RS4-2

Negative Control of the GATA-Type Transcription Factor NsdD Requiring for Sexual Development by Protein Interactions with IndB and IndD in *Aspergillus nidulans*

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The filamentous ascomycete *Aspergillus nidulans* is a homothallic organism that contains two reproductive cycles: asexual and sexual reproductions. During differentiation several distinct morphological changes occur. The asexual reproductive structure is the conidiophore, which produces conidiospores. Many genes including *fadA, flbA, brlA, abaA,* and *wetA* were involved in this process and sophisticated regulation also was required to achieve proper conidiation (Adams *et al.*, 1998). The reproductive unit of the sexual cycle is the cleistothecium that contains red-pigmented ascospores. During the sexual development, small hyphal nests and Hulle cells were developed. Later, the meiotically derived ascospores were produced within a rigid, round, and black cleistothecium.

A minimum time of 16-20 vegetative hyphal growth is required to achieve developmental competence after germination of conidia. The competent mycelia become susceptible to environmental signals that control induction and balancing of the asexual and sexual reproduction processes (Axelrod *et al.*, 1973). However, the regulatory systems and genes that drive the initial developmental decisions in *A. nidulans* are hardly known. External conditions which disturb the programmed internal reproduction cycles of *A. nidulans* has been studied (Axelrod *et al.*, 1973, Champe *et al.*, 1994, Han *et al.*, 1990). Preference of sexual differentiation was seen in hypoxic condition and in a medium containing lactose as a sole carbon source. On the other hand, in high osmotic environment, asexual reproduction preferentially occurred. Light direct the differentiation towards the asexual cycle, whereas absence of light favours sexual propagation (Mooney and Yager, 1990).

The *veA* gene product is involved in light regulation and acts as a regulator that activates sexual development and represses asexual development (Kim *et al.*, 2002). The *veA1* mutation has been traditionally included in most of *A. nidulans* strains, because *veA1* mutants produced more conidiospores than with wild type. Strains harboring the *veA1* mutation caused much reduction of cleistothecia (Champe *et al.*, 1981; Yager, 1992). The *veA gene* was cloned and characterized with proposed transcription factor function affecting sexual differentiation positively and asexual processes negatively (Kim *et al.*, 2002). However, molecular mechanism of *veA* in reproductive differentiation processes is totally unknown.

A. nidulans mutants defective in distinct stages of sexual development have been identified (Han *et al.*,

1998). In addition, several mutations required for asexual conidial differentiation also affect sexual reproduction processes. No sexual structures were produced in *ΔnsdD* and *ΔstuA* (Wu and Miller, 1997; Han *et al.*, 2001) mutants, while *ΔsteA, ΔcsnD,* or *ΔmedA* propagate Hulle cells but no cleistothecium (Clutterbuck, 1969). Others exhibited only microcleistothecia without mature ascospores.

The *nsdD* gene encodes a GATA-type transcription factor with the type IVb zinc finger DNA-binding domain found only among lower eukaryotes and plants but not in animal kingdom (Fig. 1). Null mutation of *nsdD* resulted in no sexual structure formation, even under the conditions that preferentially promoted sexual development. In contrast, when the *nsdD* gene was over-expressed, sexual-specific organ of Hulle cell was appeared even in submerged culture, which normally completely blocked sexual as well as asexual development, and the number of cleistothecia was also dramatically increased on solid medium. However, the knowledge about the molecular mechanism and target genes of NsdD was scare.

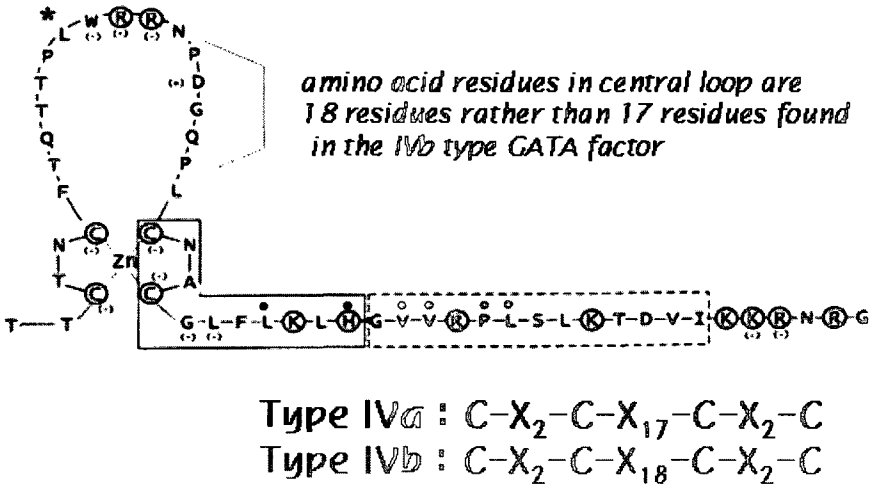

Fig. 1. Structure of C2-C2 zinc-finger motif of the type IVb GATA factor.

To clarify the NsdD function, proteins interacting with NsdD have been screened using the yeast two-hybrid system. Two Ind (interactor of NsdD) proteins, IndB and IndD were isolated and characterized further. Determination of *indB and indD* cDNA sequences revealed open reading frames of 804 bp and 846 bp, interrupted by a 50 bp and a 257 bp intron, encoding polypeptides of 268 and 282 amino acids, respectively. IndB and IndD showed 42% amino acid sequence identity and also shared similarities to an ORF in *Neurospora crassa*.

NsdD-IndB and NsdD-IndD interactions were confirmed *in vitro* using the GST-pull down assay (Fig. 2). *In vivo* interactions were also confirmed by immunoprecipitation. Various truncated NsdD proteins were tested for the abilities of interactions with IndB and IndD using the yeast two-hybrid assay. The C-terminus carrying the zinc-finger region of NsdD was sufficient for the interactions. Mutant NsdD proteins carrying change(s) either at the conserved first cysteine residue or at the two residues located between the first two cysteines in the zinc-finger motif failed to interact with IndB and IndD, indicating the requirement of the intact zinc-finger domain for the interactions. The homology regions between IndB and IndD were

responsible for interaction with NsdD. IndB and IndD interacted to make hetromultimer as well as homomultimer.

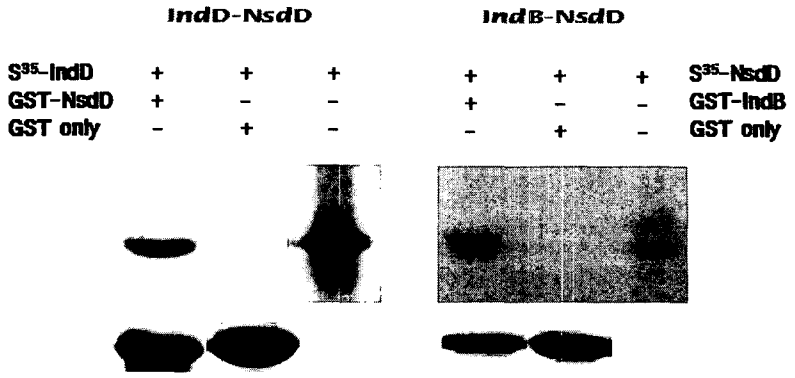

Fig. 2. *In vito* bindings of NsdD with IndB and IndD by the GST-pull down assay.

In the case of *Neurospora crassa*, a heterothallic Ascomycete, a *N. crassa* homolog of Ind proteins, IND-1 interacted with the Zn-finger region of a *N. crassa* NsdD homolog, NSD-1. Moreover, protein-interactions of IND-1 with NsdD, IndB, and IndD were also observed. However, NSD-1 failed to interact with IndD, while making heteromer with IndB.

Two other putative proteins having the same type IVb of zinc-finger domains as NsdD were also investigated for interactions with IndB and IndD. The Zinc-finger regions of two putative *Aspergillus* homologs of *Neurospora* WC-1 and WC-2 failed to interact with IndB and IndD. In addition, the type IVa zinc-finger domain of AreA of *Aspergillus* also failed to interact with IndB and IndD. These results indicate that NsdD-IndB and NsdD-IndD interactions are very specific and mediated by the NsdD zinc-finger domain.

Disruption of *indB* and *indD* were carried out by targeted gene replacement and were confirmed by PCR, Southern, northern, and western analysis. Strains carrying single null mutation of *indB* or *indD* did not show any phenotypic differences in reproduction cycles except retarded growth phenotype of *ΔindD* in *veA1* background.

In Northern analysis, 1.2 kb transcripts were detected for *indB* and *indD* genes. The *indB* transcripts expressed to similar levels during asexual development, but increased at 20-30 hour after induction of sexual development, then decreased. In contrast, the *indD* transcript was the most abundant at the initial asexual developmental stage. Furthermore, both kinds of transcripts were highly produced in *veA1* and *Δ veA* mutant strains, but repressed in veA^{+} wild type background, indicating *veA*-dependent repressions.

Overexpression of either IndB or IndD in *veA*+ resulted in block sexual differentiation (Fig. 3). This suggested that increased expressions of IndB and IndD in *veA*+ background might affect NsdD function negatively by binding to the Zn-finger region of NsdD to block NsdD's target DNA bindings.

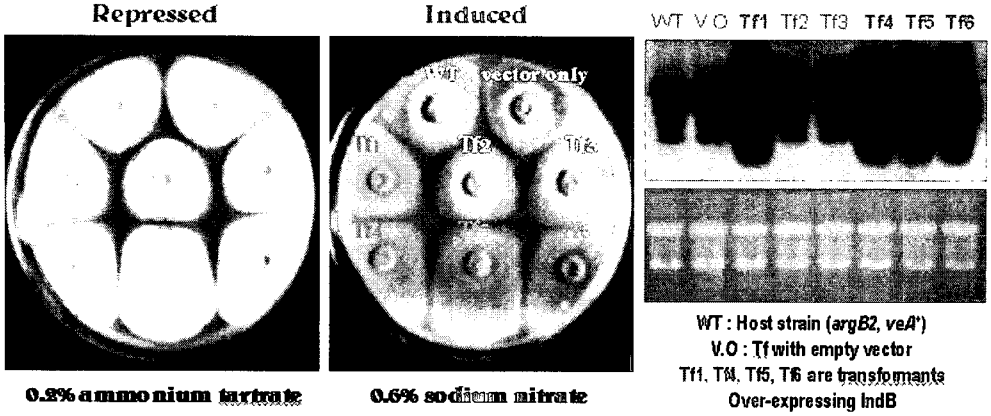

Fig. 3. Over-expression of IndB resulted in block sexual differentiation of *veA*+ strains.

Overall, IndB and IndD proteins are possibly function on sexual differentiation negatively via binding to the Zn-finger region of NsdD and inhibit DNA binding of NsdD which is positive regulator of sexual differentiation. Furthermore, IndB and IndD link VeA with NsdD in sexual differentiation pathway in *A. nidulans*.

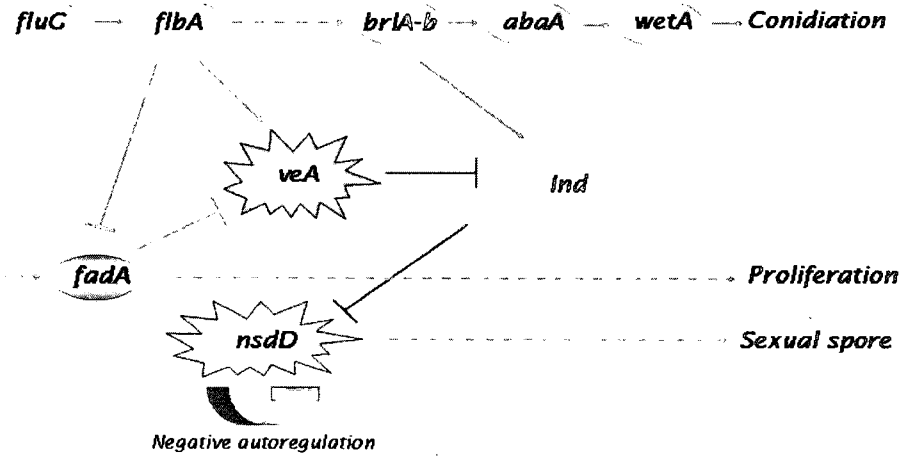

Fig. 4. Negative regulatory role of Ind proteins on sexual differentiation. Ind proteins modulate NsdD function by interacting with the Zn-finger of NsdD.

Acknowledgements

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