they are statistically robust and provide a higher resolution in identifying differentially expressed genes than designs that use a universal reference (Townsend, 2003). *N. crassa* conidia obtained from cultures grown for 7 days at 25 °C under constant light (Kasuga et al., 2005) were germinated in Vogel's minimal medium (10^7 cells/ml) at 30 °C with strong agitation. STS (12.5 μM) was added after 5 h. At different times, mycelium samples were collected by quick filtration, frozen in liquid nitrogen and kept at −70 °C.