Glycogen Synthase Kinase-3 Is Required for Efficient Dictyostelium Chemotaxis

Regina Teo,* Kimberley J. Lewis,† Josephine E. Forde,‡ W. Jonathan Ryves,* Jonathan V. Reddy,* Benjamin J. Rogers,* and Adrian J. Harwood*

*Cardiff School of Biosciences, Cardiff University, CF10 3AX Cardiff, United Kingdom; †Cardiff School of Medicine, Cardiff University, CF14 4XW Cardiff, United Kingdom; and ‡Swansea University, SA2 8PP Swansea, United Kingdom

Submitted October 26, 2009; Revised May 25, 2010; Accepted June 1, 2010
Monitoring Editor: Carole Parent

Glycogen synthase kinase-3 (GSK3) is a highly conserved protein kinase that is involved in several important cell signaling pathways and is associated with a range of medical conditions. Previous studies indicated a major role of the Dictyostelium homologue of GSK3 (gskA) in cell fate determination during morphogenesis of the fruiting body; however, transcriptomic and proteomic studies have suggested that GSK3 regulates gene expression much earlier during Dictyostelium development. To investigate a potential earlier role of GskA, we examined the effects of loss of gskA on cell aggregation. We find that cells lacking gskA exhibit poor chemotaxis toward cAMP and folate. Mutants fail to activate two important regulatory signaling pathways, mediated by phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and target of rapamycin complex 2 (TORC2), which in combination are required for chemotaxis and cAMP signaling. These results indicate that GskA is required during early stages of Dictyostelium development, in which it is necessary for both chemotaxis and cell signaling.

INTRODUCTION

Glycogen synthase kinase-3 (GSK3) is a multifunctional protein kinase that is highly conserved throughout the eukaryotes. In animals, including humans, it is associated with important embryological and physiological signaling processes, such as Wnt and insulin signaling (Cross et al., 1995; Papkoff and Aikawa, 1998). As a consequence it is linked to major clinical conditions, including cancer, diabetes, and Alzheimer’s disease. At the cellular level GSK3 mediates processes, such as regulation of gene expression, control of proteolysis, metabolism, and control of the cytoskeleton (Harwood, 2001; Kim et al., 2002). The question of how GSK3 exerts its effects on these multiple processes is important for understanding the integration of cell signaling within the cellular context.

The social amoeba Dictyostelium grows and divides in the presence of nutrients in a unicellular form, but when starved it undergoes a developmental program to form a multicellular and differentiated structure, termed the fruiting body (Harwood, 2001). Dictyostelium development comprises of two major phases. First, during aggregation phase, cells migrate together by chemotaxis in a process mediated by cAMP pulses. Second, the multicellular aggregate, or mound, undertakes a series of differentiation events and morphogenetic movements to ultimately form a fruiting body, which comprises spore cells within a spherical head, supported by a stalk of vacuolated, cellulose encased stalk cells.

Dictyostelium possesses a single homologue of GSK3, gskA (Harwood et al., 1995). It was discovered previously that GskA regulates the processes that control cell fate (Harwood et al., 1995). This initially occurs during early stages of multicellular development, when cells first come together to form a multicellular mound. At this point, cells differentiate into three basic cell types; prespore cells that eventually form spores, and prestalk cells (pst) comprising pstA cells that give rise to the stalk and the majority of its associated structures and pstB cells that form the basal disk that anchors the stalk to the substratum (Williams et al., 1989). In wild-type cells, 80% of the cells form prespore cells and the remainder form pst cells, of which the majority are pstA. However, in a gskA null mutant, the pstB population expands at the expense of the prespore population, so that the final fruiting body of the basal disk is greatly enlarged and the spore head reduced (Harwood et al., 1995). During later stages of fruiting body formation, GskA regulates a second cell fate decision by controlling the differentiation from pstA cells to pstAB cells (Schilde et al., 2004), which progress to form the main structural element of the stalk.

In the mound, activation of GskA is mediated by high concentrations of cAMP via the cAMP receptors carC and carD, which control tyrosine phosphorylation of GskA by the kinase ZakA (Kim et al., 1999) and an unidentified tyrosine phosphatase (Kim et al., 2002). Downstream of GskA lies a β-catenin homologue, Aardvark (Aar; Grimson et al., 2000), and a signal transducer and activator of transcription (STAT) transcription factor, STATa (Ginger et al., 2000), although the full role of these proteins is currently unclear. Although the phenotypes described for gskA null mutants occur during multicellular stages, GskA expression and ac-
tivity is present throughout Dictyostelium development (Plyte et al., 1999). A proteomic and microarray analysis of cells at 5 h of development, several hours before the first multicellular developmental stages, revealed ~150 genes with altered patterns of expression (Strmecki et al., 2007). Curiously, of those genes identified that had been characterized previously, all are associated with growth and aggregation phase rather than multicellular development. This raises the question of additional roles of GskA during these earlier stages of Dictyostelium development.

Here, we report an in-depth investigation of aggregation and chemotaxis of gskA null mutants. We find that gskA null mutant cells have defective chemotaxis toward cAMP and folate, a growth phase chemoattractant. The chemotaxis defect is accompanied by suppression of phosphatidylinositol 3,4,5-trisphosphate (PIP3) levels. However, we observe that loss of gskA causes a much greater effect on cAMP-mediated chemotaxis than can be accounted for by loss of PIP3 signaling alone (Lee et al., 2005; Hoeller and Kay, 2007; Takeda et al., 2007). We find that protein kinase B (PKB)R1 is not phosphorylated in gskA mutants, a protein kinase related to PKB but regulated in a PIP3-independent manner. Loss of both PIP3 signaling and PKBR1 phosphorylation has a strong effect on chemotaxis. Finally, we show that gskA null mutant cells do not generate cAMP pulses during aggregation, demonstrating that GskA plays a major role in the regulation of chemotaxis and early Dictyostelium development.

MATERIALS AND METHODS

Strains

The gskA null strain (ID Strain: DB92236114) was obtained from the Dicty Stock Centre (http://dictybase.org/) and was originally generated in the Dictyostelium discoideum strain AX2 background. The wild-type strain refers to AX2 and is the same strain denoted in Bloomfield et al. (2008). Cells were cultured axenically on plates in HL5 with glucose medium (Formedium HLG0102) before experiment. gskA null mutants were grown in media supplemented with 10 μg/ml blastidin, and gskA null transformants carrying GskA-green fluorescent protein (GFP) (gskA-GSKA) and immobilized metal assay of phosphorylation (IMPA)-GFP (gskA-IMPA) were grown in medium supplemented with 40 μg/ml G418 (catalog no. 11811–023; Invitrogen). All assays were carried at 22°C.

Plasmids

The GskA-GFP2 rescue protein for gskA was constructed by inserting the coding regions of GskA-GFP2 into pDXA GFP2 at the KmR and Nell restriction sites, such that the fusion protein was expressed under the control of a constitutive Actin15 promoter. By introducing point mutations into the coding region of the pDXA GskA-GFP2, the kinase-dead construct pDXA GskA-GFP2 K83R was made with the QuickChange site-directed mutagenesis kit (Strategene, La Jolla, CA) as per the manufacturer’s direction. The coding insert of the IMPA gene was amplified from the cDNA clone FCBP15, obtained from the Japanese D. discoideum cDNA project (Morio et al., 1998). The insert was subsequently ligated into the pTX-GFP vector.

Cell Signaling Assays

cAMP was measured as described previously (Snaar-Jagalska and Van Haastert, 1994) by using isotope dilution assay kits (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Cells were starved in KK2 buffer (6.5 mM KH2PO4, 3.8 mM KH2PO4, pH 6.2) for 5 h in shaking culture at a concentration of 1 × 106 cells/ml. To determine cAMP levels, cells were resuspended at 5 × 105 cells/ml, and 100 μl of cells was stimulated with 5 μM 2-deoxy-cAMP in the presence of 5 mM dithiothreitol. Reactions were terminated by the addition of 100 μl of 3.5% (vol/vol) perchloric acid on ice. Samples were neutralized with 50 μl of KHCO3 (50% saturated), followed by centrifugation for 2 min at 14,000 × g at 4°C. Finally, 50 μl of each sample was added to the cyclic AMP H’ assay system (GE Healthcare).

Wild-type cells transformed with the plasmid WFS8 (PHCRAC-GFP; Parent et al., 2004) were pulsed with cAMP for 5 h with an end concentration of 100 nM. Cells were subsequently stimulated by the addition of 1 μM cAMP, and protein translocation was recorded by fluorescence videomicroscope with a 60× objective.

Time-Lapse Analysis

For chemotaxis to folate, cells were grown axenically to a density of 1 × 106 and 4 × 106/cm2 cells were washed twice in 20% media/KK2 and replated in a LabTek coverglass chambered well (Nunc 155359; Nalg Nunc International, Rochester, NY) with 2 ml of 20% media/KK2. Folate (25 mM) was dissolved in 200 μl of Fektopip (52221; Sigma-Aldrich) and resuspended at 5 × 105 cells/ml, and images were recorded for every 20 s under 10× phase objective. For aggregation, 5 × 105 cells were washed and plated on KK2 agar. Images were taken every 30 s for cAMP pulse movies under 4× objective for at least 24 h. Postprocessing was carried out with Image (National Institutes of Health, Bethesda, MD). For chemotaxis, 5 × 105 cells were shaken in KK2 buffer (Formedium KK29907) for 5 h while being pulsed every 6 min with cAMP to an end concentration of 10−7 M. The chemotaxis-competent cells were placed in a Zigmond chamber (ZiO; Neuro Probe, Gaithersburg, MD) in a cAMP gradient source at 10−10 M cAMP, sink with no cAMP. Differential interference contrast images of cells were captured with a 20× objective at 6 s intervals for 15 min. Cell movement was analyzed using Dynamic Image Analysis System (DIAS), version 3.4.1 (Soil Technologies, Iowa City, IA; Curreli et al., 2001). Statistical analysis was carried out using the nonparametric Kruskal-Wallis test, with a post hoc Dunn’s multiple comparison test using Prism 4 (GraphPad Software, San Diego, CA).

Phospholipid Radiolabeling and Thin Layer Chromatography (TLC) Analysis

Cells (1 × 107) were starved and pulsed for 4 h with 100 nM cAMP. At the four-hour time-point, cells were pelleted and resuspended in 600 μl of KK2. Radioactive [γ-32P]ATP was added (1.2 MBq), and the cells were pulsed with cAMP for a further hour (100 nM/6 min). Phospholipids were extracted as described in Konig et al. (2008): cells were added to 80% methanol and acidified with A (chloroform/methanol/HCl/C150 (v/v)) and sonicated. A further 250 μl of chloroform and 450 μl of 1 M HCl were added to the cells, and then the cells were vortexed and spun, and the lower phase was collected. The process was repeated two more times, once with chloroform/methanol/HCl/C150 and the second time with methanol/EDTA (100 mM) (ratio: 1:0.9) and subsequently dried. Samples were then resuspended in chloroform/methanol (ratio: 2:1) and spotted onto a TLC plate (Cole-Parmer Instrument, Vernon Hills, IL). Plates were developed with a chloroform/acetone/methanol/acidic acid/double-distilled H2O solvent system (ratio 46:17:15:14:16) and phospholipids were visualized by spraying with molybdenum Blue spray reagent (Sigma Chemical, Poole, Dorset, United Kingdom). Radiolabeled lipid spots were imaged by exposing the plate to Hyperfilm (GE Healthcare) for 3 d. PIL, measurements were carried out according to manufacturer’s instructions (K-2500s PIL, Mass ELISA kit; Echelon Biosciences, Salt Lake City, UT). Anti-phospho protein kinase C (PKC; pan) antibody used for detection of PKB and PKBR1 was purchased from Cell Signaling Technology (190D; Danvers, MA) and analyzed as described previously (King et al., 2009).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Dictyostelium cells were starved and shaken with pulses of 100 nM cAMP for 5 h. Cells (2 × 107) were then harvested and pellets were snap-frozen. RNA was isolated using an illustra RNA spin mini kit (25-0500-72; GE Healthcare), and its integrity was checked on a 1% formaldehyde agarose gel. Genomic DNA contamination was checked by PCR using existing primers for a known gene. cDNA was prepared using the First-Strand cDNA kit (1148318801; Roche Diagnostics, Mannheim, Germany) using 1 μg of RNA and random primers. A minus-reverse transcriptase control for all genes was carried out on a Chromo4 machine (Bio-Rad laboratories, Hercules, CA) with the Opticon2 detection software. The fluorophore used for detection was SYBR Green. i SYBR Green master mix (170-8884; Bio-Rad Laboratories) was used. The reactions were performed with 100 μg of cDNA. Housekeeping genes (GAPDH, actA, carA) were used for normalization. The primers used for quantification were ig7 forward, TCCAACACTATCCATTCCTTACCATC; ig7 reverse, TCCAAGAGGAAGAGGAGAACTGC and ipmk1 reverse, AACTGCCCATGATGGATAGGT. Data analysis was carried out using the qbase software by Vandesompele and J. Hellemans (Hellemans et al., 2007). Standard curves were created for all genes by using a 3-point 10-fold dilution series of Ax2 0 h cDNA, and all samples also were normalized to the Ax2 0 h cDNA. Primer sequences are as follows: ig7 forward, TCCAAGAGGAAGAGGAGAACTGC; ig7 reverse, GCCAGAAGTCTCATTACCACTACCC; ipmk1 forward, GGCGAAGCTTACCATCCTTACCATCAC; ipmk2 forward, TGGATGATTTTTTTGATGTGCAAGCC and ipmk2 reverse, TGATGATGTTGTTGGTGTTGTTGAT; carA-1 forward, ATGTTGGGTGTTGTTGTTGTTGTTGTTGTTGTTGT and carA-1 reverse, GCAGGAAACCATCCATTGCAGAC; actA forward, CCTACTGAGGGGGTATTGCCG and actA reverse, GCCAGAAGATGTCATGGGATTG.

Vol. 21, August 1, 2010
RESULTS

gskA Null Mutants Have Aberrant Aggregation

We observed previously that gskA null mutant cells had unusual aggregation, forming small mounds ~2 h before wild-type strains. This was independent of parental background, being seen in mutants created from both DH1 and AX2 parental wild-type strains (Harwood et al., 1995; Schilde et al., 2004). Small mounds also were seen in cells treated with lithium, an inhibitor of GSK3 (Williams et al., 1999). This prompted a more in depth investigation of gskA null mutants.

To investigate the role of GskA during aggregation, wild-type and gskA null mutant cells were plated for development and recorded using time-lapse videomicroscopy. As seen previously, gskA null mutant cells formed small mounds ~2 h earlier than wild-type cells (Supplemental Movie 1). These small mounds formed without the multicellular streams seen in wild-type cells (Figure 1A). Furthermore, the small mounds were unstable, and they often dis-aggregated before reaggregating in different positions on the substratum (Figure 1B). Restoring GskA activity via expression of the wild-type gskA cDNA in gskA null mutants, rescued aggregation to that seen in the wild type (Figure 1C). However, no phenotypic rescue was seen after expression of a mutant gene that lacks kinase activity (GskAK85R), indicating a requirement for kinase activity to mediate GskA function (Figure 1C).

GSK3 Is Required for Chemotaxis to cAMP and Folate

We tested the ability of gskA null cells to undergo chemotaxis to cAMP. Cells were pulsed with cAMP for 5 h and then placed in a gradient formed from a 1 μM cAMP source. gskA null mutant cells had a complete loss of chemotaxis. Cell chemotaxis can be measured as the chemotactic index (CI), where +1 represents maximally accurate chemotaxis toward the cAMP source, −1 represents chemotaxis away from the cAMP source, and 0 is random movement neither toward nor away from the cAMP source. We also have calculated the percentage of cells that respond positively toward cAMP (% cells respond), and this represents the proportion of cells that have CI values of ≥0.7, with cos−1 (0.7) = 45° and cos−1 (1.0) = 0°. Hence, cells with CI = 1.0, chemotax in a straight line to cAMP, whereas any cell that deviates 45° on either side of this would exhibit a value of 0.7.

Wild-type cells possessed a mean CI of 0.9, with 88.7% of wild-type cells exhibiting chemotaxis toward cAMP compared with 31.5% of gskA mutant cells. This is a slightly higher percentage (25%) than expected from cells when they randomly move. The gskA null mutant cells have a strongly decreased mean CI value of 0.18 and in addition exhibited an approximate 50% decrease in cell speed and directionality, a measure of cell turning as they migrate toward the cAMP source. Re-expression of GskA in the gskA null mutant restores CI, cell speed, and directionality to that of the

Figure 1. Analysis of Dictyostelium development. (A) Wild-type cells form streams ~8 h after starvation, but gskA null mutants bypass this stage and form small mounds that are unstable. (B) Mounds of gskA null mutant cells are constantly disaggregating and reaggregating. Arrows indicate mounds exhibiting this process. (C) gskA null mutant cells carrying a rescue plasmid proceed normally through development (bottom left), but a kinase-dead version (GskAK85R) in the gskA null mutant had similar development to the original null, indicating a requirement for kinase activity for normal development.
defect toward cAMP in the wild-type cells. These results indicate a strong chemotaxis (1.0) /H11005 of Figure 2.
carA induction for the developmental state of those used in our chemotaxis of the microarray but failed to detect diferences in gene ex-
test between wild type and gskA cAMP sensing and some downstream events. GskA (gskA
carA we used qRT-PCR to confirm that there was no diference in expressed during aggregation (Sun and Devreotes, 1991), and
Vol. 21, August 1, 2010 2791 gskA mM folate. Wild-type cells take on average 140 min to show clustering at the micropipette compared with
gskA fold induction. (C) we confirmed that induction of pdsA pdsA (Figure 2C), demonstrating that the loss of
gskA microarray (Figure 2B). This may have been below the threshold to detect on the cyclase
gene was induced to only 30% of the wild type.

To exclude the possibility that the cells could not sense cAMP, we examined expression of genes involved in che-
mataxis and cell signaling. The earlier microarray was carA the major receptor
out in cells that had been starved for 5 h and matched the developmental state of those used in our chemotaxis ex-
periments (Strmecki et al., 2007). We reexamed the results of the microarray but failed to detect diferences in gene ex-
ression of the cAMP receptor genes. carA is the major receptor expressed during aggregation (Sun and Devreotes, 1991), and
we used qRT-PCR to confirm that there was no diference in carA gene expression between wild-type and gskA null mutant
cells (Figure 2B). The microarray showed altered expression of pdsA and pdIA genes, all which play a role in aggregation and we confirmed that induction of pdsA, which encodes a cAMP phosphodiesterase, is indeed substantially reduced in gskA null cells (Figure 2B). Finally, a survey of major genes expressed
during aggregation showed that the expression of the adenyl cyclase acaA gene was induced to only 30% of the wild type. This may have been below the threshold to detect on the microarray (Figure 2B).

To confirm that gskA null cells can sense and respond to cAMP, we examined the response of phosphatase and tensin
homologue (PTEN) to cAMP stimulation. In wild-type cells, an accumulation of PTEN protein was seen on the cell membrane
but was lost to the cytosol within seconds of cAMP accumulation. This relocalization was still present in gskA null cells
(Figure 2C), demonstrating that the loss of gskA did not block cAMP sensing and some downstream events.

wild-type cells. These results indicate a strong chemotaxis defect toward cAMP in the gskA null mutant (Figure 2A).

To exclude the possibility that the cells could not sense cAMP, we examined expression of genes involved in che-
mataxis and cell signaling. The earlier microarray was carA the major receptor
out in cells that had been starved for 5 h and matched the developmental state of those used in our chemotaxis ex-
periments (Strmecki et al., 2007). We reexamed the results of the microarray but failed to detect diferences in gene ex-
ression of the cAMP receptor genes. carA is the major receptor expressed during aggregation (Sun and Devreotes, 1991), and
we used qRT-PCR to confirm that there was no diference in carA gene expression between wild-type and gskA null mutant
cells (Figure 2B). The microarray showed altered expression of pdsA and pdIA genes, all which play a role in aggregation and we confirmed that induction of pdsA, which encodes a cAMP phosphodiesterase, is indeed substantially reduced in gskA null cells (Figure 2B). Finally, a survey of major genes expressed
during aggregation showed that the expression of the adenyl cyclase acaA gene was induced to only 30% of the wild type. This may have been below the threshold to detect on the microarray (Figure 2B).

To confirm that gskA null cells can sense and respond to cAMP, we examined the response of phosphatase and tensin
homologue (PTEN) to cAMP stimulation. In wild-type cells, an accumulation of PTEN protein was seen on the cell membrane
but was lost to the cytosol within seconds of cAMP accumulation. This relocalization was still present in gskA null cells
(Figure 2C), demonstrating that the loss of gskA did not block cAMP sensing and some downstream events.

To examine chemotaxis in a context independent of cAMP signaling, we investigated the ability of the gskA null mutant to chemotax toward folate. This occurs during growth of Dictyostelium where cells are attracted to folic acid and pterins (Pan et al., 1972, 1975), substances that are secreted by bacteria, the Dictyostelium food source. We recorded the chemotaxis response of growth phase cells to 25 mM folic acid, delivered via a micropipette. We again observed a substantial chemotaxis defect in gskA null cells compared with wild type (Figure 2D). Together, these results indicate a requirement of GskA in Dictyostelium chemotaxis.

GSK3 Is Required for PIP3 Signaling

To investigate the chemotaxis defcit in gskA null mutant cells during early development, we examined PIP$_3$, a down-
stream effector of cAMP and mediator of the chemotaxis response. To do this, we monitored phosphorylation of
PKBA, the Dictyostelium homologue of PKB. As in its animal homologues, PKBA contains a pleckstrin homology (PH)domain that mediates translocation to the plasma membrane via PIP$_3$ binding, where the protein is subsequently phos-
phorylated (Kamimura et al., 2008). It therefore is a good monitor for PIP$_3$ synthesis after cAMP stimulation. In wild-
type cells, PKBA phosphorylation occurred within seconds of cAMP stimulation, with a peak between 10 and 20 s, and
then declined to basal levels by 60 s. No cAMP stimulated elevation of PKBA occurred in gskA null mutant cells (Figure
3A). We can exclude a failure to express the PKBA gene because no diference in expression between mutant and
null mutant cells. gskA PH was recruited to the membrane within 10 s of cAMP addition.
**gskA Null Mutants Have Impaired cAMP Synthesis**

Although it restored chemotaxis, IMPA overexpression did not rescue aggregation or any other aspect of morphogenesis (Figure 5A and Supplemental Movie 2). This indicated that the aggregation defect was not a simple consequence of loss of chemotaxis, and may arise from an additional signaling deficiency. To test whether these cells can generate cAMP signals, cells were developed for5hi n suspension and stimulated with the cAMP analogue 2-deoxyadenosine 3,5-monophosphate (dcAMP), and cAMP synthesis was measured. There was almost undetectable cAMP synthesis in gskA null- and gskA null-expressing IMPA cells (Figure 5B).

**DISCUSSION**

Here, we report that loss of GskA has substantial effects on Dictyostelium aggregation, causing a major defect in chemotaxis and cAMP signaling. This phenotype correlates with the presence of GskA activity during early stages of development and the results of previous microarray and proteomic analysis (Plyte et al., 1999; Strmecki et al., 2007). These results reveal an unexpectedly strong chemotaxis phenotype, which has been previously missed, and indicates that the terminal developmental phenotype of Dictyostelium mutants may not be a good indicator of defective chemotaxis.

Loss of gskA has a substantial effect on chemotaxis toward both cAMP and folate. These two chemotactic responses are mediated by different receptors and G proteins (Kumagai et al., 1989; Hadwiger et al., 1994; Kim et al., 1998). Together with the expression of the cAMP receptor carI and the
gskA chemotaxis to response (Kortholt et al., 2007), explaining how this restores PKBA phosphorylation without an effect on PKBR1. We noted however that a small amount of PKBA phosphorylation occurs in the absence of TORC2. This is evident in the report from (Kamimura and Devreotes, 2010) where PKBA phosphorylation is observed in pia mutant cells, which lacks the Dictyostelium homologue of TORC2 component Rictor. We also observed PKBA phosphorylation in a rip3 null mutant, lacking a second component of TORC2 (Supplemental Figure 1, top), but an absence of PKBR1 phosphorylation. We propose that elevation of PIP_3 as a consequence of IMPA overexpression, brings more PKBA to the membrane where it is phosphorylated through a TORC2-independent kinase. The identity of this kinase is not known, but a possible candidate could be the PDK1 homologue PdkA (Kamimura and Devreotes, 2010, Liao et al., 2010). Although both Pdka and its parologue can function in the cytosol, Pdka can translocate to the plasma membrane in response to cAMP through binding to PIP_3 (Kamimura and Devreotes, 2010), and so IMPA overexpression could enhance PKBA phosphorylation by increasing the concentration of both PKBA and PdkA on the membrane. Furthermore, PKBR1 null cells undergo normal chemotaxis (Meli et al., 2000), indicating that PKBA phosphorylation alone is sufficient for chemotaxis.

Further apparent complexity relates to earlier results concerning the effects of lithium on Dictyostelium chemotaxis. Lithium inhibits both GSK3 and IMPA (Van Looijeren Campagne et al., 1998; Van Dijken et al., 1996; Ryves and Harwood, 2001) and therefore would be expected to produce the strong chemotaxis effect seen in the gskA null mutant. However, previously we found that lithium treatment of aggregation competent wild-type cells suppressed PIP_3 signaling, but left PKBR1 phosphorylation and cAMP synthesis unaffected (King et al., 2009). We demonstrated that this result arose from inhibition of IMPA, leading to a decrease in PIP_3 and subsequent PIP_3 synthesis. Arguing

Figure 6. Proposed mechanism of GskA action on both PIP_3 and PKBR1. Phosphorylation of PKBA probably relies on both the action of TORC2 and another kinase, most likely PDK (Kamimura and Devreotes, 2010), whereas GskA also acts upstream of TORC2, hence affecting subsequent phosphorylation of PKBR1.
against the possibility that GskA becomes insensitive to lithium, we found that nuclear export of Dd-STATa (Ginger et al., 2000), which is mediated by GskA phosphorylation, is suppressed by lithium in aggregation competent cells (Supplemental Figure 1, bottom left). We therefore propose that these differences arise through the timing of GskA action. Acute inhibition of GskA in aggregation competent cells, practically defined as cells pulsed with cAMP for 4 h before addition of lithium for 1 h, has no effect on chemotaxis or cAMP synthesis. In contrast, loss of GskA activity at the beginning of development or in growth phase cells causes the block to chemotaxis and signaling. In support of this hypothesis, we found that long-term treatment with lithium present from the initiation of cell development caused a substantial suppression of cAMP synthesis (Supplemental Figure 1, bottom right). This suggests that GskA activity acts as a permissive signal to initiate or set up chemotaxis but is not required continuously either as a direct requirement within the chemotaxis response or for maintenance of a chemotactic competent state. How GskA may achieve this is unclear; however, possibilities could range from constitutive phosphorylation of a protein that acts upstream of PI3P, and TORC2 signaling to regulation of gene expression of a component, or components, required for chemotaxis and signaling.

What is clear is that GskA is required in the regulation of some aggregation-specific gene expression. Here, we have shown decreased expression in both pdsA, a cAMP phosphodiesterase, and acacA, the adenylly cyclase required for generation of cAMP during aggregation. Loss of expression of these genes could account for loss of cAMP signaling during aggregation, but not the GskA requirement for chemotaxis. The earlier proteomic and microarray analysis of gskA null mutant cells (Strmecki et al., 2007) indicates several genes that may contribute to the chemotaxis phenotype. Loss of gskA reduces expression of sodC, a superoxide dismutase, which has been shown to be required for chemotaxis toward cAMP (Veeranki et al., 2008). The sodC mutant causes an increase in RasG activity even in the absence of cAMP stimulation, a known upstream activator of PI3-kinase.

ACKNOWLEDGMENTS

We thank Peter D. Watson for invaluable help in creating the dark-field movies and advice related to microscopy. We also thank Gerry Weeks and Parvin Boulourani for advice regarding the folate experiments and Tsuyoshi Araki and Jeff Williams for the Dictyostelium monoclonal antibodies against STAa (D4). This work was funded by a Wellcome Trust Programme grant to A.J.H.

REFERENCES

Bloomfield, G., Tanaka, Y., Skelton, J., Ivers, A., and Kay, R. R. (2008). Widespread duplications in the genomes of laboratory stocks of Dictyostelium discoideum. Genome Biol. 9, R75.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995). Inhibition of glycerokinase synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.

Curreli, N., Sollai, F., Massa, L., Comandini, O., Rufo, A., Sanjurst, E., Rinaldi, A., and Rinaldi, A. C. (2001). Effects of plant-derived naphthoquinones on the growth of Pleurotus sajor-caju and degradation of the compounds by fungal cultures. J. Basic Microbiol. 41, 253–259.

Ehrenman, K., Yang, G., Hong, W. P., Gao, T., Jiang, W., Brock, D. A., Hatton, R. D., Shoemaker, J. D., and Comer, R. H. (2004). Disruption of aldehyde reductase increases group size in Dictyostelium. J. Biol. Chem. 279, 837–847.

Ginger, R. S., Dalton, E. C., Ryves, W. J., Fukuzawa, M., Williams, J. G., and Harwood, A. J. (2000). Glycerokinase synthase kinase-3 enhances nuclear export of a Dictyostelium STAT protein. EMBO J. 19, 5483–5491.

Grimson, M. J., Coates, J. C., Reynolds, J. P., Shipman, M., Blanton, R. L., and Harwood, A. J. (2000). Adherens junctions and beta-catenin-mediated cell signalling in a non-metazoan organism. Nature 408, 727–731.

Hadwiger, J. A., Lee, S., and Firtel, R. A. (1994). The G alpha subunit G alpha 4 couples to pertin receptors and identifies a signaling pathway that is essential for multicellular development in Dictyostelium. Proc. Natl. Acad. Sci. USA 91, 10566–10570.

Harwood, A. J. (2001). Signal transduction and Dictyostelium development. Protoplasma 152, 17–29.

Harwood, A. J., Pyle, S. E., Woodgett, J., Strutt, H., and Kay, R. R. (1995). Glycerokinase synthase kinase 3 regulates cell fate in Dictyostelium. Cell 80, 139–148.

Helleman, J., Martier, G., De Paepe, A., Spelman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.

Hoeller, O. and Kay, R. R. (2007). Chemotaxis in the absence of PIP3 gradients. Curr. Biol. 17, 813–817.

Kamimura, Y., and Devreotes, P. N. (2010). Phosphoinositide-dependent protein kinase (PDK) activity regulates phosphatidylinositol 3,4,5-trisphosphate-dependent and -independent protein kinase B activation and chemotaxis. J. Biol. Chem. 285, 7938–7946.

Kamimura, Y., Xiong, Y., Iglesias, P. A., Hoeller, O., Boulouarni, P., and Devreotes, P. N. (2008). PIP3-independent activation of TORC2 and PFKB at the cell’s leading edge mediates chemotaxis. Curr. Biol. 18, 1034–1043.

Kim, J. Y., Borleis, J. A., and Devreotes, P. N. (1998). Switching of chemottractant receptors programs development and morphogenesis in Dictyostelium: receptor subtypes activate common responses at different agonist concentrations. Dev. Biol. 197, 117–128.

Kim, L., Harwood, A., and Kimmel, A. R. (2002). Receptor-dependent and -independent protein kinase B activation and chemotaxis. J. Biol. Chem. 277, 306–312.

Kim, L., Liu, J., and Kimmel, A. R. (1999). The novel tyrosine kinase ZAK1 activates GSK3 to direct cell fate specification. Cell 99, 399–408.

King, J. S., Teo, R., Ryves, J., Reddy, J. V., Peters, O., Orabi, B., Hoeller, O., Williams, R. S., and Harwood, A. J. (2009). The mood stabiliser lithium suppresses PIP3 signalling in Dictyostelium and human cells. Dis. Model Mech. 2, 306–312.

Kolsch, V., Charest, P. G., and Firtel, R. A. (2008). The regulation of cell motility and chemotaxis by phospholipid signaling. J. Cell Sci. 121, 551–559.

Konig, S., Hoffmann, M., Mosblech, A., and Heilmann, I. (2008). Determination of content and fatty acid composition of unlabeled phosphoinositide species by thin-layer chromatography and gas chromatography. Anal. Biochem. 378, 197–201.
Kortholt, A., King, J. S., Keizer-Gunnink, I., Harwood, A. J., and Van Haastert, P. J. (2007). Phosphopholipase C regulation of phosphatidylinositol 3,4,5-trisphosphate-mediated chemotaxis. Mol. Biol. Cell 18, 4772–4779.

Kumagai, A., Pupillo, M., Gundersen, R., Miake-Lye, R., Devreotes, P. N., and Firtel, R. A. (1998). Regulation and function of G alpha protein subunits in Dictyostelium. Cell 77, 265–275.

Lee, S., Comer, F. I., Sasaki, A., McEved, I. X., Duong, Y., Okumura, K., Yates, J. R., 3rd, Parent, C. A., and Firtel, R. A. (2005). TOR complex 2 integrates cell movement during chemotaxis and signal relay in Dictyostelium. Mol. Biol. Cell 16, 4572–4583.

Liao, X. H., Buggey, J., and Kimmel, A. R. (2010). Chemotactic activation of Dictyostelium AGC-family kinases Akt and PKB1 requires separate but coordinated functions of PDK1 and TORC2. J. Cell Sci. 123, 983–992.

Meili, R., Ellsworth, C., and Firtel, R. A. (2000). A novel Akt/PKB-related kinase is essential for morphogenesis in Dictyostelium. Curr. Biol. 10, 708–717.

Meili, R., Ellsworth, C., Lee, S., Reddy, T. B., Ma, H., and Firtel, R. A. (1999). Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in Dictyostelium. EMBO J. 18, 2092–2105.

Morio, T., et al. (1998). The Dictyostelium developmental cdNA project: generation and analysis of expressed sequence tags from the first-finger stage of development. DNA Res. 5, 335–340.

Pan, P., Hall, E. M., and Bonner, J. T. (1972). Folic acid as second chemotactic substance in the cellular slime moulds. Nat. New Biol. 237, 181–182.

Pan, P., Hall, E. M., and Bonner, J. T. (1975). Determination of the active portion of the folic acid molecule in cellular slime mold chemotaxis. J. Bacteriol. 122, 185–191.

Papkoff, J., and Aikawa, M. (1998). WNT-1 and HGF regulate GSK3 beta activity and beta-catenin signaling in mammary epithelial cells. Biochem. Biophys. Res. Commun. 247, 851–858.

Parent, C. A., Blacklock, B. J., Froehlich, W. M., Murphy, D. B., and Devreotes, P. N. (1998). G protein signaling events are activated at the leading edge of chemotactic cells. Cell 95, 81–91.

Plyte, S. E., O'Donovan, E., Woodgett, J. R., and Harwood, A. J. (1999). Glycogen synthase kinase-3 (GSK-3) is regulated during Dictyostelium development via the serpentine receptor cAR3. Development 126, 325–333.

Ryves, W. J., Fryer, L., Dale, T., and Harwood, A. J. (1998). An assay for glycogen synthase kinase 3 (GSK-3) for use in crude cell extracts. Anal. Biochem. 264, 124–127.

Ryves, W. J., and Harwood, A. J. (2001). Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. Biochem. Biophys. Res. Commun. 280, 720–725.

Sasaki, A. T., Chun, C., Takeda, K., and Firtel, R. A. (2004). Localized Ras signaling at the leading edge regulates PKB, cell polarity, and directional cell movement. J. Cell Biol. 167, 505–518.

Schild, C., Araki, T., Williams, H., Harwood, A., and Williams, J. G. (2004). GSK3 is a multifunctional regulator of Dictyostelium development. Development 131, 4535–4565.

Snaar-Jagalska, B. E., and Van Haastert, P. J. (1994). G-protein assays in Dictyostelium. Methods Enzymol. 237, 387–408.

Stirmecki, L., Bloomfield, G., Araki, T., Dalton, E., Selkton, J., Schild, C., Harwood, A., Williams, J. G., Ivens, A., and Pears, C. (2007). Proteomic and microarray analyses of the Dictyostelium Zak1-GSK-3 signaling pathway reveal a role in early development. Eukaryot. Cell 6, 245–252.

Sun, T. J., and Devreotes, P. N. (1991). Gene targeting of the aggregation stage cAMP receptor cAR1 in Dictyostelium. Genes Dev. 5, 572–582.

Takeda, K., Sasaki, A. T., Ha, H., Seung, H. A., and Firtel, R. A. (2007). Role of phosphatidylinositol 3-kinases in chemotaxis in Dictyostelium. J. Biol. Chem. 282, 11874–11884.

Tomchik, K. J., and Devreotes, P. N. (1981). Adenosine 3',5'-monophosphate waves in Dictyostelium discoideum: a demonstration by isotope dilution-fluorography. Science 212, 443–446.

Van Dijken, P., Berguma, J. C., Hiemstra, H. S., De Vries, B., Van Der Kaay, J., and Van Haastert, P. J. (1996). Dictyostelium discoideum contains three inositol monophosphatase activities with different substrate specificities and sensitivities to lithium. Biochem. J. 314, 491–495.

Van Looikeren Campagne, M. M., Erneux, C., Van Eijk, R., and Van Haastert, P. J. (1988). Two dephosphorylation pathways of inositol 1,4,5-trisphosphate in homogenates of the cellular slime mould Dictyostelium discoideum. Biochem. J. 254, 343–350.

Varney, T. R., Casademunt, E., Ho, H. N., Petty, C., Dolman, J., and Blumberg, D. D. (2002). A novel Dictyostelium gene encoding multiple repeats of adhesion inhibitor-like domains has effects on cell-cell and cell-substrate adhesion. Dev. Biol. 243, 226–248.

Veeranki, S., Kim, B., and Kim, L. (2008). The GP1 anchored superoxide dismutase SodC is essential for regulating basal Ras activity and for chemotaxis of Dictyostelium discoideum. J. Cell Sci. 121, 3099–3108.

Williams, J. G., Duffy, K. T., Lane, D. P., McRobbie, S. J., Harwood, A. J., Traynor, D., Kay, R. R., and Jermy, K. A. (1989). Origins of the prestalk-sheath pattern in Dictyostelium development. Cell 59, 1157–1163.

Williams, R. S., Eames, M., Ryves, W. J., Viggars, J., and Harwood, A. J. (1999). Loss of a prolyl oligopeptidase confers resistance to lithium by elevation of inositol (1,4,5) trisphosphate. EMBO J. 18, 2734–2745.