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A SET/MYND chromatin re-modelling protein regulates *Dictyostelium* prespore patterning

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ABSTRACT SmdA is a *Dictyostelium* orthologue of the SET/MYND chromatin re-modelling proteins. In developing structures derived from a null mutant for *smdA* (a smdA- strain), prestalk patterning is normal, but using a prespore lacZ reporter fusion, there is ectopic accumulation of β-galactosidase in the prestalk region. As wild type slugs migrate, there is continual forward movement and re-differentiation of prespore cells into prestalk cells. Thus, a potential explanation for the ectopic reporter localization in *smdA* null prestalk cells is an increased rate of re-differentiation and anterior movement of prespore cells. In support of this notion, analysis of an unstable lacZ reporter, driven by the prespore promoter, reveals a normal staining pattern in the smdA- strain. We suggest that one or more genes regulated by SmdA acts to repress prespore re-specification.

KEY WORDS: *Dictyostelium, SET/MYND, prespore, cell sorting*

Developing *Dictyostelium* cells face two ultimate choices, to differentiate as spore cells or as stalk cells. These fates are presaged in the slug,that is formed halfway through development, by the differentiation of precursor cell types: prestalk cells and prespore cells. Most of the prestalk cells are situated in the anterior one-fifth of the slug, the prespore cells occupy the rear. These differentiation states are at the slug stage only quasi-stable. This can most clearly be demonstrated by dissecting the slug into its component prestalk and prespore regions. If sufficient time is allowed for re-differentiation, each part will form a proportioned culminant. This regulation mechanism presumably helps explain how *Dictyostelium* is able to maintain a remarkably constant stalk-spore ratio, over a >1,000 range of cell number.

PspA is a very commonly used marker of prespore cell differentiation. The transcription factors that regulate its expression have not been identified but a promoter region of 216nt, that contains essential regulatory sequences, has been defined (Early and Williams, 1989). We synthesized this sequence in segments and used each in affinity chromatography. Two of the sub-regions purified a SET/MYND orthologue that we have named SmdA. The SET/MYND domain sub-family of SET proteins is conserved in organisms ranging from yeast to vertebrates but has not hitherto been studied in *Dictyostelium*. The SET domain of vertebrate Smyd1 and Smyd3 catalyse methylation of histone H3 on lysine K4 (Hamamoto *et al.*, 2004; Tan *et al.*, 2006). Smyd2 dimethylates H3-K36 and also methylates the p53 tumour suppressor protein (Brown *et al.*, 2006; Huang *et al.*, 2006). In general Smyd proteins are thought to bind indirectly by interaction with other transcription factors, e.g. the oestrogen receptor (Kim *et al.*, 2009), but Smyd3 acts as a direct DNA binding protein (Hamamoto *et al.*, 2004).

We generated a null strain for SmdA but can find no effect on the total level of *pspA* expression, suggesting either that the purification of SmdA was serendipitous or that any effect is very subtle. There is a marked effect on prespore patterning but this aberration is only observed using a stable *pspA* reporter. This leads us to suggest a role for SmdA in stablilising prespore cell differentiation.

Results

Identification of SmdA

When the *pspA* promoter was subjected to deletion analysis a region, between -338 and -122, was defined that contained essential regulatory elements near its boundaries (Early and Williams, 1989). In order to identify transcription factors that interact with the -338 to -122 region, it was synthesised as 4 sub-regions and each was used in affinity chromatography (B.N-C., J. B. and J. G. W., unpublished results). Among the proteins purified by two

Abbreviations used in this paper: SMD, SET/MYND protein.

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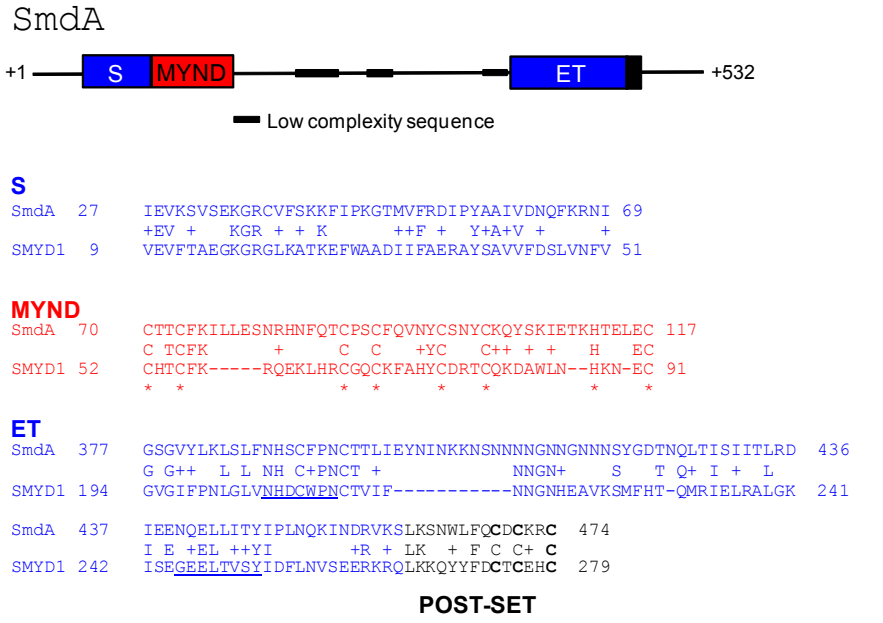

smdA: SET and MYND domain containing protein 1, *D. discoideum* DDB_G0288495
SMYD1:SET and MYND domain containing 1,isoform CRAa [H.Sapiens] Acc.no. EAW77077

Fig. 1. Domain organization of the SmdA protein and sequence alignment of its conserved domains with Smyd1. *The top panel is a representation of the domain organization of the* Dictyostelium *SmdA protein (dictyBase ID: DDB_G0288495). The split SET domain is shown in blue; the MYND domain is represented in red and the post-SET domain is displayed in black. The lower panel is a sequence alignment of the three conserved domains present in* Dictyostelium *SmdA with human Smyd1 (isoform CRAa, acc no: EAW77077).*

of the regions (-291 to -163 and -122 to -168) was a protein annotated as a Smyd ortholog at dictyBase (no. DDB_G0288495). We name the protein SmdA and Fig. 1 is an alignment of SmdA with human Smyd1.

Smyd proteins contain a SET domain that is split into two parts, (designated S and ET in Fig. 1) by the MYND domain. The SET domain of Smyd1 spans positions 24 to 454 and mediates lysine-directed H3 methylation. Histone methyltransferase activity is dependent on the amino acid sequence NHSCXPN and the presence of the cysteine-rich post SET domain at the C terminus (Rea *et al.*, 2000). Both domains are conserved in SmdA. The MYND domain spans from amino acid position 70 to 116 in Smyd1. It is a predicted zinc-binding domain, defined by 7 cysteine residues and a single histidine residue, arranged in a C4-C2HC configuration. All are conserved in SmdA (asterisked in Fig .1). The *Dictyostelium* genome encodes six other, annotated Smyd orthologs. The database of gene expression profiles, at dictyBase ("dictyExpress"), indicates that the *smdA* mRNA is strongly developmentally regulated, with a peak at about 16hr of development and it is approximately three-fold enriched in prespore over prestalk cells.

SmdA is dispensable for growth, morphological development and prestalk patterning

smdA was inactivated, by replacement of the SET and MYND domains with a blasticidin resistance cassette using homologous gene replacement (Supplementary Fig. 1). Disruption was confirmed by PCR of genomic DNA and by RT-PCR on RNA from growing cells. One of the resultant null strains, Ko2, was designated the smdA- strain and was used in subsequent experiments. The strain grows and develops apparently normally (data not shown). It was transformed with prestalk-specific lacZ reporter constructs (Gaudet *et al.*, 2008): ecmAO:gal (a marker of pstA, pstO and pstO/ALC differentiation) ecmO:gal (a marker of pstO and pstO/ALC differentiation), ecmA:gal (a marker of pstA differentiation), and ecmB:gal (a marker of pstAB and pstB differentiation). The smdA- strain expresses all four reporters with a normal pattern (Fig. 2).

The patterning of a stable prespore marker becomes aberrant in the smdA- strain

Since SmdA was purified using sequences from within the *pspA* promoter, we quantitated *pspA* expression in parental and smdA- slug cells by Q-PCR. The value for the mutant is normalized to that of Ax-2 and the mean is shown with the Standard Deviation (n=3). There is no significant difference. The smdA- strain and parental Ax-2 cells were transformed with pspA:lacZ, to determine whether there is a patterning defect. In Ax-2 the construct is, as expected, predominantly expressed in the prespore region (Fig. 3B). However, in the smdA- strain high-level expression extends to the very tip of the slug. This is a clonal population of a reporter transformant but the same pattern was observed in two independently generated transformant pools (data not shown).

We initially suspected that SmdA might be required, as a co-represssor, to prevent *pspA* expression in prestalk cells at the slug stage. However, the explanation is more complex. Parental slugs

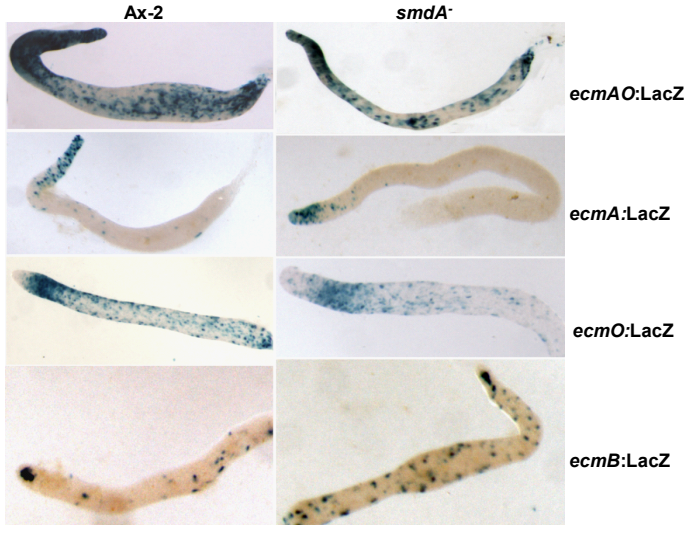

Fig. 2. Analysis of prestalk-specific gene expression in parental and smdA- slugs. *AX-2 and smdA- cells, transformed with the indicated prestalk specific markers were developed to the slug stage and stained for β-galactosidase.*

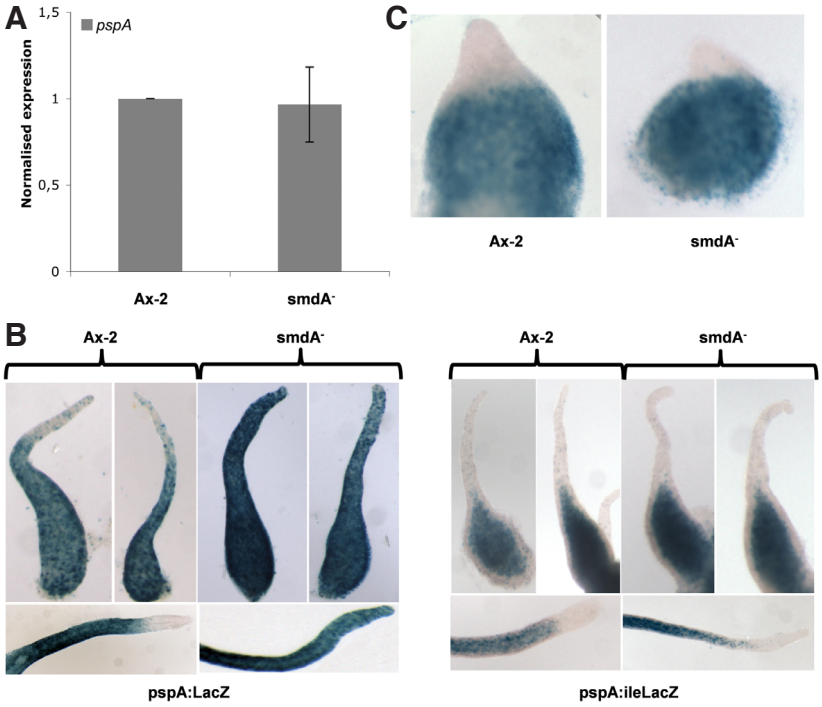

Fig. 3. Analysis of prespore-specific gene expression in smdA- slugs. *AX-2 and smdA- cells were developed to the slug stage. RNA was extracted and analysed by Q-PCR to determine the level of* pspA *expression. The value for the mutant is normalized to that of Ax-2 and the mean is shown with the standard deviation (n=3). AX-2 and smdA- cells, transformed with the prespore markers pspA:gal (left) or pspA:ile-gal (right) were developed and stained for β-galactosidase. For both reporters the four images at the top are of first fingers while the two at the bottom are of migrating slugs. AX-2 and smdA- cells, transformed with pspA:gal were developed to the tipped mound stage and stained for β-galactosidase.*

expressing stable prespore-specific reporter proteins show a clear demarcation of unstained prestalk cells from stained prespore cells but after a time of migration stained cells appear in the prestalk region; reflecting a natural turnover process, whereby a cluster of anteriorly located prestalk cells, the pstAB cells, periodically commit prematurely to stalk cell differentiation, are discarded from the rear of the slug and replaced by the forward movement and re-differention of prespore cells (Abe *et al*., 1994; Harwood *et al*., 1991; Sternfeld, 1992).

This cellular flow was discovered using vital dyes and stable reporter proteins but was elegantly verified using unstable reporter constructs (Detterbeck *et al*., 1994). PspA:ile-gal is similar in structure to pspA:gal, except that it encodes a mutant form of β-galactosidase that is processed in the cell to reveal an ile residue at the N terminus (Detterbeck *et al*., 1994). Such a protein has a lower half-life than the parental form of the protein. When this reporter construct is transformed into cells and analysed during slug migration the clear demarcation between prepore and prestalk cells is maintained. The logical conclusion is that when prespore cells move forward they re-specify as prestalk cells, cease to transcribe *pspA* and the unstable reporter protein rapidly disappears from the cell. When the pspA:ile-gal construct is expressed in smdA- slugs staining is confined to the prespore region (Fig. 3B). One strong prediction from this model is that the mis-localisation be progressive; that using the stable reporter patterning should be normal at very early stages of prestalk-prespore segregation. This is fully borne out in that tipped smdA- mounds expressing pspA:gal display correct patterning (Fig. 3C).

Discussion

We isolated SmdA by affinity chromatography using two separate regions of the *pspA*, prespore-specific promoter. However, many proteins were identified as binding to each fragment and our subsequent analyses tend to suggest that the isolation of SmdA was serendipitous. We tested the possibility that SmdA, like Smyd3 (Hamamoto *et al*., 2004), binds directly to DNA, by expressing SmdA in *E. coli*. We could not detect binding to *pspA* promoter sequences in a gel retardation assay (unpublished results). Nor was there any discernible effect of the *smdA* null mutation on total *pspA* gene expression at the slug stage. Analysis of the *smdA* null did, however, reveal an apparent topological change in *pspA* reporter gene expression.

In the smdA- strain, just as in the *ampA*- mutant of Varney *et al.,* (2002), stable β-galactosidase reporter protein is present, ectopically, in the prestalk region of standing and migrating slugs. This seemed to suggest that SmdA might be a co-repressor, interacting with a negative regulator of transcription, However at early stages of slug formation, or in mature slugs expressing an unstable prespore marker, staining is restricted to the prespore region. A likely explanation for these observations is that a gene regulated by SmdA is essential to stabilise the differentiation state of the prespore cells; so that in the absence of SmdA cells rapidly re-differentiate as prestalk cells that then sort to the prestalk region. A very similar function has been posited for the product of the triA gene (Jaiswal *et al*., 2006). If correct, it would be of interest to profile gene expression patterns in the parental and null strains for both *triA* and *smdA* to identify genes involved in stabilising prespore cell differentiation.

Materials and Methods

Cell growth, transformation, development and β-galactosidase staining

Dictyostelium discoideum cells strain Ax-2 (Gerisch isolate) were grown in axenic medium at 22C. Cells growing at a density of 2 x 10^6 cell/ml were transformed by electroporation. For development cells were harvested and washed in KK2 (16.1 mM KH_2PO_4, 3.7mM K_2HPO_4 pH 6.2) and plated on 1.2% water agar plates at a density of 6 x 10^5 cells/cm^2. Transformant pools, transformed with lacZ markers, were fixed and stained for β-galactosidase at the first finger or migratory slug stages.

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