Combinatorial cell-specific regulation of GSK3 directs cell differentiation and polarity in *Dictyostelium*

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SUMMARY

In *Dictyostelium*, the interaction of secreted cAMP with specific cell surface receptors regulates the activation/de-activation of GSK3, which mediates developmental cell patterning. In addition, *Dictyostelium* cells polarize in response to extracellular cAMP, although a potential role for GSK3 in this pathway has not been investigated. Previously, we had shown that ZAK1 was an activating tyrosine kinase for GSK3 function in *Dictyostelium* and we now identify ZAK2 as the other tyrosine kinase in the cAMP-activation pathway for GSK3; no additional family members exist. We also now show that tyrosine phosphorylation/activation of GSK3 by ZAK2 and ZAK1 separately regulate GSK3 in distinct differentiated cell populations, and that ZAK2 acts in both autonomous and non-autonomous pathways to regulate these cell-type differentiations. Finally, we demonstrate that efficient polarization of *Dictyostelium* towards cAMP depends on ZAK1-mediated tyrosine phosphorylation of GSK3. Combinatorial regulation of GSK3 by ZAK kinases in *Dictyostelium* guides cell polarity, directional cell migration and cell differentiation, pathways that extend the complexity of GSK3 signaling throughout the development of *Dictyostelium*.

KEY WORDS: cAMP, Cell polarity, Receptors, Protein phosphorylation, Chemotaxis, *Dictyostelium*

INTRODUCTION

The regulation of cell specification by GSK3 may be universal (Angers and Moon, 2009; Harwood, 2008; Kimmel et al., 2004; McNeill and Woodgett, 2010). For metazoans, this generally involves the functional repression of GSK3 by Wnt-mediated canonical signaling. The Wnts are a family of secreted glycoproteins, the canonical signals of which are transduced intracellularly by co-receptors of seven-transmembrane Frizzled (Fz) and LDL receptor-related proteins (LRP) 5/6 (Angers and Moon, 2009; Bhanot et al., 1996; McNeill and Woodgett, 2010; Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000; Yang-Snyder et al., 1996). There are, however, at least two multicellular systems, *C. elegans* and *Dictyostelium*, that use both activating and inhibitory pathways for GSK3 regulation of cell fate determination (Angers and Moon, 2009; Hardin and King, 2008; Harwood, 2008; Kimmel et al., 2004; McNeill and Woodgett, 2010; Moon et al., 2002). GSK3 activation during endoderm/mesoderm specification in *C. elegans* is Wnt/Fz dependent. *Dictyostelium* use a distinct signal, secreted cAMP, that targets a family of specific cell-surface receptors (CAR1, CAR2, CAR3, and CAR4) and regulates GSK3 activity (Harwood, 2008; Kimmel et al., 2004). Signaling by both cAMP and Wnt also regulates cell polarity (Hardin and King, 2008; Harwood, 2008; Kimmel and Firtel, 2004; Kimmel and Parent, 2003; Kimmel et al., 2004; Schlesinger et al., 1999; Veeman et al., 2003; Walston et al., 2004).

Although in certain aspects the cAMP/CAR and Wnt/Fz pathways appear functionally related, they are mechanistically distinct. GSK3 activity per se is not altered upon Wnt stimulation; rather, Wnt/Fz functions to disrupt association of GSK3 with the specific substrate β-catenin. By contrast, in *Dictyostelium*, signaling through distinct cAMP receptors serves to activate or inhibit the enzymatic activity of GSK3. These actions, respectively, involve the tyrosine phosphorylation of GSK3 via ZAK1 or de-phosphorylation by a PTPase (Harwood et al., 1995; Kim et al., 2002; Kim et al., 1999; Schilde et al., 2004; Strmecki et al., 2007).

*Dictyostelium* development is characterized by a succession of distinct phases. Early events regulate cell polarization and directed cell migration toward centers of cAMP signaling where cells form multicellular aggregates that differentiate into progenitor prespore and prestalk cells (Kimmel et al., 2004; Williams, 2006). After aggregation, precursor populations sort asymmetrically along a body axis. The anterior 20% is primarily prestalk, whereas the posterior 80% is highly enriched in prespore cells. However, the prestalk population is not homogeneous; prestalk A (pstA) and prestalk B (pstB) cell populations are identified by the expression of specific genes. During the commitment to terminal differentiation, the prepro and prestalk precursors differentiate into mature spores and stalk cells (Gaudet et al., 2008; Kimmel and Firtel, 2004; Williams, 2006).

We had shown that the cAMP/CAR3/ZAK1/GSK3 cascade positively regulates prespore gene expression and spore differentiation, but suppresses prestalk differentiation (Kim et al., 2002; Kim and Kimmel, 2000; Kim et al., 1999; Kimmel and Firtel, 2004). *car3*, *gsk3* and *zak1* nulls have impaired prespore/spore differentiation, and resistance to cAMP-mediated repression of pstB cell and stalk formation (Harwood et al., 1995; Kim et al., 2002; Kim and Kimmel, 2000; Kim et al., 1999; Kimmel and Firtel, 2004; Plyte et al., 1999; Schilde et al., 2004). CAR3 stimulation will activate ZAK1, which, in turn, will tyrosine phosphorylate and activate GSK3. However, biochemical and...
genetic data indicate that additional components immediately upstream of GSK3 must be involved; limited, but reproducible, tyrosine phosphorylation and activation of GSK3 are evident in zak1 nulls, suggesting the presence of an additional tyrosine kinase (Kim et al., 2002). Furthermore, regulation of pstA cells by ZAK1 and GSK3 is not identical (Harwood et al., 1995; Kim et al., 1999). We have identified a new activating tyrosine kinase, ZAK2, in the GSK3 pathway. Although both ZAK1 and ZAK2 can phosphorylate and activate GSK3, they function distinctly in control of the different cell populations. ZAK2 and ZAK1 regulate separate prestalk populations through the common target GSK3. Both kinases are required to activate prespore/spore differentiation via GSK3, but ZAK2 also appears to have an additional non-autonomous function. Finally, we extended our studies to examine the regulation of cell polarity in Dictyostelium by cAMP- and GSK3-mediated signaling. Results indicate that activation of GSK3 by ZAK1 is required for cell polarization and migration.

MATERIALS AND METHODS

Dictyostelium culture, development and differentiation

Dictyostelium wild-type and mutant cells were grown, developed on nitrocellulose filters and differentiated in shaking culture or in monolayers as described previously (Kim et al., 2002; Kim et al., 1999). Developing organisms with cell-specific lacZ reporter plasmids were fixed and stained as described previously (Richardson et al., 1994). Relevant DictyBase gene numbers are DDB0185150 for GSK3, DDB0185184 for ZAK1 and DDB0229958 for ZAK2.

Isolation of ZAK2 cDNA and generation of zak2 nulls

ZAK2 cDNA was isolated as described (Kim et al., 1999). The blasticidin-resistance cassette was subcloned into the single EcoRV site (GATATC) at nucleotide 1506 within the C-terminal tyrosine kinase domain coding region of the ZAK2 cDNA. Disruptants were screened by PCR using a 5′ primer at nucleotide 1440 (GTTGTTCTAATCTTTATGCGACCAGAG) and a 3′ primer at nucleotide 1883 (CCACTACCATAGTTGAATGAG). Disruption was confirmed by genomic Southern blot and loss of expression by a developmental northern blot hybridized with a full-length probe to ZAK2. Disruption was confirmed by genomic Southern blot and loss of expression was confirmed by a developmental northern blot hybridized with a full-length probe to ZAK2.

GSK3 kinase assay

The GSK3 peptide kinase assays and in vitro phosphorylation were as described previously (Kim et al., 2002; Kim et al., 1999). Developing organisms with cell-specific lacZ reporter plasmids were fixed and stained as described previously (Richardson et al., 1994). Relevant DictyBase gene numbers are DDB0185150 for GSK3, DDB0185184 for ZAK1 and DDB0229958 for ZAK2.

Prespore and prestalk A cell purifications

Wild-type cells expressing GFP with either the cotB or ecmA promoters were developed on the nitrocellulose filter for 15 hours. Slugs were harvested and mechanically dissociated in PBS and 40 mM EDTA (Chen et al., 2004). Cells were washed, resuspended at 10^6 cells/ml and sorted by fluorescence intensity using the FACS VantageSE (BD Biosciences). Cells with strongest signal (<5%) were positively selected as prespore specific (Chen et al., 2004). The positive and negative pools were confirmed by fluorescence microscopy and RT-PCR using cotB primers.

Sporulation in chimeras

Wild-type and zak2-null cells expressing act15/lacZ were grown and mixed at varying cells ratios with growing unmarked wild-type zak2-null cells. Cell mixes were plated for development and sori collected from terminal structures and disrupted. Cells were visualized under bright-field and fluorescence microscopy. Mature spores exhibited characteristic ovoid and phase bright properties.

Chemotaxis

For submerged experiments, log phase cells were analyzed at various cell densities. After 12 hours at 20°C, cell migration, streaming and aggregation were scored. For chemotaxis, log phase cells were differentiated with 50 nM pulses of cAMP for 7 hours and plated at 6 x 10^4 cells/cm^2. An Eppendorf Patchman micromanipulator with a glass capillary needle (Eppendorf Femtopip) filled with 100 nM cAMP solution was used to test cell responses, followed by time-lapse, digital recording and analysis by DIAS (Brzostowski et al., 2004; Soll, 1999).

RESULTS

Tyrosine kinase ZAK2 regulates GSK3 and cell pattern formation

We previously described the screening of bacterial expression libraries with anti-phosphotyrosine sera to isolate cDNAs for Dictyostelium tyrosine kinases (Kim et al., 1999). The procedure yielded multiple tyrosine kinases. We characterized the biochemical and cellular properties of tyrosine kinase ZAK1 in considerable detail (Kim et al., 2002; Kim et al., 1999) and also identified another tyrosine kinase, ZAK2, that has a very high amino acid sequence identity with ZAK1 (Fig. 1). ZAK2 was independently identified as DPYK4 (Adler et al., 1996), but its sequence was only partially characterized. Given its sequence and functional kinship with ZAK1, we suggest that ZAK2 is a more consistent nomenclature.

Both ZAK2 and ZAK1 contain an N-terminal serine/threonine-type (PSK) kinase domain, as well as a separate C-terminal tyrosine kinase (PTK) domain (Fig. 1A). Both domains possess all the conserved residues essential for their specific activities. This distinguishes the ZAK tyrosine kinase group from that of the Janus Kinases (JAKs). JAKs have active C-terminal PTKs, but inactive N-terminal PSKs (Hou et al., 2002). The PTK and PSK domains of ZAK1 and ZAK2 are highly related (Fig. 1A), although there is an AAT, tri-nucleotide expansion in ZAK1 that is absent in ZAK2. This repeat encodes an asparagine block [N(40)] within the PSK activation loop of ZAK1 that causes low activity (Kim et al., 1999).

ZAK2 is expressed during the growth phase of Dictyostelium and throughout the major developmental stages (Fig. 1B). To assess the role of ZAK2 during the Dictyostelium life cycle, we disrupted the ZAK2 gene by homologous recombination. ZAK2 disruption was confirmed by genomic Southern (data not shown) and northern blot hybridizations (Fig. 1D) and RT-PCR (Fig. 1C) using gene-specific ZAK2 and ZAK1 probes.

We compared the development of wild-type and zak2-null cells. Gross morphology is largely similar between the two strains through ~12 hours of development. However, as differentiation and morphogenesis proceed, we observed dramatic defects in zak2 nulls. The terminal fruiting body structure of wild-type cells is characterized by a sorus, or large spore mass, atop an elongated stalk of vacuolated cells. Terminally differentiated zak2 nulls do not form as prominent sori and exhibit expanded stalk structures (Fig. 1D), indicating that ZAK2 plays a central and reciprocal role in control of spore and stalk cell differentiation. These morphological characteristics are similar to those of zak1 and gsk3 nulls, and suggest that ZAK2, like ZAK1 and GSK3, is required in a pathway for spore formation, and perhaps suppresses stalk cell differentiation (Kim et al., 1999). The sequence, structural and functional similarities between ZAK1 and ZAK2 also suggest that GSK3 is a regulatory target of ZAK2 in the control of development.

Dictyostelium and mammalian GSK3s have high sequence identity and are identically phosphorylated by ZAK1 in vitro (Kim et al., 2002; Kim et al., 1999). We, therefore, examined if rabbit
We next compared the relative expression of cell specific patterns of gene expression development. GSK3β were also tyrosine phosphorylated by the purified, bacterially expressed tyrosine kinase domain of ZAK2. GSK3β was incubated with or without ZAK2 in the presence of γ-32P]ATP and tyrosine-specific phosphorylation monitored (Fig. 2A). Although there is an inherent tyrosine auto-phosphorylation activity in GSK3β, this assay has proven effective for identifying tyrosine trans-phosphorylation of GSK3 by heterologous protein tyrosine kinases (Kim et al., 1999). Indeed, GSK3 tyrosine phosphorylation is significantly enhanced by the presence of ZAK2 similar to that observed with ZAK1 (Kim et al., 1999).

The ability of ZAK2 to phosphorylate GSK3 in vitro suggests that ZAK2 also regulates GSK3 in vivo. We, thus, examined the kinase activity of GSK3 throughout development of zak2 nulls. Wild-type, zak2-null and gsk3-null cells were developed and whole-cell extracts prepared at representative time points. GSK3 kinase activity was measured (Fig. 2B) using a pre-phosphorylated peptide that behaves as a GSK3-specific substrate (Plyte et al., 1999); activity was normalized to GSK3 levels by western blotting using α-GSK3 antibody. As has been previously observed (Kim et al., 2002; Kim et al., 1999; Plyte et al., 1999), wild-type cells exhibit a twofold activation of normalized GSK3 activity during development. GSK3 activation during development was significantly compromised in zak2 nulls compared with wild type. We had previously shown that zak1 nulls also had comparably reduced levels of GSK3 activation during development (Kim et al., 1999). The data indicate that the remaining elevated GSK3 activity observed in zak2 nulls is the result of ZAK1 expression and its ability to phosphorylate and activate GSK3; unfortunately, we were unable to establish a cell line that was deficient for both ZAK1 and ZAK2. Nonetheless, these data suggest that ZAK1 and ZAK2 function collectively to regulate GSK3 during Dictyostelium development.

Cell specific patterns of gene expression

We next compared the relative expression of ZAK2 and ZAK1 mRNAs in prespore and prestalk cells, the major progenitor cell types of Dictyostelium development. Specificity and relative efficiency of ZAK2 and ZAK1 mRNA amplification by RT-PCR was established using RNA from growing wild-type, zak2-null and zak1-null strains as controls (see Fig. 1C).

Prespore cells were isolated by FACS from a population of differentiated Dictyostelium that expressed GFP from the prespore-specific cotB promoter (cotB/GFP cells), and relative levels of prespore ZAK2 and ZAK1 mRNA were assayed by semi-quantitative RT-PCR (Fig. 3A). We show that ZAK1 mRNA Fig. 1. Tyrosine kinase ZAK2 has a high sequence identity with ZAK1 and similarly regulates cell fate decisions. (A) A schematic diagram of the molecular architectures of ZAK2 and ZAK1. Both kinases possess an N-terminal serine/threonine kinase (PSK) domain, a short linker region and a C-terminal tyrosine kinase (PYK) domain. Asparagine repeat-block (N) in the activation loop of ZAK1 does not exist in ZAK2. (B) ZAK2 is expressed throughout development. Equal amounts of total RNA for each developmental stage were analyzed by northern blot using a ZAK2-specific probe. (C) ZAK2 mRNA is not expressed in ZAK2 mutant cells. RNA was prepared from growing wild-type, zak1-null and zak2-null cells, and ZAK1- and ZAK2-specific primers were used for RT-PCR. (D) ZAK2 mRNA is not expressed in ZAK2 mutant cells. Equal amounts of total RNA for each developmental stage of zak2-null cells and from growing (0 hours). Wild-type cells were analyzed by northern blot using a full-length ZAK2 probe. (E) Terminal differentiation (arrows) of wild-type and zak2-null strains after plating on bacterial lawns for 10 days.

Fig. 2. GSK3 is phosphorylated and activated by ZAK2. (A) Purified mammalian GSK3β was incubated in the absence (−) or presence (+) of purified, recombinant ZAK2 tyrosine kinase domain and γ-32P]ATP, and then analyzed by gel electrophoreses. SDS gels were then incubated with 1 N KOH at 55°C for 90 minutes, which hydrolyzes phosphoserine and phosphothreonine, but does not affect phosphotyrosine (Kim et al., 1999). (B) Whole-cell lysates were prepared from wild-type, zak2-null and gsk3-null cells at each developmental stage, and GSK3 kinase activities were measured using a GSK3-specific peptide substrate. Values were normalized by western blot assay using α-GSK3 (lower panel). Results are mean±s.e.m.
expression in prespore cells is enriched ~10-fold in comparison with ZAK2 mRNA. The quality of the RT-PCR design was confirmed using sequentially diluted prespore RNA samples to amplify mRNAs from ZAK1 and the prespore marker pspA (Fig. 3B). In agreement with these conclusions, recently published data of global gene expression in Dictyostelium using RNA-seq (Parikh et al., 2010) predict more than a 10-fold enrichment of ZAK1 mRNA sequences in prespore cells in comparison with ZAK2 (pyk4) mRNA in prespore cells at the threshold of RNA-seq detection.

Relative quantification of ZAK2 and ZAK1 expression in prestalk cells is technically more complex. In agreement with RNA-seq analyses (Parikh et al., 2010), ZAK2 and ZAK1 mRNA levels are very similar in the total prestalk cell population (Fig. 3C). However, differences are observed in the subpopulations of prestalk (pst) cells, which are characterized by distinct gene expression patterns. The pstA cells are defined by the presence of ecmA mRNA expression and represent the largest pst cell population. pstA cells were isolated by FACS from a population of differentiated Dictyostelium expressing GFP from the ecmA promoter (ecmA/GFP cells), and relative levels of ZAK1 and ZAK2 mRNA assayed by semi-quantitative RT-PCR (Fig. 3D). Here, we see that ZAK2 expression in the pstA cells is significantly enriched in comparison with that of ZAK1; the prestalk ZAK1 mRNA expression is, thus, primarily restricted to cells that do not express ecmA (e.g. prestalk B cells). Although we were unable to obtain a sufficiently pure population of pstB cells for analyses, these conclusions are consistent with our previous data that indicate that ZAK1 has a significant function in the regulation of ecmB expression, but not of ecmA gene expression (Kim et al., 1999).

ZAK1 and ZAK2 regulate distinct prestalk subpopulations

Despite the restriction of ZAK2 and ZAK1 expression to different pst subpopulations, the similarity of the terminal phenotypes of zak1- and zak2-null cells, and their shared capacity to regulate GSK3 activity during late development suggest some commonality in control of development. ZAK1 activation of GSK3 suppresses prestalk differentiation, but promotes prespore differentiation (Kim et al., 1999). Data from Fig. 1D suggest that zak2 nulls are also hyperactive for stalk differentiation. We, thus, investigated whether ZAK2, like ZAK1, was involved in a signaling pathway that represses stalk differentiation. Dictyostelium will form stalk cells in monolayer culture when treated with the DIF-1 inducing factor, but stalk differentiation is inhibited by cAMP (Berks and Kay, 1988). The majority of wild-type, zak2-null and zak1-null cells form stalk cells in monolayer culture in the presence of DIF-1 (Fig. 4A). However, only wild-type cells were sensitive to inhibition by cAMP (Fig. 3A). zak2, zak1 (Kim et al., 1999) and gsk3 nulls (Harwood et al., 1995; Schilde et al., 2004) were all resistant to cAMP inhibition of stalk differentiation (Fig. 4A), indicating a related signaling path.

Multicellular development of Dictyostelium is characterized by specific cell pattern formation. At the slug stage, the differentiated cells are organized along an anterior-posterior axis, with the anterior zone comprised primarily of prestalk cells; the prespore cells dominate the posterior region. We were interested to determine the roles of ZAK1 and ZAK2 in the spatial regulation of gene expression in the two primary prestalk sub-groups, prestalk A (pstA) and prestalk B (pstB) cells, which are distinguished, respectively, by the expression patterns of ecmA and ecmB (Gaudet et al., 2008; Williams, 2006).

In wild-type cells, ecmA (as visualized with a lacZ reporter) is expressed throughout the entire anterior zone (Fig. 4B), with minimal expression through the posterior of the slug. ecmB expression is restricted to a narrow cone within the center of the anterior prestalk region (Fig. 4C). gsk3 nulls have aberrant patterning of both prestalk subtypes (Harwood et al., 1995; Schilde et al., 2004). In gsk3 nulls, both ecmA and ecmB expression is expanded from the anterior zone throughout the posterior region (Fig. 4B,C). zak2 nulls have a disrupted ecmA expression pattern that is similar to that of gsk3 nulls, but an ecmB pattern like that of wild type (Fig. 4B,C). By contrast, zak1 nulls have a completely reciprocal phenotype (Fig. 4B,C); ecmB expression in zak1 nulls is expanded, as with gsk3 nulls, but ecmA expression is similar to the wild-type pattern. Thus, although ZAK2 and ZAK1 both negatively regulate prestalk differentiation, they are selective for the different subtypes: the pstA gene ZAK2, which is preferentially expressed in pstA cells, regulates expression of ecmA, while the ZAK1 regulates expression of ecmB and is preferentially expressed in pstB cells.

We next examined the role of ZAK2 on overall levels of prestalk gene expression (Fig. 5A). Wild-type and zak2-null cells were differentiated in shaking culture under conditions where exogenous CAMP induces the expression of prestalk genes ecmA and ecmB, and prespore genes pspA and cotB. RNA was isolated prior to and post cAMP-induction and probed on northern blots. Consistent with the expansion of ecmA-expressing cells in zak2 nulls, we see a dramatic increase in global levels of ecmA mRNA compared with wild type or internally normalized to ecmB expression (Fig. 5A), highlighting the preferential role of ZAK2 in prestalk A determination. We conclude that ZAK1 and ZAK2 regulate distinct prestalk cell pathways. In addition, zak2 nulls correspondingly exhibited a dramatic relative reduction in prespore gene expression (Fig. 5A), in agreement with the reduction of spore differentiation during normal development (Fig. 1D).

Although ZAK2 and ZAK1 expression is enriched in different pst populations, we have postulated that they have similar mechanistic kinase functions. We were, thus, interested to determine whether...
ZAK1 could substitute for ZAK2 in pstA cells. zak2 nulls were engineered to overexpress ZAK1 using the ecmA promoter (zak2 [ecmA/ZAK1] cells). ZAK1 expression would track the induction of endogenous ecmA gene expression. Cells were developed on solid substrata and ecmA mRNA expression was monitored by northern blot assay. Expression level differences between parental and engineered strains are not always easily interpretable. However, clear temporal differences between the zak2 and zak2 [ecmA/ZAK1] strains are observed. The zak2-null strain shows continuous accumulation of ecmA mRNA through late development. However, once ZAK1 becomes induced in the zak2 [ecmA/ZAK1] cells, ecmA expression is rapidly repressed.

Fig. 5. ZAK2 regulation of gene expression in prestalk A and prespore cells. (A) mRNA expression patterns. Log phase wild-type and zak2-null cells were washed, resuspended, treated with 50 nM cAMP pulses for 5 hours, and then incubated with 300 μM cAMP for 4 hours. RNA was isolated before and after treatment with 300 μM cAMP. ecmA and ecmB are prestalk- and cotB and pspA are prespore-specific markers used for northern analyses. (B) ZAK1 can substitute for ZAK2 in control of ecmA expression. zak2 nulls and zak2 nulls expressing ecmA/ZAK1 (zak2 [ecmA/ZAK1]) were developed on solid substrata and ecmA and pspA expression was determined by northern blot hybridization.

(Zig Fig. 5B). Thus, ZAK1 is functionally equivalent to ZAK2 to negatively regulate ecmA. In parallel, we show that prespore pspA gene expression is not disrupted in ZAK1-expressing cells. Data may suggest that overexpression of ZAK1 in pstA cells promotes prespore gene expression (see below).

ZAK2 regulates prespore/spore differentiation

Although ZAK2 is expressed very poorly in prespore cells, data from Fig. 1D and Fig. 5A nonetheless indicate that ZAK2 regulates prespore/spore differentiation. To examine this directly, we differentiated wild-type and zak1-null cells into spores in monolayer culture at low cell density. Under these conditions, cell-cell interactions are minimized and spore formation requires stimulation by 8-Br-cAMP (Kay, 1987). Although wild-type cells form spores at a high efficiency when treated with 8-Br-cAMP, spore differentiation of zak2-nulls is largely refractory (Fig. 6A). Similar results were observed with zak1 nulls (Fig. 5A) (Kim et al., 1999), as well as with gsk3 nulls (Harwood et al., 1995; Kim et al., 1999; Schilde et al., 2004).

Developmental northern blot analyses were used to examine expression of the prespore-specific makers pspA and cotB throughout the developmental cycle of *Dictyostelium* on solid substrata. Compared with wild type, zak2 nulls have severely reduced expression of both prespore genes during development on solid substrata (Fig. 6B), data that are qualitatively similar to that observed when cells are differentiated in shaking culture using exogenous cAMP (Fig. 5A). These data are also similar and consistent with that previously observed for zak1- and gsk3-null strains (Harwood et al., 1995; Kim et al., 1999). Thus, ZAK2, ZAK1, and GSK3 are required to direct prespore/spore fates during *Dictyostelium* development.

Non-autonomous regulation of prespore/spore differentiation by ZAK2

We have previously shown that kinase-inactive variants of GSK3 (GSK3-DN) will inhibit endogenous GSK3 signaling and function as dominant-negative mutants (Kim et al., 2002; Kim et al., 1999). We were interested to determine how disruption of GSK3-signaling in pstA or psp cells impacted developmentally regulated gene in all cells.
car4-nulls exhibit enhanced prespore gene expression and reduction in expression of prestalk markers (Fig. 6C) (Ginsburg and Kimmel, 1997), but ubiquitous expression of GSK3-DN using the act15 promoter reverses these developmental patterns; prestalk ecmA gene expression is activated, whereas expression of prespore markers psA and cotB is repressed (Fig. 6C) (Kim et al., 2002; Kim et al., 1999). Not surprisingly, a similar phenotype is observed when GSK3 signaling is specifically inhibited in prespore cells using cotB/GSK3-DN. Prespore gene expression (i.e. pspA and cotB) is inhibited and there is a cell-autonomous enhancement of prestalk ecmA gene expression and parallel decrease in prespore gene expression (Fig. 6C) (Kim et al., 1999).

To mimic the specific inhibition of ZAK2 function in pstA cells, we used the ecmA/DN-GSK3 construct (Fig. 6C). As expected, inhibition of GSK3 signaling in prestalk A cells autonomously activated ecmA expression, but remarkably, specific inhibition of GSK3 in prestalk A cells also caused the repression of prespore genes. It is well established that prespore/spore differentiation is regulated through an interplay of cell-autonomous and non-autonomous signaling (Aubry and Firtel, 1999; Kimmel, 2005; Kimmel and Firtel, 2004). The ecmA/DN-GSK3 results suggest a potential function for ZAK2/GSK3 to regulate a non-autonomous pathway for prespore/spore differentiation that is mediated by the action of pstA cells. These conclusions are consistent with the observed expression pattern of ZAK2, which has relatively low levels of expression in prespore cells.

We next studied these postulated contributions of autonomy/non-autonomy by lineage tracing of GFP-marked cells during terminal differentiation of chimeras with varying mixtures of wild type or zak2 nulls. Approximately 80% of wild-type cells form spores during normal development, whereas sporulation efficiency of developed zak2 nulls is only ~30% (Table 1). zak2 nulls that express GFP (zak2 [act15/GFP]) sporulate at similarly poor efficiencies (~35%) during chimeric development with a large (~40-fold) excess of unmarked zak2-nulls (Table 1). However, when GFP-marked zak2-null cells are co-developed with unmarked wild-type cells, the zak2-null sporulation efficiency was enhanced approximately twofold (Table 1). When normalized (Table 1), these sporulation efficiencies approximate those of wild type and suggest strongly that ZAK2 in wild-type cells regulates a non-autonomous pathway that stimulates the spore differentiation of cells that lack ZAK2.

We were also interested to determine the potential impact on wild-type sporulation in the presence of a large population of cells that lack ZAK2. In controls, GFP-marked wild-type cells (WT[act15/GFP]) sporulate at very high efficiency (~80%), but when mixed with a 90-fold excess of zak2 nulls, wild-type sporulation is reproducibly (P<0.01) suppressed by ~25-30% (Table 1). These data are consistent with a dependency of wild-type sporulation on a ZAK2-regulated, non-autonomous signaling pathway.

**Table 1. Non-autonomous regulation of spore differentiation by ZAK2**

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<th>% Spores</th>
<th>% GFP cells as spores</th>
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<th>% Spores</th>
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<th>Relative wild-type sporulation efficiencies</th>
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<td>31±3%</td>
<td>61±3% (B)</td>
<td>0.76 in zak2 (P&lt;0.01)</td>
</tr>
</tbody>
</table>

*Numbers derive from a minimum of 500 cells and multiple experiments and are expressed as means±s.e.m. Values A and B are used to calculate relative sporulation efficiencies.

Relax sporulation efficiency for control, GFP cells within each homogeneous [(GFP-zak2)+zak2] or [(GFP-wild type)+wild type] mixture is set at 1.00.

Relative sporulation efficiency for GFP cells within each heterologous [(GFP-zak2)+wild type] or [(GFP-wild type)+zak2] mixture is calculated as B/A (see ¹ above). NA, not applicable.
Still, the wild-type sporulation values in the presence of \textit{zak2} nulls are not fully suppressed to that of \textit{zak2} nulls. Although we suggest that ZAK2 functions significantly in a non-autonomous pathway for prespore differentiation, ZAK2 in wild-type cells probably has an inherent, cell-autonomous potential for spore differentiation. ZAK2 is expressed during growth and early development, and we do not exclude the possibility that ZAK2 can function in an autonomous pathway during the initial phases of prespore/pseudopore fate choice.

**Tyrosine phosphorylation of GSK3 regulates cell polarity**

In addition to its role in regulating cell differentiation, cAMP is required to organize multi-cell formation during early \textit{Dictyostelium} development. Although a dependent role for GSK3 has not been evaluated, the recent linkage of GSK3 function to cell polarization and migration (Ciani et al., 2004; Eickholt et al., 2002; Etienne-Manneville and Hall, 2003a; Gartner et al., 2006; Jiang et al., 2005; Schlessinger et al., 2007; Shi et al., 2004; Yoshimura et al., 2005; Zhou et al., 2004) makes this an interesting line of investigation.

Directional cell migration constitutes an essential and early stage for multicellular development in \textit{Dictyostelium}. During early development, cells polarize and migrate directionally toward signaling centers that produce cAMP. Cells ‘stream’ in coordinated groupings toward these cAMP centers and aggregate into multicellular structures, the precursors for later development. The ability of cells to stream and aggregate at low cell density is characteristic of their capacity to polarize directionally and chemotax towards the cAMP signal. During careful comparison of the early developmental phenotypes of wild-type, \textit{gsk3-null}, \textit{zak1-null} and \textit{zak2-null} cells, we noticed differences in the ability of cells to form multicellular aggregates.

To examine aggregation more directly, cells were placed in submerged culture at varying cell densities. Wild-type and \textit{zak2-null} cells establish territorial streams at densities as low as \(2 \times 10^4\) cells/cm\(^2\) (Fig. 7A), ~1000 times lower cell density than that normally used during standard development on solid surfaces. However, \textit{gsk3} and \textit{zak1} nulls were both dramatically defective in directional cell movement and in chemotactic streaming at these cell densities. Time-lapse imaging confirmed that \textit{gsk3} and \textit{zak1} nulls do not coalesce by forming migration streams, but rather aggregate through random cell collision and adhesion (Fig. 7A).

We also examined the functional link between GSK3 and ZAK1 in chemotaxis and aggregation using \textit{gsk3} nulls that express either the GSK3 wild-type form or the Y214F variant (Kim et al., 2002). GSK3\(^{3214F}\) is not phosphorylated or activated by ZAK1, has an inherent low activity compared with wild type, and acts as a partial dominant-negative during \textit{Dictyostelium} development (Kim et al., 2002). At a density of \(10^4\) cells/cm\(^2\) (Fig. 7B), \textit{gsk3} nulls are unable to establish territorial streams. Re-expression of wild-type GSK3 in \textit{gsk3-null} results in a near complete rescue of chemotaxis in submerged cultures (Fig. 7B), whereas cells that express GSK3\(^{3214F}\) remain defective (Fig. 7B).

Chemotactic aggregation is dependent upon both signal production and signal response. We therefore examined whether cells responded to an exogenous cAMP gradient. Consistent with the streaming/aggregation data (Fig. 7A,B), wild-type and \textit{zak2-null} cells became highly polarized and migrated robustly toward a cAMP point source. By contrast, the \textit{gsk3} and \textit{zak1} nulls had extreme defects in polarity organization and in chemotactic movement (Fig. 7C), indicating a defect in chemotactic response.

To characterize the chemotactic defects more precisely, we dynamically imaged cellular movement toward a cAMP point source using DIAS software (Fig. 8). Chemotaxing wild-type cells are highly polarized and migrate in a directed manner (Fig. 8).
Wild-type cells have a dominant anterior pseudopod that is regulated by F-actin polymerization and that extends persistently in the direction of the gradient; myosin II (myo II) assembly at the posterior and sides of the cells suppresses spurious lateral pseudopod formation and promotes contraction towards the gradient source (see Kimmel and Parent, 2003; Kimmel et al., 2004). By contrast, zak1 nulls are very poorly polarized and their movement is characterized by the continuous formation of lateral pseudopods that often directs cells away from the gradient source (Fig. 8). As would be expected if GSK3 were in a regulatory path for chemotaxis downstream of ZAK1, gsk3 nulls exhibit the most severe defects in migration (Fig. 8). gsk3 nulls are minimally polarized and have highly restricted directional movement. They exhibit significant lateral pseudopod extension and difficulty in retraction of the cell posterior, a phenotype that further supports a role for ZAK1/GSK3 in control of polarity. By contrast, zak2 nulls polarize and migrate well towards a cAMP source.

**DISCUSSION**

The terminal structure of asexual development of *Dictyostelium* is comprised of a sorus (a spore mass) atop a stalk of vacuolated cells. Stalk-like cells also form the base of the organism and the ‘cups’ that surround the sorus. These terminally differentiated cells derive from non-committed prespore and prestalk precursors. The pioneering work of Williams, Kay, Firtel and others have shown that progenitors to both prestalk and prespore populations are heterogeneous (Gaudet et al., 2008; Williams, 2006; Yamada et al., 2010). Spatially restricted expression of gene patterns has been used to define very specific classes of prestalk cells (e.g. pstA, pstB, pstO and pstU cells). Here, we have focused on three main progenitor classes, prestalk A, prestalk B and prespore cells, but further subdivisions within each can be made (Gaudet et al., 2008; Kimmel, 2005; Kimmel and Firtel, 2004; Williams, 2006). We show that regulation of tyrosine phosphorylation of GSK3 in *Dictyostelium* underscores a paradigm for cellular differentiation and chemotactic response. Thus, aspects of development from the formation of multicellular structures through terminal differentiation are dependent upon the precise regulation of GSK3.

Prestalk A cells express *ecmA* and differentiation is negatively regulated autonomously by a ZAK2/GSK3 pathway. Loss of ZAK2 or GSK3 or expression of the kinase-inactive GSK3 (DN-GSK3) in prestalk A cells increases *ecmA* expression (Fig. 4B, Fig. 5B, Fig. 6C). Although overexpression of ZAK1 in prestalk A cells of zak2 nulls represses *ecmA* expression (Fig. 5B), ZAK1 normally has a limited function in prestalk A differentiation (Fig. 3D, Fig. 4B). Conversely, prestalk B differentiation is negatively regulated autonomously by ZAK1/GSK3. Loss of ZAK1 or GSK3 expands *ecmB* expression (Fig. 4C) and relieves inhibition by cAMP (Kim et al., 1999). Although re-expression of ZAK1 in prestalk B cells of zak1 nulls represses *ecmB* expression, *ecmB* expression remains elevated in gsk3 nulls that overexpress ZAK1 in prestalk B cells (Kim et al., 1999).

cAMP inhibition of stalk differentiation in monolayer culture has been previously shown to require functional GSK3 signaling. We suggest that zak2 nulls cells preferentially differentiate into a prestalk A population in these monolayers. As these zak2 nulls cells would be unable to activate GSK3-signaling, they would be insensitive to inhibition by cAMP. Likewise, pstB cells may be the most dominant population during differentiation of the zak1 null cells; they would also lack normal GSK3 signaling and would also be insensitive to inhibition by cAMP.

Prespore differentiation is positively regulated autonomously by ZAK1/GSK3. Loss of ZAK1 or GSK3 diminishes *pspA* and *cotB* expression (Kim et al., 1999). Expression of DN-GSK3 in wild-type prespore cells represses *cotB* expression (Fig. 6C). Re-expression of ZAK1 in prespore cells of zak1 nulls induces *cotB*; however, *cotB* expression remains low in gsk3 nulls that overexpress ZAK1 in prespore cells (Kim et al., 1999), indicating that GSK3 is required for ZAK1 regulation of prespore gene expression. Prespore differentiation is also positively regulated, but non-autonomously via the ZAK2/GSK3 pathway in prestalk A cells. ZAK2 is expressed at relatively low levels in prespore cells, but loss of ZAK2 or expression of DN-GSK3 in prestalk A cells represses *cotB* expression (Figs 5, 6). The non-autonomous regulation of prespore differentiation by ZAK2/GSK3 signaling was substantiated by comparing relative efficiencies of sporulation during chimeric development of wild-type and zak2 null cells. However, as ZAK2 is expressed in cells prior to the prespore differentiation, ZAK2 may function autonomously during the initial phases cell differentiation.

The loss of GSK3 signaling not only impairs cell fate determination, but also chemotactic movement. However, data indicate that these are functionally separate events. zak1 nulls, which have severe defects in cAMP-mediated chemotaxis, establish proper anterior prestalk A patterning, whereas prestalk A patterning is aberrant in chemotactically competent zak2 nulls. These data argue strongly that defects in cell pattern formation, as a consequence of restricted GSK3 signaling, do not result from simple abnormalities in chemotaxis towards cAMP.

Proteomic and microarray data indicate that ZAK1/GSK3 signaling regulates certain gene expression patterns during early *Dictyostelium* development (Strmecki et al., 2007). However, transcriptional misregulation in zak1- and gsk3-null cells is unlikely
to account for the entirety of their chemotaxis defects. Indeed a
cotemporaneous study suggests that multiple chemoattractant
signaling pathways are disrupted in Dictyostelium that lack GSK3
(Toe et al., 2010).

A common GSK3-dependent pathway that regulates both cell
fate determination and cell polarity as in Dictyostelium may not be
shared broadly among the metazoa. Canonical Wnt signaling is
mediated by the effective inhibition of GSK3, although regulation
is not by alterations in enzymatic activity as in Dictyostelium,
but rather by disassembly of GSK3/substrate complexes (Angers
and Moon, 2009; McNeill and Wooodgett, 2010). Although Wnts can
also direct planar cell polarity, the mechanisms involved are
distinct from canonical signaling and do not generally involve
coordinated GSK3 regulation.

Still, changes in GSK3 activity per se can influence cell
polarization and specification in some developmental contexts.
Mis-expression of GSK3 in Drosophila directs polarity defects
(Tomlinson et al., 1997). In addition, localized inhibition of
GSK3β can establish and maintain neuronal specification by
altering microtubule assembly that can polarize the formation
of axons and dendrites (Ciani et al., 2004; Eickholt et al., 2002;
Gartner et al., 2006; Jiang et al., 2005; Schlessinger et al., 2007;
Shi et al., 2004; Yoshimura et al., 2005; Zhou et al., 2004).
Recent data indicate that a novel GSK3-mediated pathway
involving Wnt signaling can polarize the microtubule
cytoskeleton (Schlessinger et al., 2007). Microtubule polarization
in C. elegans is similarly specified by Wnt regulation of GSK3
(Hardin and King, 2008; Schlessinger et al., 1999; Walston et al.,
2004).

Although neuronal and astrocyte polarization and chemotactica
cell movement in Dictyostelium appear unrelated, their shared
dependence upon GSK3 may suggest some mechanistic
commonality. Persistent directed movement of Dictyostelium
toward a chemoattractant source involves antagonistic activities
that localize specifically to the leading edge or rear of the
chemotaxing cell (Kay et al., 2008; Kolsch et al., 2008; McMain
et al., 2008). Continuous cell polarization is required to maintain
directionality and to suppress spurious lateral movement away
from the directional signal.

Non-autonomous regulation of cell patterning has been widely
observed throughout Dictyostelium developmental cycle. The
most mechanistically well described pathway involves prestalk
control of a PKA-activity cascade within prespore cells that
drives sporulation (Anjard and Loomis, 2005; Cabral et al.,
2008; Wang et al., 1999). Prespore cells produce and secrete the
SDF-2 (spore differentiation factor) precursor AcbA, which is
inactive. Full-length AcbA is 84 amino acids, but is processed
into the small active SDF-2 peptide by prestalk-specific
transmembrane proteases. SDF-2 binding to DkHA surface
receptors on prespore cells initiates the pathway for PKA
activation and sporulation. Dictyostelium that lack the prestalk
proteases cannot sporulate (Anjard and Loomis, 2005; Cabral et
al., 2006; Wang et al., 1999). The non-autonomous role of ZAK2
in control of sporulation is more subtle. Subpolarity persists in
cells lacking ZAK2, but to a significantly lesser degree
compared with wild-type controls. The defect, however, is
primarily by prestalk A marker, the data suggest the importance
for narrow regulation of prestalk function. Expansion of prestalk
cells may interfere with the regulatory balance that helps define
normal prespore/prestalk ratios in Dictyostelium that are optimal
for terminal differentiation.

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