GSK3 is a multifunctional regulator of *Dictyostelium* development

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Accepted 22 June 2004

Development 131, 4555-4565
Published by The Company of Biologists 2004
doi:10.1242/dev.01330

Summary

Glycogen synthase kinase 3 (GSK3) is a central regulator of metazoan development and the *Dictyostelium* GSK3 homologue, GskA, also controls cellular differentiation. The originally derived *gskA*-null mutant exhibits a severe pattern formation defect. It forms very large numbers of pre-basal disc cells at the expense of the prespore population. This defect arises early during multicellular development, making it impossible to examine later functions of GskA. We report the analysis of a *gskA*-null mutant, generated in a different parental strain, that proceeds through development to form mature fruiting bodies. In this strain, Ax2/gskA–, early development is accelerated and slug migration greatly curtailed. In a monolayer assay of stalk cell formation, the Ax2/gskA– strain is hypersensitive to the stalk cell-inducing action of DIF-1 but largely refractory to the repressive effect exerted by extracellular cAMP. During normal development, apically situated prestalk cells express the *ecmB* gene just as they commit themselves to stalk cell differentiation. In the Ax2/gskA– mutant, *ecmB* is expressed throughout the prestalk region of the slug, suggesting that GskA forms part of the repressive signalling pathway that prevents premature commitment to stalk cell differentiation. GskA may also play an inductive developmental role, because microarray analysis identifies a large gene family, the 2C family, that require *gskA* for optimal expression. These observations show that GskA functions throughout *Dictyostelium* development, to regulate several key aspects of cellular patterning.

Key words: GSK-3, *Dictyostelium*, cAMP, DIF, DIF-1, 2C gene

Introduction

Glycogen synthase kinase 3 (GSK3) was discovered as a metabolic enzyme, that regulates the response of glycogen synthase to insulin; however, it is now known to be active in a wide range of cellular processes, ranging from apoptosis to embryonic development (Cohen and Frame, 2001; Frame and Cohen, 2001; Harwood, 2001). In animal development, it is best known for its activity in Wnt signalling, where it regulates cellular differentiation, migration and growth (Miller, 2002). In the canonical Wnt/β-catenin pathway, cell stimulation by Wnt protein ligands leads to the accumulation and nuclear translocation of β-catenin, which acts as a transactivator for genes regulated by T-cell factor/lymphoid enhancer factor (TCF/LEF) (reviewed by Brantjes et al., 2002).

Dictyostelium has a single GSK3 homologue, GskA, that has 78% amino acid identity to mammalian GSK3β (Harwood et al., 1995). Upon starvation, neighbouring *Dictyostelium* amoebae aggregate, using cAMP as chemoattractant. The cells then differentiate into prestalk and prespore cells, which coordinately move and terminally differentiate to form the stalk and spore head of the fruiting body. Cell fate is determined by diffusible signalling molecules; extracellular cAMP induces prespore differentiation (Kay et al., 1978; Mehdy et al., 1983; Oyama et al., 1988), while differentiation inducing factor 1 (DIF-1) directs prestalk-specific gene expression (Williams et al., 1987). Prestalk cells comprise a number of subpopulations and one of these, the pstB cells, ultimately forms the basal disc; the structure that anchors the stalk to the substratum (Jermyn et al., 1996).

The *gskA*-null mutant, which is generated in the auxotrophic strain DH1, develops to form a fruiting body with a greatly enlarged basal disc and tiny spore head (Harwood et al., 1995). The strain displays a highly aberrant pattern of expression of both the prestalk/stalk-specific gene *ecmB* and the prespore-specific gene *psA*. *ecmB* mRNA is detected 4 hours earlier and at a much higher level in the mutant than in DH1 parental cells. By contrast, expression of the prespore marker gene *psA* is reduced to 5% of control levels in the *gskA*-null mutant. Consistent with this gene expression pattern, prespore cell numbers are severely reduced and the mound is almost entirely filled with cells that, based upon their patterns of expression of the *ecmA* and *ecmB* genes, are pstB cells. In monolayer culture experiments, DH1-derived *gskA*-null cells do not display the cAMP repression of stalk cell induction, which is normally seen in wild-type cells, and are only poorly induced to form spores.

Consistent with a role in regulating cell fate, GskA activity is upregulated about twofold at the mound stage and the cAMP-receptor cAR3 mediates this activation (Plyte et al., 1999). The dual specificity tyrosine kinase ZAK1 tyrosine phosphorylates GskA and this activates it (Kim et al., 1999; Kim et al., 2002). The activity of ZAK1 is developmentally regulated, with kinetics that are approximately coincident with the activation profile of GskA. Furthermore, there is no peak of GskA kinase activity in a *zakA*-null strain (Kim et al., 1999).

Likewise, the GskA activation peak during development is missing in the *cAR3*-null strain (Plyte et al., 1999). All three strains, the *gskA*-null, the *zakA*-null and the *cAR3*-null, are insensitive to the inhibitory effect that cAMP exerts on the induction of stalk cell differentiation by DIF-1 (Harwood et al., 1995; Kim et al., 1999).

The only known substrate for GskA is the STAT transcription factor Dd-STATa (Kawata et al., 1997). Phosphorylation of Dd-STATa by GskA enhances nuclear export of Dd-STATa (Ginger et al., 2000). Although upstream regulators of GskA, and a target of its action, have been identified, it is unclear precisely how GskA exerts its effects on cell fate. We report an analysis of a *gskA* mutant strain, made in a different genetic background, that significantly modifies understanding of the developmental roles played by GskA.

Materials and methods

Generation of a *gskA* gene disruptant

The region encoding *gskA* was amplified from AX2 genomic DNA using the oligonucleotide primers gskA-1-fw (5′-ATGAGTT-CAAAGGATCAGATATTGGAGAAAGATA-3′) and gskA-1289-rev (5′-GGAGGAGGTAGTTGAAGGTGAAGCAGAA-3′). The amplified sequence contains the 1289 bp coding region of *gskA* and the five *gskA* introns. The gene was disrupted (Abe et al., 2003) by an in vitro transposition technique, using an artificial transposon carrying a Blasticidin S-resistance cassette for selection in *Dictyostelium*. The integration position of the transposon was determined by sequencing from the transposon-internal Sq-Fw and Sq-Rv primers (Epicentre, USA). Several disruption constructs were obtained, differing in the flanking *gskA* sequences. A construct where the *gskA* gene was disrupted at an approximately similar position to the construct used in the original *gskA* knockout (Harwood et al., 1995) was chosen for the disruption of *gskA* in AX2 cells. As part of the analysis procedure for the disruptant, the kinase activity of GskA against the mouse glycogen synthase-derived substrate GSM [RRRPASVPPSPSLSRHS(pS)HQRR] was determined as described (Ryves et al., 1998).

Cell culture and development

Axenic *Dictyostelium* strains were grown at 22°C in HL5 medium (Watts and Ashworth, 1970). For selection of transfected strains expressing dominant markers, the medium was supplemented with 10 μg/ml Blasticidin S (Cayla) or 20-200 μg/ml G418 (Sigma). For development on a bacterial lawn, cells were spread together with a suspension of *Klebsiella aerogenes* on a 160 mm culture dish with SM-agar (0.5% Bacto-peptone (Difco), 0.05% yeast extract (Oxoid), 0.5% glucose, 0.23% KH_2PO_4, 0.13% K_2HPO_4, 1.5% bacto-agar (pH 6.4) and allowed to develop at 22°C.

In order to obtain synchronous development, cells were harvested during logarithmic growth, washed twice in 16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4 (pH 6.2; KK_2), resuspended to 1×10^9 cells/ml in H_2O and spread at a density of 2.5×10^6 cells/cm^2 on 1.5% water agar plates. If the formation of slugs was desired, the cell pellet was resuspended in an equal volume of H_2O to give ~1×10^9 cells/ml. Spots of cell suspension were placed on 1.5% water agar plates. The agar plates were incubated for 16 hours in the dark with a unidirectional light source. Slime trails and developing structures were transferred to overhead projector films (Punchline) and stained with Coomassie staining solution. For development on filters, the washed cells were spotted onto nitrocellulose filters sitting on a KK_2-soaked filter pad (Whatman). The development of *Dictyostelium* in monolayer culture, and stalk and spore cell inductions were as described previously (Harwood et al., 1995).

Spore viability assay

Spore viability was determined essentially as described (Dynes et al., 1994). In brief, spores were suspended in 10 mM EDTA/0.1% Nonidet P-40 in KK_2, incubated at 42°C for 45 minutes, washed three times in KK_2, serially diluted plated on *Klebsiella aerogenes*. The number of colonies was scored after 3-4 days.

Immunostaining of prespore vesicles

Slugs were dissociated by trituration through a syringe, fixed in methanol and immunostained with a polyclonal rabbit, anti-prespore vesicle antibody. Immunofluorescence was generated using an Alexaflour 488-coupled anti-rabbit antibody (Molecular Probes).

lacZ reporter gene expression

Clones of an Ax2/gskA– mutant and a control random integrant clone were transformed by electroporation (Howard et al., 1988) with *lacZ* fusion constructs and selected at 20 μg/ml G418. Slugs were developed on 1.5% water agar, transferred onto glass cover slips and then processed for *lacZ* gene expression as described (Dingermann et al., 1989).

Induction of prespore cell differentiation in suspension culture

Logarithmically growing cultures were harvested, washed twice with KK_2, plated at 1×10^8 cells per 9 cm plate on 1.5% water agar and allowed to develop until aggregation centres formed. Cells were harvested, resuspended to a density of 1×10^7 cells/ml in KK_2 and shaken in suspension at 300 rpm with pulses of 300 μM cAMP every hour. Every 2 hours, 0.5 ml of cell suspension were harvested and frozen. Cells were lysed by repeated freeze-thawing and 100 μl aliquots of cell lysate were incubated in microtitre plates with 30 μl 2.5× Z buffer (Dingermann et al., 1989). The reaction was started by addition of 20 μl ONPG (10 mg/ml), incubated at 22°C until the colour changed and the increase in absorption was measured at 420 nm. β-galactosidase activity was normalised against protein content of the samples and is expressed as gain in absorption per hour per mg of protein.

Western transfer and northern transfer analyses

Western Transfer analysis was performed as described previously (Araki et al., 1998). For detection of GskA the anti-GSK-3 antibody 4G-1E (Upstate Technology) was used. Northern transfer analysis was performed as described previously (Fukuzawa et al., 1997).

Expression profiling

Microarray analysis was performed as described previously (Araki et al., 2003) but using the PCR products from 5568 cDNA clones (Morio et al., 1998) (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html). The results were normalised for signal intensities, signal-to-noise ratios (>3), and Lowess-normalised for different dye intensity and analysed using ‘GeneSpring’ (Silicon Genetics).

Results

When developing clonally on a bacterial lawn Ax2/gskA– cells form functional fruiting bodies

Targeted disruption of the *gskA* gene in the strain AX2 was achieved using a construct in which a transposon, carrying a blasticidin-resistance cassette, was inserted into the region encoding the catalytic domain of GskA (Abe et al., 2003). Inactivation of GskA was initially demonstrated by western transfer, using as controls ‘random integrants’; transformant clones where the integrating DNA inserted into the genome at loci other than *gskA* (Fig. 1A). As a further check, the level of GskA activity was determined by enzymatic assay. GskA

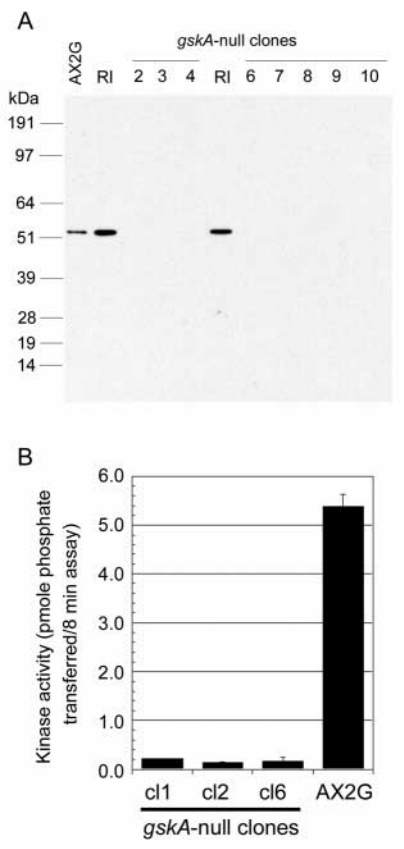

Fig. 1. (A) Western transfer analysis of transformant clones generated using the *gskA* disruption construct. Axenically growing cells were harvested and GskA protein was analysed as described in methods. Protein loading and transfer onto the membrane was normalised by staining with Ponceau S. (B) GSK3 kinase activity measured in cell extracts from AX2 cells and *gskA*-null clones. Growing cells were harvested and GSK3 levels assayed as described (Ryves and Harwood, 1998). Each sample was assayed in triplicate and means and standard deviations are shown.

activity is undetectable in the three Ax2/gskA– clones but is readily detected in control, random integrant cells (Fig. 1B).

When developed clonally on a bacterial lawn, many Ax2/gskA– cells fail to undertake multicellular development. Those that do enter development form fruiting bodies (Fig. 2A,B) but the stalks of the fruiting bodies are significantly shorter (191±48 μm) than those of random integrants (272±117 μm). Nearly every stalk carries a spore head but the spore heads are often malformed (Fig. 2B). As a confirmation that these morphological traits, i.e. a shortened stalk and a misshapen spore head, are indeed due to inactivation of *gskA*, we showed that both phenotypic defects can be reverted by expressing *gskA* in Ax2/gskA– cells under the control of a semi-constitutive promoter (data not shown).

In order to determine whether the malformed spore heads contain normal spores, they were examined in the light microscope and their viability was determined. The spores of the Ax2/gskA– strain have a normal appearance (Fig. 2C) and are as viable and as resistant to heat treatment as spores derived from random integrant cells (Fig. 2D).

Development of the AX2-derived gskA-null strain on non-nutrient agar is clonally variable but the migratory slug stage is always greatly curtailed

Although many Ax2/gskA– cells complete development relatively normally when allowed to develop asynchronously, on a bacterial lawn, their development is significantly more aberrant when developing synchronously. Thus, when Ax2/gskA– cells growing in axenic medium are removed from their food source and allowed to develop on non-nutrient agar, mounds appear ~2 hours earlier than in parallel random integrant controls (data not shown). Furthermore, many of the Ax2/gskA– structures terminate development as mound-shaped structures or very small fruiting bodies (Fig. 3A). In the Ax2/gskA– mutant migration of slugs, away from the point of aggregation, is also greatly decreased relative to controls (Fig. 3B).

The extent of the developmental defects described above is variable between different growth regimes and between different disruptant clones. Thus, if Ax2/gskA– cells are pre-grown on bacteria rather than in axenic medium, fewer cells arrest development as mounds (Fig. 3A). This difference may reflect a metabolic defect, caused by the absence of GskA but

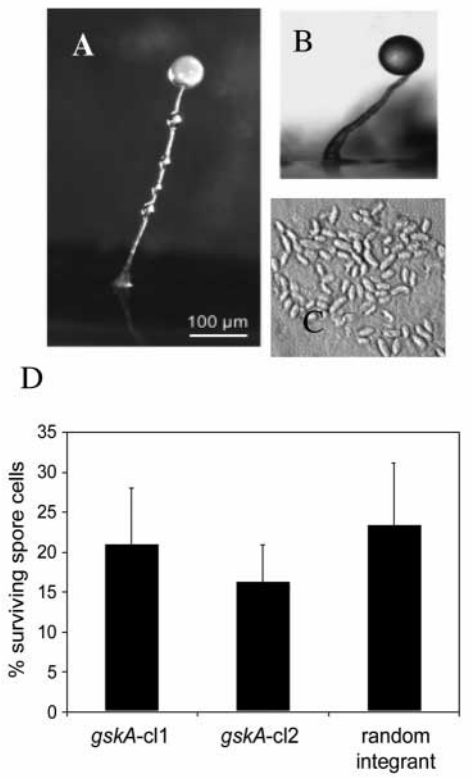

Fig. 2. Terminal morphology and spore characteristics of a random integrant and an Ax2/gskA– clone developing on a bacterial lawn. (A) A random integrant control is shown at same magnification as B, an Ax2/gskA– fruiting body. The beads on the stalk of the random integrant fruiting body are water droplets. Scale bar: 100 μm. (C) The spore head of an Ax2/gskA– clone was squashed under a cover slip. (D) The temperature sensitivity of spores, derived from fruiting bodies that were generated on KK2-agar, was determined as described in the Materials and methods. The percentage of surviving spores, relative to the total number of germinating untreated spores, was determined, and means and standard deviations are shown.

only fully manifest when cells are growing axenically. Furthermore, cells from some Ax2/gskA– clones develop more normally than others. Cells from such clones form short slugs that migrate a small distance away from the point of aggregation (data not shown). The typical behaviours are, however, as described; when pre-grown and developed synchronously, Ax2/gskA– strains are developmentally aberrant at post-mound stages but they are not totally developmentally compromised. This residual developmental potential has allowed us to investigate the role of GskA in multicellular development.

Ax2/gskA– cells precociously express cell-type specific markers

An axenically grown Ax2/gskA– clone and a control random integrant clone were developed on non-nutrient agar and the expression levels of *ecmA*, *ecmB* and *psA* were compared by northern transfer. In the Ax2/gskA– mutant there is a lower apparent expression level of all three genes (Fig. 4). This general reduction in gene expression levels varies from experiment to experiment, and probably reflects the fact that a variable proportion of the mutant cells are left behind as mounds (Fig. 3A); such cells never enter late development and consequently do not express late developmental genes. Although there is a lower peak level of *psA* and *ecmB* gene expression in the Ax2/gskA– mutant, both genes start to be expressed about 2 hours earlier in the mutant than in the random integrant. This also holds true for the *ecmA* gene. The fact that developmental gene expression is brought forward by about 2 hours in the Ax2/gskA– strain is consistent with the precocious mound formation mentioned above.

Prespore genes are expressed in the correct spatial pattern in the Ax2/gskA– mutant

To analyse the spatial pattern of prespore gene expression in the Ax2/gskA– strain, a clone of the mutant and a random integrant clone were transformed with a *lacZ* reporter gene under the control of the *psA* promoter. The psA-*lacZ* construct is expressed at the same approximate level in the prespore region of the slug in both random integrant and an Ax2/gskA– clone (Fig. 5A). CotC-*lacZ*, another prespore marker, displays a similar pattern of expression in the two strains (Fig. 5A).

In order to obtain a measure of prespore differentiation in the Ax2/gskA– strain that is not dependent upon the expression of a reporter gene and associated possible copy number effects, slugs of the Ax2/gskA– strain, developed on non-nutrient agar, were dissociated and immunostained with an anti-prespore vesicle antibody. This revealed approximately equivalent amounts of prespore vesicle and spore coat material in the *gskA*-null strain and the parental AX2 cells (Fig. 5B). Thus, for those Ax2/gskA– cells that proceed past the mound stage, there is no apparent impairment of prespore differentiation.

GskA is not essential for the induction of prespore or spore differentiation

Prespore cell differentiation can be induced in isolated cells by exposure to extracellular cAMP and the DH1-derived *gskA* null strain is highly defective in this response (Harwood et al., 1995). We therefore determined the cAMP-responsiveness of the *psA* promoter in the Ax2/gskA– mutant, by monitoring the induction of β-galactosidase activity in cells transformed with psA-*lacZ*. Exposure to pulses of cAMP induces expression of *lacZ* in the mutant and random integrant cells to comparable extents (Fig. 6A). Induction of the endogenous *psA* gene by extracellular cAMP was also monitored, by northern transfer analysis, and it too is fully cAMP inducible in the Ax2/gskA– mutant (data not shown).

Prespore differentiation is inducible by extracellular cAMP but an increase in intracellular cAMP is required for activation of cAMP-dependent protein kinase and resultant spore formation (Kay, 1989). In the *gskA*-null derived from DH1, a greatly reduced number of spore cells are formed in monolayer culture in the presence of the membrane-permeant cAMP analogue 8-bromo-cAMP (Harwood et al., 1995). In the AX2G/gskA– strain, there is an approximate threefold reduction in the efficiency of spore formation, relative to a random integrant (Fig. 6B). Therefore, in the Ax2/gskA– strain prespore differentiation, in response to elevated extracellular cAMP, appears normal, whereas terminal spore differentiation, in response to elevated intracellular cAMP levels, is reduced but not eliminated.

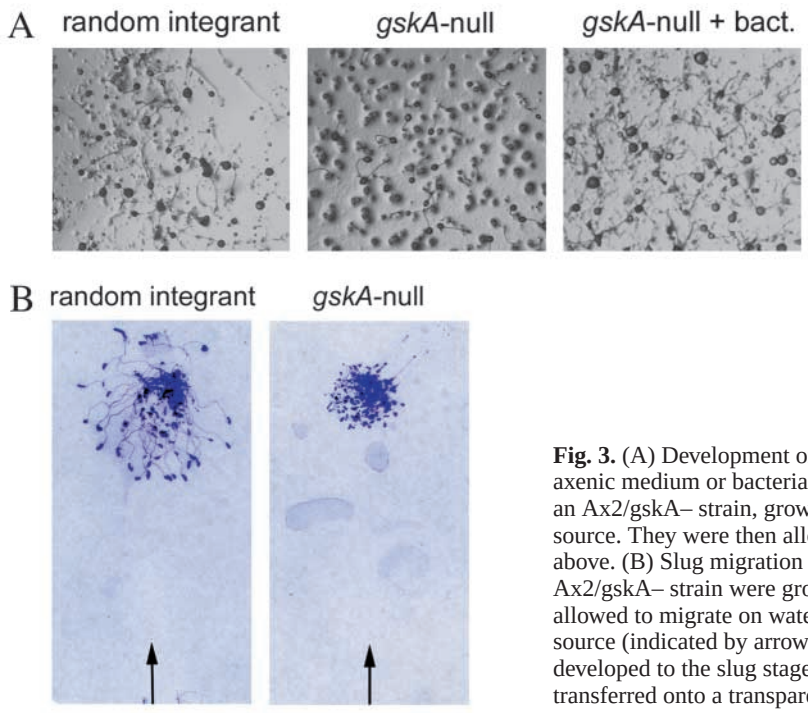

Fig. 3. (A) Development on non-nutrient agar of Ax2/gskA– cells pregrown in either axenic medium or bacteria. Cells of a random integrant control were compared with an Ax2/gskA– strain, grown either axenically or in the presence of a bacterial food source. They were then allowed to develop on KK_2-agar and photographed from above. (B) Slug migration on water agar. Cells from a random integrant clone and an Ax2/gskA– strain were grown in axenic medium, developed to the slug stage and allowed to migrate on water agar for 24 hours and towards a unidirectional light source (indicated by arrows). Approximately 80% of the *gskA*-null structures developed to the slug stage. The structures and their associated slime trails were transferred onto a transparent membrane and visualised by protein staining.

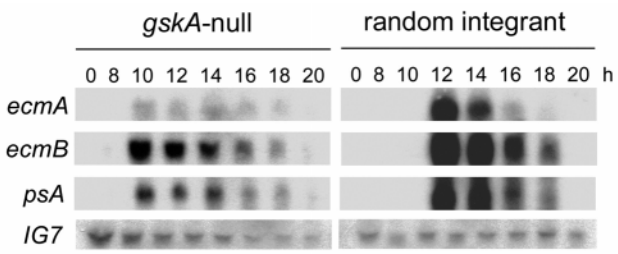

Fig. 4. Northern transfer assay of developmentally regulated genes in a random integrant and an Ax2/gskA– mutant. Cells were developed on KK_2 agar and RNA was prepared from samples taken at 2 hour intervals over a period of 24 hours. The blot was hybridised with an *ecmB*- and a *psA*-specific gene probe. Detection of *ecmA* mRNA was by its cross-hybridisation with the *ecmB* probe. Re-probing with the constitutively expressed *IG7* gene acts as a control for loading and transfer.

At the slug stage *ecmB* expression is aberrant in the Ax2/gskA– strain

We next investigated prestalk differentiation, again using *lacZ* reporter gene constructs. The slug contains several different prestalk populations that are defined by their patterns of expression of reporters driven by *ecmA* and *ecmB* promoter fragments. PstA cells comprise the front 1/3 of the prestalk region, pstO cells occupy the remainder of the prestalk region (Early et al., 1993) and there are scattered cells in the prespore region that show a similar gene expression pattern to pstO cells. These are termed the anterior-like cells or ALC (Sternfeld and David, 1982). The *ecmA*-derived prestalk markers, ecmAO-*lacZ* (a marker for pstA cells, pstO cells and ALC), ecmA-*lacZ* (a marker for pstA cells) and ecmO-*lacZ* (a marker for pstO cells and ALC), are expressed in an apparently identical manner in mutant and control random integrant cells (Fig. 5A).

The *ecmB* gene is expressed at a relatively low level at the slug stage, but its expression increases when culmination begins (Jermyn et al., 1987). In marked contrast to the DH1-derived *gskA*-null strain, ecmB-*lacZ* is not overexpressed at the mound stage in Ax2/gskA– cells (data not shown). However, there is a very significant difference in ecmB-*lacZ* expression at the slug stage. In the random integrant, ecmB-*lacZ* is expressed normally, i.e. in the small cone of pstAB cells in the slug tip (Fig. 5A), but in the Ax2/gskA– strain ecmB-*lacZ* is expressed throughout the prestalk region.

The Ax2-derived gskA-null strain is hypersensitive to DIF and shows a greatly reduced sensitivity to extracellular cAMP as an inhibitor of stalk cell differentiation

The above results show that *ecmB* is mis-expressed at the slug stage in the Ax2/gskA– strain. Therefore, we analysed the response of the AX2-derived null mutant to the extracellular signals that control *ecmB* gene expression. In a monolayer assay, stalk cell differentiation and *ecmB* gene expression are induced by the addition of DIF-1 (Berks and Kay, 1990). Hence, we first compared the DIF inducibility of parental AX2 cells and the Ax2/gskA– strain. There is a major difference in sensitivity; the concentration that induces half-maximal stalk cell differentiation is about 10-fold lower in the Ax2/gskA– strain (Fig. 7A). In addition, the maximal extent of stalk cell differentiation is approximately halved in the parental strain.

If, in a stalk-induction assay, cAMP is added at the same time as DIF-1 stalk cell differentiation and *ecmB* gene expression are greatly repressed (Berks and Kay, 1990). In the DH1-derived *gskA*-null strain cAMP is not inhibitory to these processes (Harwood et al., 1995). We analysed the repressive effect of cAMP on DIF-induced stalk cell differentiation in AX2G/gskA cells, in a random integrant and in parental Ax2 cells (Fig. 7B). The number of stalk cells formed in the *gskA*-null is very weakly repressed by extracellular cAMP but it is strongly repressed by cAMP in the random integrant and AX2 cells (Fig. 7B). We also analysed an ecmB:*lacZ*-expressing strain and found that cAMP repression of ecmB expression is severely abrogated in the null strain (data not shown).

As the *gskA*-null mutant is hypersensitive to DIF-1 (Fig. 7A), it seemed possible that loss of cAMP repression of stalk cell formation may result from saturating concentrations of DIF-1 overriding the inhibition by cAMP. We therefore determined the effect of extracellular cAMP at subsaturating levels of DIF-1. There is a slight increase in the relative inhibitory effect of cAMP in the *gskA*-null cells as DIF-1 levels are lowered, but this is a very small effect in comparison with the dramatic inhibitory effect that cAMP exerts on control cells at low DIF-1 concentrations (Fig. 7C). We conclude, therefore, that the cAMP repression pathway is defective in the *gskA*-null strain, but that this not a consequence of elevated DIF sensitivity.

Gene expression profiling of random integrant and Ax2/gskA– strains at the slug stage identifies potential activation targets

In order to search for other genes that are regulated by GskA during multicellular development, we performed microarray analysis. A profile of gene expression differences in the *gskA*-null strain was obtained using RNA samples isolated at the slug stage. The cDNAs were prepared using RNA from either random integrant or Ax2/gskA– slugs and they were labelled with either Cy3 or Cy5. A mixture of the two differently labelled cDNA preparations was hybridised to an array bearing PCR products derived from 5568 ESTs (Morio et al., 1998). Based upon the estimate of VanDriessche et al. (VanDriessche et al., 2002), there should be no more than a 20% redundancy in this EST set. Hence, the array potentially accesses ~40% of the estimated 11,000 Dictyostelium genes (Glockner et al., 2002).

We are not, unfortunately, able to interpret those ESTs that appear to be over-expressed in the null mutant. This limitation arises because, when developing on non-nutrient agar, a significant proportion of the null strain cells do not reach the slug stage (Fig. 3A). Therefore, those genes that are expressed at a high level during early development, and are then switched off, will appear to be overexpressed in the mutant. We can, however, interpret the data for those genes that are underexpressed in the null strain. This was achieved using *psA* as a standard to correct for the 'dilution effect' that results from the presence of cells arrested as mounds. From the composite microarray data for *psA*, we estimate this 'dilution' effect causes an apparent underexpression of less than twofold [average value for the null/random integrant signal (±s.d.) for *psA*=0.60±0.085 (*n*=15)]. As *psA* is barely expressed at the

mound stage but is highly expressed at the slug stage, this will be the maximum extent of the dilution effect; i.e. any gene that is significantly expressed at the mound stage will cause a smaller dilution effect than is observed for *psA*. As the dilution effect creates an apparent approximate twofold underexpression, we placed a relatively severe significance cut off for the primary analysis of fourfold (Fig. 8).

Seven ESTs showed an average expression level in the null mutant of less than fourfold that of the control, and this was also duplicated in the dye swap. Strikingly, all seven ESTs proved to be highly homologous to one gene, the 2C gene (Fig. 8). There is a 2C-related gene, 7E (Corney et al., 1990; Richards et al., 1990), but the ESTs that we identified as GskA responsive are all significantly more homologous to 2C than to 7E (data not shown).

Identifying those ESTs that are strongly overexpressed on all slides automatically excludes ESTs where just one hybridisation failed or gave a high background. Finally, therefore, the entire data set was reanalysed for all ten 2C-

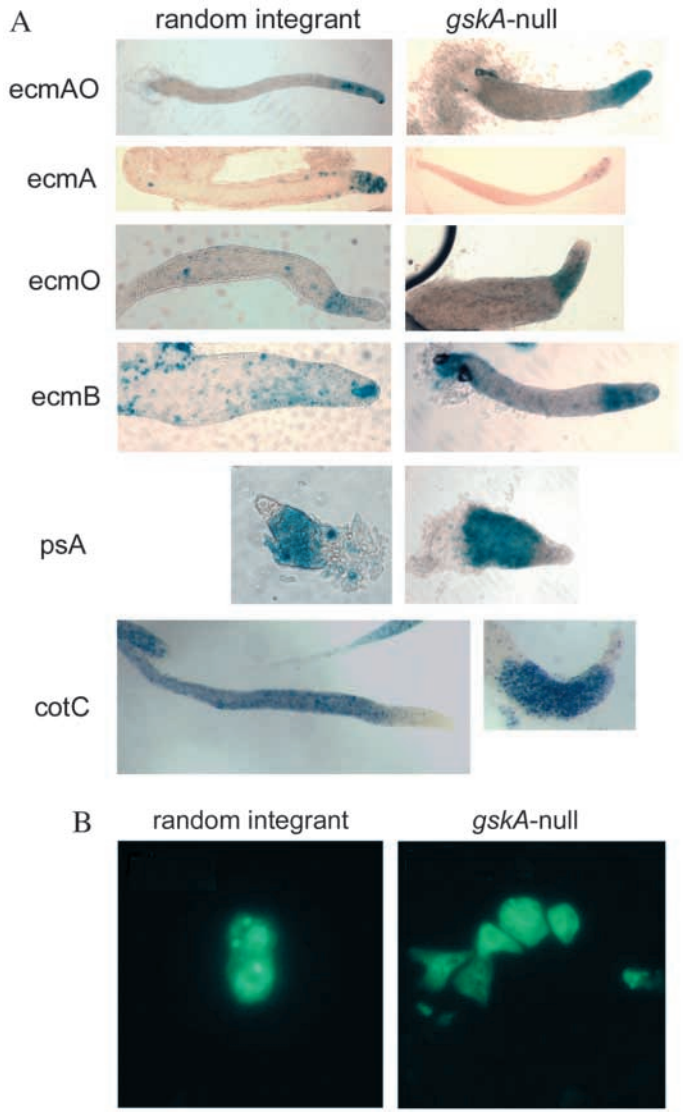

Fig. 5. (A) Analysis of the expression pattern of cell-type specific reporter constructs in a random integrant and an Ax2/gskA– strain. Stable transformants for each of the indicated *lacZ* reporter constructs were allowed to develop on water agar. The stained structure for each random integrant transformant clone is shown on the left and the equivalent *gskA*-null mutant structure is shown on the right. The developmental promoter, or promoter subfragment, that directs expression of the *lacZ* gene is indicated on the left. Slug stages are shown for strains transformed with all reporter constructs, except for strains transformed with psA-*lacZ*, where tipped aggregates are shown. All structures derive from pools of transformants. (B) Prespore vesicle staining in a random integrant and a *gskA*-null mutant. Slugs were stained for prespore vesicles as described in the Materials and methods section.

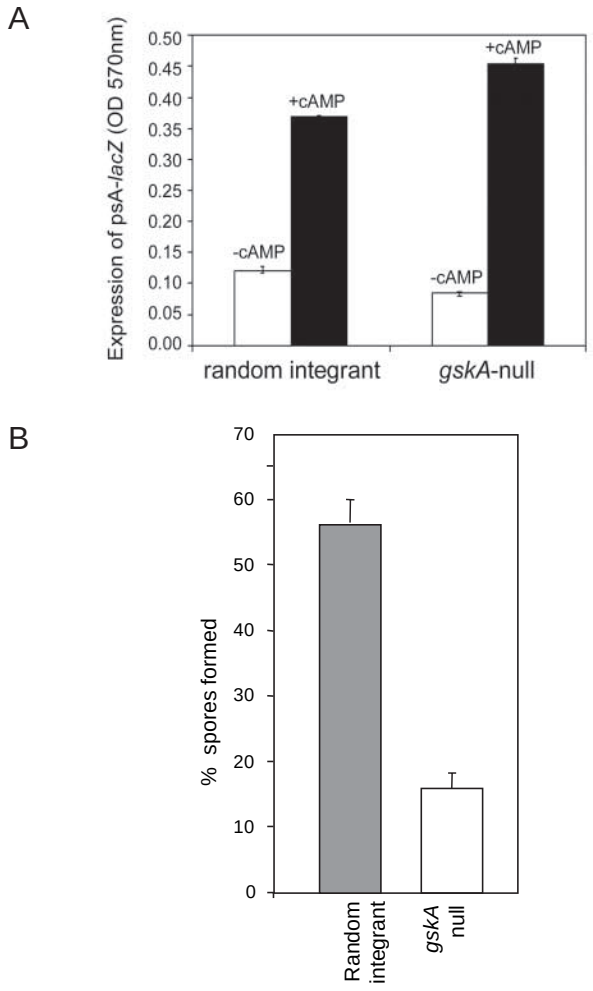

Fig. 6. (A) Induction of the prespore marker psA-*lacZ* in cells of a random integrant and an Ax2/gskA– strain. Cells were developed in suspension culture and pulsed with 300 μM cAMP. Samples were taken at t=0 (–cAMP) and t=8 hours (+cAMP), and β-galactosidase activity was determined. All samples were measured in triplicate and the means and standard deviations are presented. The accumulation of β-galactosidase is higher in the *gskA*-null mutant than in the random integrant. This is likely to be due to copy number differences for the reporter construct in the two strains. (B) Induction of spore cell differentiation by 8-Bromo-cAMP in a random integrant and an Ax2/gskA– strain. The random integrant and Ax2-derived *gskA*-null mutant were plated at low density (10^4 cells/cm^2), in spore induction medium and exposed to 15 mM 8-bromo-cAMP for 24 hours (Kay, 1989). Spores were counted and expressed as a percentage of total number plated. Data are expressed as mean number±s.e.m. (n=3).

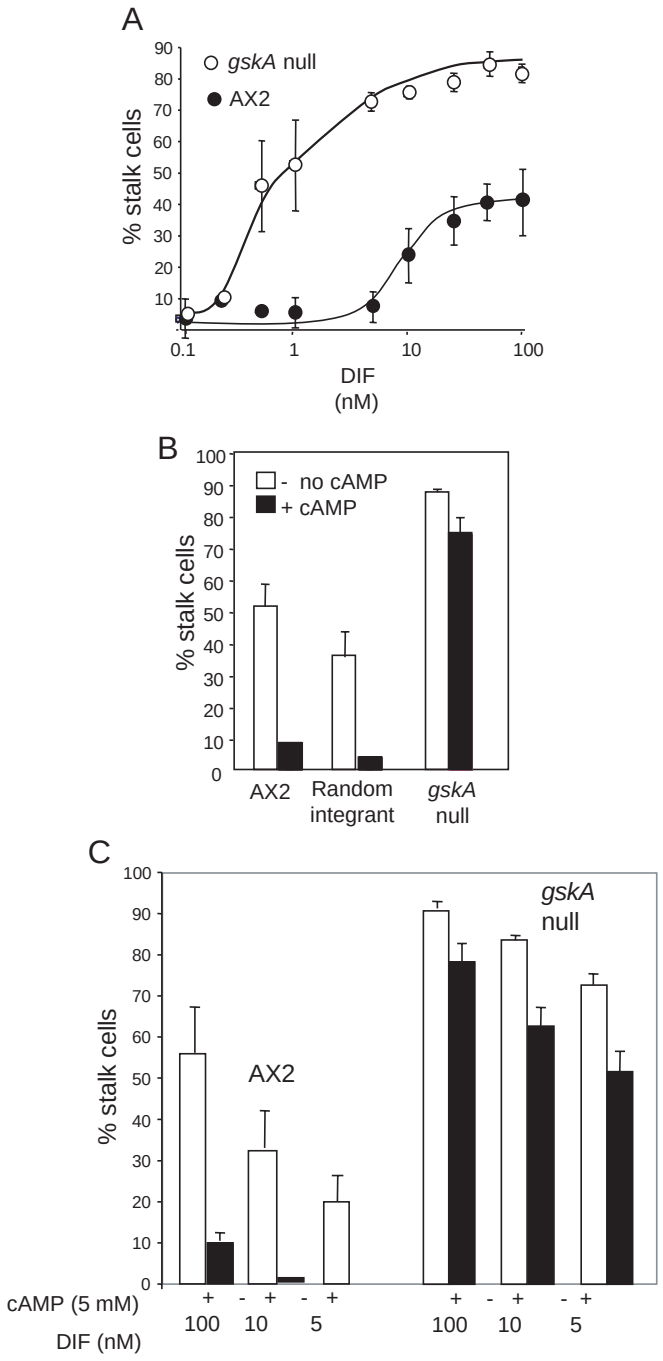

Fig. 7. Induction of stalk cell differentiation by DIF-1. Comparison of the DIF-1 sensitivity of AX2 cells and an Ax2/gskA– strain. Ax2 (black circles) and *gskA*-null mutant cells (white circles) were plated at low density (10^4 cells/cm^2) in stalk medium in 5 mM camp). After 24 hours incubation, cAMP was washed off and replaced with DIF at varying concentrations for a further 24 hours. Stalk cells were counted and expressed as a percentage of total number plated. Data are expressed as mean±s.e.m., and represent the combination of two clones analysed in triplicate. (B) Comparison of the effect of cAMP on AX2 cells, a random integrant and an Ax2/gskA– strain. Cells were treated as in A, but incubated in 100 nM DIF±5 mM cAMP, as indicated. (C) Comparison of the effect of cAMP on AX2 cells and an Ax2/gskA– strain using different inductive concentrations of DIF-1 Cells were treated as in A, but incubated in 100, 10 or 5 nM DIF ±5 mM cAMP, as indicated.

related ESTs on the array. The ratio of the 2C signal for null/random integrant is 0.19±0.088. Correction for the dilution factor, 0.60±0.085 (legend to Fig. 8), indicates that 2C is expressed at approximately one-third the wild-type level in the *gskA*-null strain.

Discussion

Strain differences between *gskA* null mutant phenotypes

The morphology of mutants arising from *gskA* gene disruption varies markedly with parental strain. Inactivation of *gskA* in the DH1 parental background gives rise to highly abnormal structures, which contain very few spore cells and a greatly expanded pre-basal disc cell population. By contrast, many clones of the AX2-derived *gskA* null mutant generate small, but normally proportioned, fruiting bodies. The parent of the original null mutant, DH1, is a uracil auxotroph of the axenic strain AX3 and inactivation of *gskA* in AX3 yields a mild phenotype resembling that of the AX2 disruptant (C.S. and J.G.W., unpublished). The phenotypic differences presumably arise, therefore, from the absence in DH1 of pyr5-6 – an enzyme of pyrimidine biosynthesis. We suggest that perturbations in nucleotide biosynthesis, caused by the absence of pyr5-6, elevate the mutation rate in DH1. There is a major variability in DIF responsiveness in DH1 stocks derived from different laboratories (C.S. and J.G.W., unpublished) and this is consistent with hyper-mutability of DH1. Hence, we further believe that the particular DH1 substrain used in the initial mutant screen contains a genetic enhancer of the gskA-null phenotype.

Paradoxically, the use of the DH1 strain in the original *gskA* study may have actually been fortuitous (Harwood et al., 1995), because the milder phenotypes seen with the AX2-derived gskA-null mutant could have been overlooked. This was the original fate of the cAR3 cAMP receptor mutant. Initial characterisation of the mutant suggested that it was morphologically normal but more detailed analysis showed that it is defective in GskA signalling (Plyte et al., 1999). The difference in severity of phenotype between the *gskA*- and *cAR3*-null mutants was originally thought to be due to receptor redundancy (Plyte et al., 1999). However, our present analysis suggests that the *gskA* and *car3* phenotypes are much more similar than previously believed.

The rate of early development is accelerated in AX2G/gskA-null cells but the slug phase is curtailed

Dictyostelium cells enter development when their food source, more specifically their amino acid supply, becomes limiting (Marin, 1976) and growth conditions are an important modulator of subsequent development. GSK3 is a metabolic enzyme and this provides one possible explanation for the fact that early development is accelerated in *AX2G/gskA*– cells. As the transition from slug migration to culmination is regulated by endogenous ammonia produced by cellular catabolism (Schindler and Sussman, 1977), a metabolic defect may also explain the greatly curtailed migratory slug phase in the Ax2/gskA– strain.

Loss of *gskA* has weak effects on spore differentiation

When the DH1-derived *gskA* null strain is allowed to develop

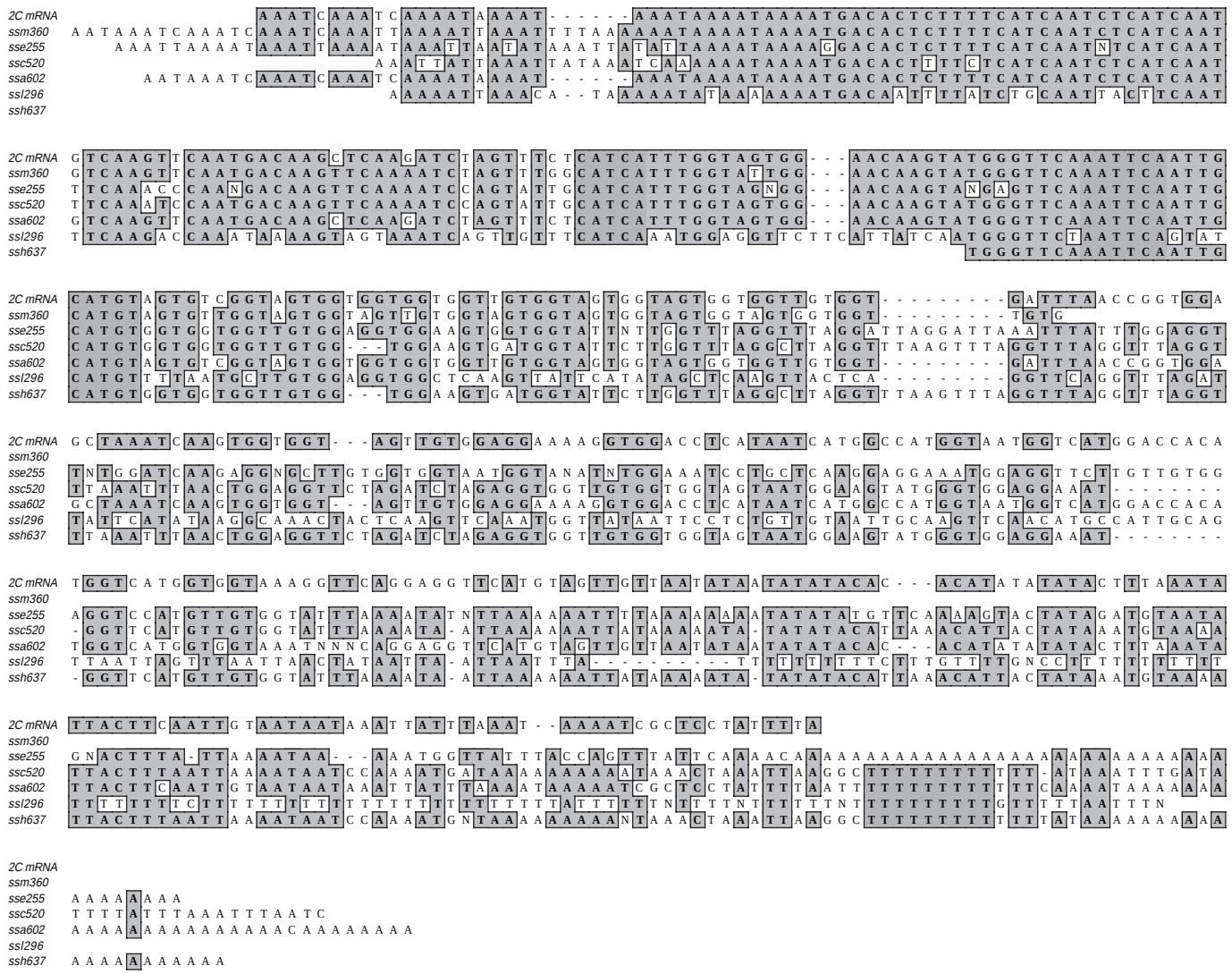

Fig. 8. Sequence comparison for seven of the ESTs that are underexpressed in the *gskA*-null strain. Microarray analysis was performed as described in the Materials and methods. This yielded seven ESTs where the sequence is known. This Clustal W analysis shows that the seven ESTs are all members of the 2C gene family.

clonally on a bacterial lawn, it exhibits a severe reduction in prespore and spore differentiation. By contrast, the AX2-derived *gskA*-null cells express prespore genes and form fruiting bodies. Although inactivation of the *gskA* gene in AX2 cells is permissive for prespore and spore differentiation, analysis under conditions of synchronous development revealed a heterogeneity in the response. In some clones of the AX2-derived null strain, pre-growth in axenic medium and development on non-nutrient agar caused a proportion of the aggregates to arrest as mounds. One possible explanation for the variable penetrance of the *gskA*-null mutation, under these particular developmental conditions, is a cryptic heterogeneity in the AX2 parental cells used to generate the null strains.

Analysis of the signalling pathways that direct prespore and spore differentiation

The fact that prespore and spore differentiation occur in the Ax2/gskA– strain led us to study cellular responses to known agonists of the two processes. Extracellular cAMP acts, via an unknown intracellular signalling pathway, to induce prespore gene expression (Gomer et al., 1986; Kay et al., 1978). In the DH1-derived *gskA*-null strain, extracellular cAMP activates prespore gene expression very poorly, but in AX2G/gskA– cells, cAMP is a potent activator. The membrane-permeant cAMP analogue 8-bromo cAMP induces spore differentiation (Kay, 1989). This and much other evidence (reviewed by Williams et al., 1993) suggests that activation of PKA, via an elevation in intracellular cAMP, is both necessary and sufficient to trigger terminal spore differentiation. This induction is greatly impaired in DH1-derived *gskA*-null cells. The induction of spore cell formation by 8-bromo cAMP is also reduced in AX2G/gskA– cells, to about 30% of control levels. However, this is a much less severe effect than in the DH1-derived strain (Harwood et al., 1995) or in the *gskA*-null derived in strain JH10 (Kim et al., 1999), where spores are

formed at only 10% of control levels. One possible explanation for this difference, for the DH1 strain, is that it is defective in prespore differentiation and that this enhances a subsequent effect of the *gskA* mutation on spore formation. Consistent with this, the DH1 strain produces very few viable spores, during normal development (A.H., unpublished) while AX2G/gskA– cells forms completely viable spores (Fig. 2D).

Temporally and spatially regulated *ecmB* expression is dependent upon GskA

PstA cell and pstO cell differentiation occur normally in the AX2G/gskA– strain but one aspect of prestalk/stalk-specific gene expression is significantly aberrant; in slugs of the AX2G/gskA– strain, the *ecmB* gene is activated, precociously and ectopically, in all cells within the prestalk region. Normally, cells within the prestalk region of migrating slugs undergo a movement and differentiation cycle that presages events at culmination (Abe et al., 1994). A subset of the pstA cells, located very near the slug tip, activate expression of *ecmB* and are then termed pstAB cells. These cells are periodically shed from the back of the slug, where they rapidly differentiate into stalk cells (Sternfeld, 1992). During culmination, the differentiation of prestalk cells into pstAB cells becomes continuous rather than sporadic. However, very similar processes of movement and *ecmB* activation occur; after first expressing *ecmB*, at the entrance to the stalk tube, the newly formed pstAB cells move down the tube and terminally differentiate as vacuolated stalk cells (Jermyn and Williams, 1991).

In the ZAK1-null and, as we have now shown, in the *gskA*-null, the *ecmB* gene is expressed throughout the prestalk region. In addition, in slugs of the original DH1-derived *gskA*-null strain, in the Ax2-derived *gskA*-null strain and in ZAK1-null slugs inhibition of stalk cell differentiation by cAMP is greatly attenuated. In combination, these data suggest that extracellular cAMP signalling activates ZAK1 and GskA to repress *ecmB* expression in pstA cells and prevent their premature differentiation into pstAB cells (Fig. 9).

The above scheme may also help explain the DIF-1 hypersensitivity of the *gskA*-null strain. As the inducing concentration of DIF-1 is lowered, in AX2 cells, there is a pronounced increase in the repressive effect that cAMP exerts on stalk cell differentiation (Fig. 7C). Thus, there seems to be an antagonism between DIF-1 and extracellular cAMP. By contrast, the *gskA*-null strain is largely refractory to the inhibitory effect of extracellular cAMP at all DIF-1 concentrations tested; the low level of cAMP repression of stalk cell formation that does occur probably derives from a previously described, gskA-independent mechanism (Coates et al., 2002). We suggest that, in AX2 cells incubated in the absence of exogenous cAMP, endogenously produced cAMP antagonises the inductive effect of DIF-1. If true, this would effectively shift the DIF-1 dose-response curve to higher concentrations. However, the shift would not occur in a *gskA*-null strain, where the extracellular cAMP inhibition mechanism is inoperative. Hence, lower DIF levels would be needed for the induction of stalk cell differentiation in the *gskA* null.

The extracellular cAMP repression pathway functions independently of Dd-STATa

Dd-STATa acts as a repressor of *ecmB* gene transcription by binding to two, mutually redundant sites within the promoter (Fig. 9) (Mohanty et al., 1999). In addition, Dd-STATa is a direct target of GskA; the kinase modifies a region near the N terminus of Dd-STATa and triggers its export from the nucleus (Ginger et al., 2000). Moreover, just as for the *gskA*-null, the Dd-STATa-null strain is hypersensitive to DIF-1 (Mohanty et al., 1999). Despite these congruencies, however, Dd-STATa cannot be the mediator of extracellular cAMP repression;

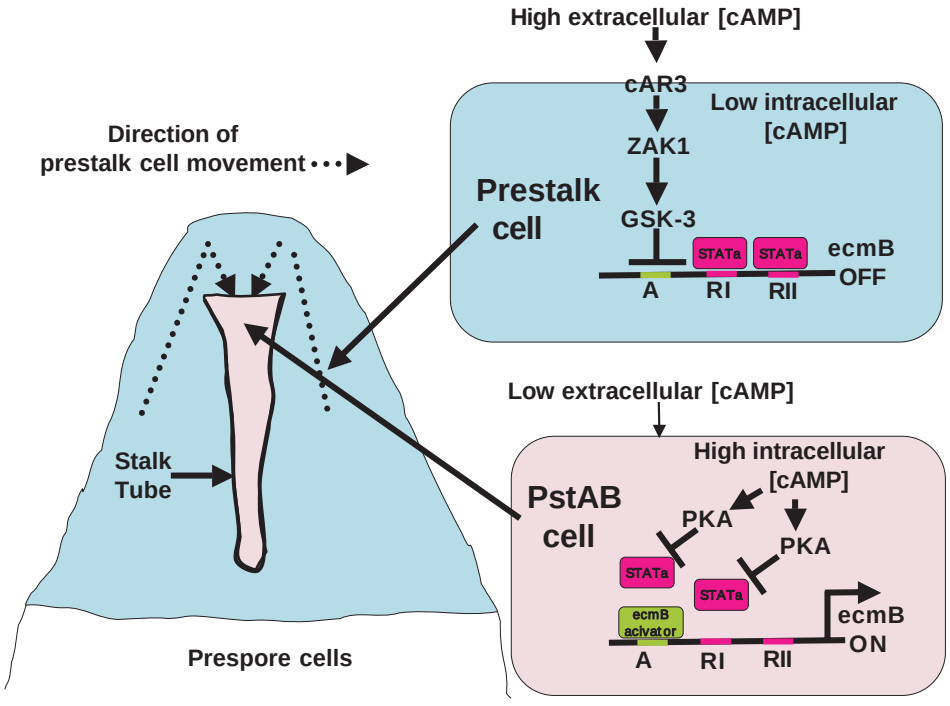

Fig. 9. GskA and the regulation of stalk cell differentiation. The structure at the left is a representation of an early culminant, with prestalk cells undertaking the 'reverse fountain' movement. As they pass the entrance to the stalk tube, the prestalk cells activate expression of the *ecmB* gene and are then termed pstAB cells (pink). Before entry to the stalk tube (blue), the *ecmB* promoter is inactive; high extracellular cAMP signalling acts (via the cAR3 cAMP receptor, the dual specificity kinase ZAK1 and GSK-3) to prevent the ecmB activator region (A) from functioning. In addition, Dd-STATa functions as a repressor, acting via two repressor elements (RI and RII) located distal to the *ecmB* cap site. At culmination, repression by Dd-STATa is also relieved by a parallel pathway involving activation of PKA.

because the Dd-STATa-null strain is hypersensitive to the repressive effect of cAMP on *ecmB* gene expression (Mohanty et al., 1999). In addition, an ecmB promoter construct lacking the repressor elements remains subject to extracellular cAMP repression (Y. Yamada and J.G.W., unpublished). It would seem, therefore, that GskA exerts its effects via the ecmB activator (Fig. 9), an as yet unidentified factor that interacts with a directly repeated sequence in the ecmB promoter (Ceccarelli et al., 2000).

Evidence that GskA also activates gene expression

To determine whether GskA has a more general role in regulating gene expression, we used expression profiling to analyse the *gskA*-null mutant. Using a microarray that monitors almost one-half of expressed genes, we discovered a gene family that is significantly underexpressed in the Ax2-GskA- strain. 2C is a very small gene, of less than 0.5 kb, that has the potential to encode an 8.8 kDa protein of unknown function (Richards et al., 1990). The 2C gene is strongly developmentally regulated, with a peak during mid-culmination (Corney et al., 1990). Analysis of the genome sequence shows that there are multiple copies of the 2C gene and their level of relatedness is so high we assume we are measuring their composite behaviour. Given the role of cAMP in GskA regulation, it is perhaps significant that the intracellular concentration of 2C mRNA is regulated by the extracellular cAMP concentration (Richards et al., 1990).

Relationship to other GSK3 regulated signalling pathways

In animal cells, GSK3 represses gene expression by causing the degradation of β-catenin. Wnt stimulation alleviates this repression by preventing GSK3 phosphorylation and subsequent degradation of β-catenin, allowing it to activate gene expression. Superficially, the results presented here mirror the metazoan pathway; however, there are many differences. *Dictyostelium* expresses a homologue of β-catenin, known as Aardvark (Aar) (Grimson et al., 2000). In the *aar*-null mutant at culmination, *ecmB* expression expands outside the stalk tube and throughout the tip (Coates et al., 2002). However, this is an indirect structural effect; it arises from a loss of adherens junctions, which require Aar protein for their formation (Coates et al., 2002).

Although we conclude that the *Dictyostelium* pathway does not conform to the canonical Wnt signalling pathway, even among the metazoa, the canonical pathway is not the only route whereby Wnt signals and GSK3 can regulate cell fate (Veeman et al., 2003). For example, during early nematode development, the Wnt signal MOM-2 acts positively on GSK3 to regulate the function of the β-catenin homologue WRM-1 (Thorpe et al., 2000). This is an interesting potential parallel with the positive effect that gskA has on expression of the 2C gene family. However, much of the mechanism of these GSK3-mediated pathways has yet to be resolved and it remains to be determined whether there is any functional overlap with the pathways used by *Dictyostelium*.

This work was supported by a Wellcome Trust Program Grant 053640/Z/98/C to J.G.W.; a Wellcome Trust Prize Studentship 057760 to C.S.; a Wellcome Trust Senior Fellowship 040846 to A.J.H.; and a Wellcome Trust Project Grant 065236 to H.W. We are very grateful to the members of the cDNA project in Japan for generously providing the ESTs.

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