Glycogen Synthase Kinase-3 Is Required for Efficient Dictyostelium Chemotaxis

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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 (PIP3) 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; Pankoff 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, STaA (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 Dictostelium 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 (Strmeci 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 Dictostelium 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 Dictostelium 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 Dictostelium discoidum strain AX2 background. The wild-type strain refers to AX2 and is the same strain denoted in Bloomfield et al. (2006). Cells were cultured axenically on plates in HL5 with glucose medium (Formedium HL50102) before experiment. gskA null mutants were grown in media supplemented with 10 µg/ml blasticidin, 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 kanamycin. All samples were maintained at 22°C.

Plasmids

The GskA-GFP2 rescue construct for gskA was constructed by inserting the coding sequence of GskA into pDXA GFP2 at the KpnI and NsiI 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 sequence of the IMPA gene was amplified from the cdna clone FCBP15, obtained from the Japanese Dictys discoidum 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 KH2P04, 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 × 106 cells/ml, and 100 µl of cells was stimulated with 5 mM 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 3.5% (vol/vol) sodium hydroxide, and cAMP was measured as described previously (Snaar-Jagalska and Van Haastert, 1998). cAMP levels were pulsed with cAMP for 5 h with an end concentration of 100 nM cAMP. 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 155379; Nalge Nunc International, Rochester, NY) with 2 ml of 20% media/KK2. Folate (25 mM) was delivered to a Femtopipet (520 µl) at the end of the chemotaxis phase and images were recorded for every 20 s under 10× phase objective. For aggregation, 5 × 106 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 Zipper chamber (Z02; Neuro Probe, Gaithersburg, MD) in a cAMP gradient (source at 10−10 M cAMP, sink with no cAMP). Images and 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 × 106) were starved and pulsed for 4 h with 100 nM cAMP. At the four-hour, 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 into 250 µl of acetonitrile/methanol (1:1) 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. TLC analyses were carried out according to manufacturer’s instructions (K-2500 PIP, 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 (190D10; Danvers, MA) and analyzed as described previously (King et al., 2009).

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

Dictostelium cells were starved and shaken with pulses of 100 nM cAMP for 5 h. Cells (2 × 106) 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 (11483188001; Roche Diagnostics, Mannheim, Germany) using 1 µg of RNA and random primers. A minus-reverse transcriptase control was used, and qRT-PCR 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. iQ SYBR Green master mix (170-8884; Bio-Rad Laboratories) was used. The reactions were set up with 100 µg of CDNA. Housekeeping genes comprising of transcripts for β-actin (www.dictybase.com; ID Strain: DDB_G0294034), impk1, and impk2. The genes of interest were carA-1 (DDB_G0273937), acaA (DDB_G0281545), and pdks (DDB_G0285995). Data analysis was carried out using the qbase software (Vandesompele and Hellemans, 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 were also normalized to the Ax2 0 h cdna. Primer sequences are as follows: β-actin forward, TCAACAGAGGAGAGAGAACTGC and β-actin reverse, TGGGAGGCTTCTACCATC; impk1 forward, GCAGGTTCAACCATTATA; impk2 forward, TGGTAGGTTTGGATTGTCACGC and and impk2 reverse, TGATGATGGTTGTTGTTGTTAG. The genes of interest were carA-1 and carA-2, and carA-1 forward, ATGTTGGTGGTTAGGGAACACCTATG and carA-2 forward, CTTGATTTCGCTTTGACGC and carA reverse, AACTCCCGATGAGTATAGGT.
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 disaggregated 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 (GskA K85R), 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 mM 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. Cells were pulsed with cAMP for 5 h and then placed in a gradient formed from a 1 mM 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 null 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 normal wild type.

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 (GskA K85R) in the gskA null mutant had similar development to the original null, indicating a requirement for kinase activity for normal development.

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wild-type cells. These results indicate a strong chemotaxis deficit 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 chemotaxis and cell signaling. The earlier microarray was carried out in cells that had been starved for 5 h and matched the developmental state of those used in our chemotaxis experiments (Strmecki et al., 2007). We reexamined the results of the microarray but failed to detect differences in gene expression 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 difference 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 of 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 folate, delivered via a micropipette. Wild-type cells take on average 140 min to show clustering at the micropipette compared with gskA null mutants, taking an average of 270 min. Figures here show a representative of one replicate experiment. Control, buffer used to dissolve folate.

**Figure 2.** Chemotaxis of gskA null mutant cells. (A) Chemotaxis parameters for wild-type, gskA null mutant, and gskA null cells expressing GskA (gskA-GskA). % cells respond to cAMP indicates the proportion of cells that have a CI values of ≥0.7, with cos−1 (0.7) = 45° and cos−1 (1.0) = 0°. Values are means and SDs based on at least 140 cells, compiled from three independent experiments. Dunn’s multiple comparison’s test between wild type and gskA null mutant showed a significant difference at p < 0.001 for all three parameters. (B) qRT-PCR showed gene induction for carA, pdsA, and aca are to similar levels to that observed in the earlier microarray study (Strmecki et al., 2007). The y-axes denote fold induction. (C) gskA null mutant cells are able to cause PTEN-GFP relocalization within 10 s of cAMP stimulation. (D) Chemotaxis to 25 mM folate. Wild-type cells take on average 140 min to show clustering at the micropipette compared with gskA null mutants, taking an average of 270 min. Figures here show a representative of one replicate experiment. Control, buffer used to dissolve folate.
with a global cAMP signal, PIP₃ (Figure 3B) and when levels of fluorescence were quantified, gskA membrane translocation was observed in the cytosol to the membrane. In wild-type cells, PH was recruited to the membrane within 10 s of cAMP addition (Strmecki et al., 2007). The wild-type cells was observed either in the microarray (PI3-kinase) substrate phosphatidylinositol 4,5-bisphosphate (PIP₂, King et al., 2009). We used IMPA overexpression to elevate PIP₃ in gskA null cells and examined its effects. Overexpression of IMPA in gskA null mutant cells not only rescued chemotaxis, restoring mean cell speed, directionality, and CI values of gskA null mutant cells to wild-type control values (Figure 4A), it also restored PKBA phosphorylation (Figure 4B). This not only indicated that GskA plays a major role in PIP₃ signaling, it again demonstrated that gskA null mutant cells could sense sufficient cAMP to restore chemotaxis when this downstream effector is restored.

We used IMPA overexpression to probe how loss of gskA affects PIP₃ signaling. One possibility is that GskA regulates IMPA activity or other targets on the inositol–PIP₂ pathway. A lack of PIP₂ would then lead to a decrease in PIP₃ synthesis, in a similar mechanism to that seen with lithium treatment (King et al., 2009). In contrast, elevation of PIP₃ may bypass a block in an alternative pathway, such as activation of PI3-kinase. We measured synthesis of phosphatidyl inositol phosphates (PIPs) in wild-type, gskA null mutant, and gskA null mutant IMPA-overexpressing cells.

We observed no difference in phosphatidylinositol (PI), phosphatidylinositol phosphate (PIP), and PIP₂ synthesis between wild-type and gskA null mutant cells (Figure 4C). This argued against a requirement for GskA for PIP₂ synthesis. In contrast, we saw an increase in PIP₂ in gskA null mutant IMPA-overexpressing cells (Figure 4C). This suggested that IMPA overexpression bypasses a deficit in PIP₂ signaling by elevating PIP₂ and hence PIP₃ synthesis.

Finally, we measured PIP₃ directly and found that in response to cAMP, gskA null mutant cells did not synthesize PIP₃ but seemed to lose it. This response is the opposite to that observed in either wild-type or gskA null mutant IMPA-overexpressing cells (Figure 4D), which rapidly synthesize new PIP₃ under these conditions. Overall, the data showed that very little PIP₃ is formed in gskA null mutant cells.

GskA Is Required for Target of Rapamycin Complex 2 (TORC2)-mediated Signaling

We noted that the cAMP chemotaxis phenotype seen in the gskA null mutant was much stronger than that observed for loss of PIP₃ signaling alone (Hoeller and Kay, 2007; Takeda et al., 2007; King et al., 2009), and sought evidence for a further molecular defect in the gskA null mutant. Previously, it has been found that signaling via the kinase target of rapamycin (TOR) acting in the TORC2 complex was capable of mediating cAMP chemotaxis when PIP₃ synthesis was inhibited (Kamimura et al., 2008). This alternative chemotaxis mechanism acts via a second PKB homologue, known as PkgB, or PKBR1 (Meili et al., 2000). This kinase protein is associated with the plasma membrane via myristoylation and not via a PH domain–PIP₃ interaction (Meili et al., 1999) and is activated by phosphorylation from TORC2 (Lee et al., 2005). We examined PKBR1 phosphorylation in gskA null mutant cells after cAMP stimulation, and found that as seen for PKBA, PKBR1 phosphorylation was also lost (Figure 4B).

We could detect PKBR1 expression (data not shown), suggesting a signaling failure upstream of PKBR1. In contrast to PKBA phosphorylation, PKBR1 phosphorylation was not restored by overexpression of IMPA (Figure 4B), indicating that this second molecular mechanism is independent of PIP₃ signaling. These results indicated that GskA is required for two intracellular signaling processes, explaining why it possesses a very strong chemotaxis defect.
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 in 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). Dark-field optics was used to examine the cell signaling activity during aggregation. When viewed under these conditions, changes in cell shape in response to cAMP pulses can be seen as a change in light scattering (Tomchik and Devreotes, 1981). In wild-type cells, waves of light scattering propagate from the center of the aggregation field pass through the developing cell field as spiral waves (Figure 5C and Supplemental Movie 3A). In contrast to wild-type cells, no light scattering waves were observed in gskA null mutant cells or gskA mutants overexpressing IMPA (Figure 5C and Supplemental Movie 3B). These observations suggested that although PIP3 signaling is sufficient to rescue chemotaxis, it was unable to restore cAMP signaling and fully rescue aggregation.

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 carA and the
cAMP production is impaired in both type cells at \( gskA \) with lighter areas of moving cells (top left). All \( gskA \) chemotaxis to \( gskA \) et al response (Kortholt simple loss of PIP3 is sufficient for chemotaxis, it is not essential and a large drop in the CI value. We found that loss of \( gskA \) chemotaxis phenotype observed for the \( gskA \) null mutant, we also measured loss or inhibition of PI3K or PIP3 after overexpression of IMPA. Previous observations have shown that PIP3 signaling is able to elicit the chemotaxis response (Kortholt et al., 2007), explaining how this restores chemotaxis to \( gskA \) null mutant cells. Although PIP3 signaling is sufficient for chemotaxis, it is not essential and a simple loss of PIP3 cannot totally explain the severe chemotaxis phenotype observed for the \( gskA \) null mutant cells. Loss or inhibition of PI3K or PIP3 synthesis reduces speed and directionality in chemotaxing cells; however, it has only a minor effect on the CI value (Hoeller and Kay, 2007; King et al., 2009). In contrast, although we also saw reduced speed and directionality in the \( gskA \) null mutant, we also measured a large drop in the CI value. We found that loss of \( gskA \).}

![Figure 5](image)

**Figure 5.** (A) Overexpression of IMPA in the \( gskA \) null background does not rescue aggregation and multicellular development. (B) cAMP production is impaired in both \( gskA \) null mutants and \( gskA \)-IMPA cell lines. (C) Dark-field images of aggregating wild-type cells at \( \sim 3 \) h shows spirals of dark, stationary cells alternating with lighter areas of moving cells (top left). All \( gskA \) null mutant cell lines (null, \( gskA \)-GFP, and \( gskA \)-IMPA) do not show such patterning or light scattering waves. Bar, 50 \( \mu m \).

In our experiments, we monitored PKBA and PKBR1 activation through antibodies specific to motifs phosphorylated by the protein kinase PDK-1 (Kamimura and Devreotes, 2010). This in turn is dependent on phosphorylation by TOR kinase as part of the TORC2 complex after cAMP stimulation. These events occur at the plasma membrane so that \( PKBR1 \) is dependent solely on TORC2 activation, whereas PKBA phosphorylation requires simultaneous TORC2 activation and PIP3 synthesis. This presents a paradox: if TORC2 activation is required for both PKBA and PKBR1, how can overexpression of IMPA restore 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 et al., 2008; Liao et al., 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 PIP3 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 PdK1 (Kamimura and Devreotes, 2010, Liao et al., 2010). Although both PdK1 and its parologue can function in the cytosol, PdK1 can translocate to the plasma membrane in response to cAMP through binding to PIP3, (Kamimura and Devreotes, 2010), and so IMPA overexpression could enhance PKBA phosphorylation by increasing the concentration of both PKBA and PdK1 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 Lookeren Campagne et al., 1998; Van Dijken et al., 1996; Ryves et al., 1998; 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 PIP3 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 PIP3 and subsequent PIP3 synthesis.
against the possibility that GskA becomes insensitive to lithium, we found that nuclear export of Dd-STAT4 (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 PI3, 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 pdmA, a cAMP phosphodiesterase, and accA, 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 (Sasaki et al., 2004), and could contribute to the higher basal levels of PKBα phosphorylation before cAMP stimulation (Figure 3). The microarray also showed that expression of the gene alrA is reduced in gskA null mutant cells. The AlrA protein encodes an aldehyde reductase, which when disrupted also suppresses cell motility, although the mechanism is unclear (Ehrenman et al., 2004). Curiously, both GskA and AlrA have the capacity to alter glucose metabolism within the cell, but the significance of this is not known. In contrast, loss of gskA elevates expression of ampaA. The product of this gene is antiadhesion protein that accumulates during aggregation (Varney et al., 2002), and elevated levels of AmpA could explain the instability of the small mounds observed during the aggregation of the gskA null.

Together, this study indicates that GskA is an important regulator of the aggregation process and is essential for cell chemotaxis. Our observations however also indicate that the role of GskA is probably complex, acting through a combination of mechanisms that are required at a minimum for chemotaxis and signaling but that also may include metabolism and cell adhesion. These may integrate to during early development to allow aggregation proceed to the multicellular stage, in which GskA mediates cell fate determination.

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