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CreA-mediated repression in *Aspergillus nidulans* does not require transcriptional auto-regulation, regulated intracellular localisation or degradation of CreA

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Abstract

The major regulatory protein in carbon repression in *Aspergillus nidulans* is CreA. Strains constitutively over-expressing *creA* show normal responses to carbon repression, indicating that auto-regulation of *creA* is not essential for CreA-mediated regulation. In these strains, high levels of CreA are present whether cells are grown in repressing or derepressing conditions, indicating large-scale degradation of CreA does not play a key role. CreA is located in the nucleus and cytoplasm in cells when grown in either repressing or derepressing conditions, and absence of CreB, CreD or AcrB does not affect either the localisation or amount of CreA. Therefore, CreA must require some modification or interaction to act as a repressor. Deletion analysis indicates that a region of CreA thought to be important for repression in *Trichoderma reesei* and *Sclerotina sclerotiorum* CreA homologues is not critical for function in *Aspergillus nidulans*.

Keywords: *Aspergillus nidulans*; Filamentous fungi; Gene regulation in filamentous fungi; Transcriptional repressor; CreB deubiquitinating enzyme; CreC; CreD; AcrB

1. Introduction

The major repressor protein regulating the carbon catabolite repression of genes involved in carbon metabolism in *Aspergillus nidulans* is CreA. The gene encoding CreA was identified by mutations that relieve repression of genes encoding enzymes for the use of alternative carbon sources when glucose is present (Arst and Cove, 1973; Bailey and Arst, 1975; Hynes and Kelly, 1977). CreA is a Cys_2His_2 zinc finger DNA binding protein (Dowzer and Kelly, 1989, 1991), and the consensus binding motif was shown to be SYGGRG (Cubero and Scazzocchio, 1994; Cubero et al., 2000; Kulmberg et al., 1993). Both the zinc finger region of the protein and the DNA binding consensus sequence are very similar to those of Mig1p, which is involved in carbon catabolite repression in *Saccharomyces cerevisiae* (Nehlin and Ronne, 1990; Nehlin et al., 1991). It was thought that Mig1p acts by recruiting the co-repressor complex, Ssn6p/Tup1p, to promoters that are under carbon catabolite repression control (Nehlin et al., 1991; Treitel and Carlson, 1995; Tzamarias and Struhl, 1995) and that cytoplasmic translocation of Mig1p, triggered by its phosphorylation by Snf1 kinase in the nucleus, was a key regulatory step for releasing glucose repression (De Vit and Johnston, 1999; De Vit et al., 1997). However, studies on the nuclear export of Mig1p showed that glucose still regulates Mig1p-dependent repression in a mutant that no longer has export activity, indicating that Mig1p is regulated both by nuclear localisation, and by modification of its ability to repress transcription (De Vit and Johnston, 1999). It has subsequently been shown that the Ssn6p–Tup1p complex is tethered to the *GAL1* promoter DNA in both repressing and activating conditions (Papamichos-Chronakis et al., 2002), and that Mig1p is not required for the Ssn6p–Tup1p complex to be tethered to

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the promoter (Papamichos-Chronakis et al., 2002). It has also been shown that Mig1p is tethered to the *GAL1* promoter under both repressing and derepressing conditions, and although Mig1p is not essential for the binding of the Ssn6p–Tup1p complex to the promoter, it is important for Ssn6p–Tup1p mediated glucose repression (Papamichos-Chronakis et al., 2002). The Hxk2p-dependent signalling pathway is also important in glucose sensing (Moreno et al., 2005), and Hxk2p is nuclear localised in response to glucose, and Mig1p is required to keep Hxk2p in the nucleus (Ahuatzi et al., 2004), and the two proteins directly interact (Ahuatzi et al., 2007). In glucose grown cells, Hxk2p interacts with both Mig1p and Snf1p to inhibit the phosphorylation of Mig1p (Ahuatzi et al., 2007). In this system, Snf1p-dependent phosphorylation of Mig1p abolishes the interaction with Ssn6p–Tup1p, and controls transcriptional repression/derepression, and it is the regulation of this interaction, not the Mig1p cytoplasmic localisation, that is the molecular switch that controls transcriptional repression/derepression of *GAL1* (Papamichos-Chronakis et al., 2002). Hexose phosphorylating enzymes have also been shown to be important in signalling in *A. nidulans*, with strains lacking both HxkA and GlkA showing impairment of carbon catabolite repression (Flipphi et al., 2003).

In *A. nidulans,* mutations in *creB* and *creC* were also identified that shared some of the mutant phenotypes of *creA* (Hynes and Kelly, 1977) The CreB protein is a deubiquitinating enzyme, and CreC is a WD40 protein present in a complex with the CreB deubiquitinating enzyme (Kelly and Hynes, 1977; Lockington and Kelly, 2001, 2002; Todd et al., 2000). Thus it is likely that deubiquitination is involved in the carbon catabolite repression mechanism. There are no protein sequences in the *S. cerevisiae* genome that are apparent homologues of CreB and CreC, and this aspect of the regulation differs between *A. nidulans* and *S. cerevisiae*. Mutations in *creD* suppress some of the phenotypes of *creB* and *creC* mutations (Boase and Kelly, 2004; Kelly and Hynes, 1977) and CreD contains arrestin domains and PY motifs, and has been shown to interact with the HECT ubiquitin ligase, HulA in bacterial two hybrid experiments (Boase and Kelly, 2004). Like mutations in *creD*, mutations in *acrB* also suppress some of the phenotypes due to mutations in *creB* and *creC* (Boase et al., 2003), and thus AcrB may also be involved in this ubiquitination pathway. Although the components of both the deubiquitination and ubiquitination network have been identified, the direct targets of these have not been identified. It is probable that either the amount or the activity of CreA is regulated by ubiquitination, but whether this control is direct or indirect has not been demonstrated.

There has been some evidence that ubiquitination is a component of the *S. cerevisiae* carbon catabolite repression mechanism. In yeast, glucose activates the genes important for its uptake and metabolism, and this is regulated via Rgt1p (Ozcan et al., 1996). In the absence of glucose, transcription of the yeast glucose transporters, *HXT2* and *HXT4*, is prevented by Rgt1p, and at high levels of glucose, Mig1p represses the expression of *HXT2* and *HXT4*. Only at low glucose concentrations are both repressors inactive, leading to a 10- to 20-fold derepression of gene expression. The repressor function of Rgt1p is inhibited by Grr1p, a subunit of ubiquitin ligase complex (Barral et al., 1995). The SCF^{Grr} complex targets Gis4p, a factor involved in carbon signalling, and over-expression of *GIS4* suppresses the *snf1Δ,* at least on raffinose (de Rue et al., 2005).

To date, little is known about the molecular events that allow CreA function to result in carbon catabolite repression. Key unresolved questions are investigated here. Firstly, we have investigated whether the auto-regulation of *creA* transcription (Arst et al., 1990; Shroff et al., 1996) is essential for repression and derepression of genes subject to CreA-mediated repression. The second question is whether differential stability of CreA plays a crucial role in carbon catabolite repression, and thus we investigated whether there is a reduction in the overall amount of CreA in the cell in conditions that are carbon catabolite derepressing compared to conditions that are repressing when *creA* expression is deregulated and over-expressed. The third question is whether altered intracellular localisation of CreA plays a key role in carbon catabolite repression, and to address this we determined the localisation of CreA in cells grown in repressing and in derepressing conditions, and undertook preliminary experiments to locate the sequences required for nuclear localisation. We have also investigated whether the amount or intracellular localisation of CreA was affected by the absence of CreB, CreD, or AcrB. We were also able to investigate, in cells where CreA is over-expressed and present in the nucleus, whether genes subject to carbon catabolite repression can be activated appropriately when repression is removed.

2. Materials and methods

2.1. Strains

The full genotypes of strains used in this research are outlined in Table 1.

2.2. Plasmids constructed for the production of tagged versions of CreA

Various plasmids were constructed to express tagged versions of full length CreA (Fig. 1). In order to detect the CreA protein in *A. nidulans,* a PCR product containing the coding region of the *creA* gene was inserted into an expression plasmid, such that it was driven by the *gpdA* promotor sequence and contained sequences to produce a HIS tag at the 3′ end, to produce pGPD::CreA:HIS. A Myc-epitope tag was then inserted in frame at the amino-terminal end of the *creA* coding sequence to form pGPD::Myc:CreA:HIS. In order to visualise the intracellular location of CreA, a GFP tag was added to the carboxyl-terminal end of CreA to create the pGPD::Myc:CreA:GFP plasmid. The GFP fused protein was readily visualised in