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The *Dictyostelium* GSK3 kinase GlkA coordinates signal relay and chemotaxis in response to growth conditions

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ABSTRACT

GSK3 plays a central role in orchestrating key biological signaling pathways, including cell migration. Here, we identify GlkA as a GSK3 family kinase with functions that overlap with and are distinct from those of GskA. We show that GlkA, as previously shown for GskA, regulates the cell's cytoskeleton through MyoII assembly and control of Ras and Rap1 function, leading to aberrant cell migration. However, there are both qualitative and quantitative differences in the regulation of Ras and Rap1 and their downstream effectors, including PKB, PKBR1, and PI3K, with *glkA*$^-$ cells exhibiting a more severe chemotaxis phenotype than *gskA*$^-$ cells. Unexpectedly, the severe *glkA*$^-$ phenotypes, but not those of *gskA*$^-$, are only exhibited when cells are grown attached to a substratum but not in suspension, suggesting that GlkA functions as a key kinase of cell attachment signaling. Using proteomic iTRAQ analysis we show that there are quantitative differences in the pattern of protein expression depending on the growth conditions in wild-type cells. We find that GlkA expression affects the cell's proteome during vegetative growth and development, with many of these changes depending on whether the cells are grown attached to a substratum or in suspension. These changes include key cytoskeletal and signaling proteins known to be essential for proper chemotaxis and signal relay during the aggregation stage of *Dictyostelium* development.

1. Introduction

Chemotaxis, or directed cell migration up a chemical gradient, is vital for many processes in eukaryotes, including embryogenesis, wound healing, and immune cell functions (Geng, 2001; Keller, 2005; Luster et al., 2005). Investigations into the mechanisms controlling chemotaxis in amoeboid cells, such as neutrophils and *Dictyostelium* cells, have identified multiple, conserved pathways that control sensing of the direction of the chemoattractant. Feedforward and feedback loops amplify this response and lead to the formation of a highly polarized cell with an F-actin enriched leading edge, actomyosin-enriched posterior, and a lateral cortex that mediates forces involved in movement of the cell (King and Insall, 2009; Annesley and Fisher, 2009; Swaney et al., 2010; Bastounis et al., 2014).

Vegetative *Dictyostelium* amoebae grow as separate independent cells but aggregate to form multicellular structures upon starvation. Aggregation is mediated by a signal-relay pathway in which cAMP functions as an extracellular chemoattractant acting through the cAMP G-protein coupled receptor (GPCR) cAR1 and intracellularly to activate PKA (Xiao et al., 1997; Jin et al., 2000; Liu et al., 2016; Aubry and Firtel, 1999; McMains et al., 2008; Zhang et al., 2003; Maeda et al., 2004). Cells respond to extracellular cAMP by polarizing and chemotaxing towards the source and by synthesizing and secreting more cAMP, all regulated through cAR1 (Sun et al., 1990; Parent and Devreotes, 1999; Brzostowski et al., 2013; Liao et al., 2013). After stimulation, the receptors become rapidly desensitized and the chemotaxis pathways adapt (Van Haastert and Devreotes, 2004; Takeda et al., 2012). Extracellular cAMP is degraded by the major extracellular cAMP phosphodiesterase PdsA (PDE1) (Lacombe et al., 1986; Laub et al., 1998; Masaki et al., 2013). The intracellular cAMP levels are regulated by the phosphodiesterase RegA, controlled by the MAP kinase ERKB (ERK2), whose own activity is controlled through cAR1

Abbreviations: GlkA, glycogen synthase kinase-like kinase A; PI(3,4,5)P_3, phosphatidylinositol (3,4,5)-triphosphate; MyoII, Myosin II; AL, activation loop; HM, hydrophobic motif; RBD, Ras binding domain; iTRAQ, isobaric tags for relative and absolute quantification; DIAS, Dynamic Image Analysis System

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(Laub and Loomis, 1998; Kimmel and Parent, 2003; Kimmel et al., 2004; Maeda et al., 2004). These intricate activation and positive feed forward and negative feedback networks integrate signal relay and the intracellular pathways that control the cytoskeleton and cell movement enabling cells to effectively form a multicellular organism (Aubry and Firtel, 1999; McMains et al., 2008).

In developed *Dictyostelium* cells, activation of signaling pathways at the cell's cortex in response to the chemoattractant gradient leads to localized F-actin polymerization and the formation of a leading edge (Chung et al., 2001). Leading edge actin polymerization, in association with posterior Myosin II (MyoII) assembly, is critical for trailing edge retraction, and MyoI-dependent lateral cortical tension drives forward movement (Xiao et al., 1997; Jin et al., 2000; Takeda et al., 2007; Shu et al., 2012; Kortholt et al., 2013; Bastounis et al., 2014; Cai et al., 2014). The activations of the Ras GTPases RasG, RasC, and Rap1 are some of the initial steps in polarity formation and the reorganization of the cytoskeleton (Janetopoulos and Firtel, 2008; Kortholt et al., 2011; Liu et al., 2016). RasG controls chemotaxis by activating Class I PI3Ks that recruit Akt/PBK to the plasma membrane. RasC and Rap1 activate TORC2 (Target of Rapamycin Complex 2), which activates Akt/PKB and the related kinase, PKBR1 (Funamoto et al., 2002; Kae et al., 2004; Lee et al., 2005; Sarbassov et al., 2005; Loovers et al., 2006; Kamimura et al., 2008; Zhang et al., 2008; Cai et al., 2010; Khanna et al., 2016). Rap1 also regulates PI3K activation and actin assembly and MyoII disassembly at the leading edge, the latter by binding to and activating the Ser/Thr kinase Phg2 (Kortholt et al., 2006; Jeon et al., 2007; Bolourani et al., 2008; Lee and Jeon, 2012).

Of particular interest is whether there are additional global regulators that help integrate these individual signaling pathways to control pathways such as aggregation. In vertebrates, GSK3 is a multifunctional kinase that exists as two closely related isoforms, GSK3α and GSK3β, that, in humans, have been implicated in a number of diseases, including Type II diabetes, muscle wasting, inflammation, cancer, and neurological disorders (Hoeflich et al., 2000; Frame et al., 2001; Grimes and Jope, 2001; Amar et al., 2011). We, and others, found previously that *Dictyostelium* GSK3 (GskA) is required for proper chemotaxis (Teo et al., 2010; Kim et al., 2011; Kölsch et al., 2013; Sun et al., 2013), in addition to GSK3's earlier known role in controlling cell fate in *Dictyostelium* (Harwood et al., 1995; Kim et al., 2002, 1999; Schilde et al., 2004; Strmecki et al., 2007). We found that *Dictyostelium* GSK3 null (*gskA* null or *gskA*$^-$) cells exhibit aberrant chemoattractant-mediated RasC and RasG activation and misregulation of downstream PI3K, Akt/PKB, and PKBR1 and a significant reduction in MyoII assembly (Kölsch et al., 2013; Sun et al., 2013). Using phosphoproteomics, the Ras/Rap1 effector Daydreamer (DydA) and the cAMP receptor-activated Rap1 GEF GflB were identified as in vivo substrates of GskA and were found to be required for chemotaxis (Kölsch et al., 2013; Liu et al., 2016). Interestingly, in *gskA*$^-$ cells, DydA phosphorylation at the GSK3 site remains at 20% of wild-type levels, suggesting that another kinase may contribute to DydA regulation. In this context, we hypothesized that the closest *Dictyostelium* GSK3 homolog GlkA might also play an important role in the regulation of the cytoskeleton and during chemotaxis.

GlkA has been annotated as a glycogen synthase kinase-like kinase A and a probable serine/threonine-protein kinase, but the function of GlkA has not been examined. In this paper, we show that GlkA (*glkA*, DDB_G0270218) is a member of the GSK3 kinase family with functions both distinct from and overlapping with those of GskA in controlling cell responses. Like *gskA*$^-$ cells, *glkA*$^-$ cells have greatly reduced MyoII assembly; however, there are both qualitative and quantitative differences in the regulation of Ras and Rap1 and their downstream effectors, with *glkA*$^-$ cells exhibiting a more severe chemotaxis phenotype than *gskA*$^-$ cells. Unexpectedly, the developmental defects and the chemotaxis phenotypes of *glkA*$^-$ cells are only observed when cells are grown attached to a substrate and not when grown in suspension. Furthermore, these phenotypes rapidly reverse when the growth conditions are switched. Using quantitative proteomic analysis of wild-type and *glkA*$^-$ cells grown in suspension and in attachment conditions, we demonstrate that a considerable portion of the proteome is altered in *glkA*$^-$ cells, including signaling and cell adhesion components required for proper chemotaxis. These analyses also identified a cadre of proteins involved in signal relay and chemotaxis to cAMP that are particularly affected in *glkA*$^-$ cells regardless of the growth conditions. Our findings suggest that GlkA and GskA function to integrate several interacting networks that control aspects of chemotaxis to cAMP and signal relay during aggregation, and, for the first time, identify a potential regulatory role of cell attachment during growth in the ability of cells to respond to starvation.

2. Materials and methods

2.1. Culture conditions and differentiation of Dictyostelium cells

Vegetative *Dictyostelium* cells were axenically grown at 21 °C in HL5 medium under attachment conditions on 150 × 15 mm Petri dishes, and in suspension in 125 ml Erlenmeyer flasks on a gyratory shaker (355 rpm). Petri dishes and Erlenmeyer flasks containing 20 ml of HL5 were inoculated with logarithmic phase growing cells to a density of ~ 1.8×10^6 cells/ml, and incubated for 24 h before being harvested by low-speed centrifugation (1500 rpm for 3 min). For comparisons of the switch between adhesion and suspension growth conditions, and the other way around, 24 h cultures of vegetative *Dictyostelium* cells grown on Petri plates and in Erlenmeyer flasks containing 20 ml of HL5 were diluted to a density of ~ 1.8×10^6 cells/ml and incubated for different times (6, 15, and 24 h) in the other culture condition before being harvested by low-speed centrifugation (1500 rpm for 3 min). Cell density for cells grown on plates and in shaking cultures, prior to starvation and stimulation with cAMP, was equivalent. To obtain developed *Dictyostelium* cells, developmentally competent and capable of responding to cAMP as a chemoattractant, vegetative cells (~ 2.5–5 × 10^6 cells/ml) grown on plates or in suspension were harvested by centrifugation, washed twice, and suspended at a density 5×10^6 cells/ml with 12 mM Na/K phosphate buffer, pH 6.2, and pulsed with 7.5 μM cAMP solution for 5.5 h at 6-min intervals. Cells were counted with a hemocytometer. We maintained the cells carrying expression constructs and knockout cells in the same medium containing 10–20 μg/ml G418, 10 μg/ml blasticidin, or 35 μg/ml hygromycin as required.

2.2. Electroporation

We performed transformation in E buffer (12 mM Na/K phosphate, 50 mM sucrose, pH 6.1) by electroporation of 20 μg DNA per 8×10^6 cells using a Bio-Rad Gene Pulser II set at 1 kV and 3 μF. After overnight recovery in HL5 in a 100 mm Petri dish, cells were selected in the appropriate antibiotic for transformants.

2.3. Plasmid construction and gene disruption

We amplified a 1752 bp fragment containing the *glkA* gene from wild-type genomic DNA using primers JL1 and JL4. The cleaned PCR product was digested with *Spe*I/*Xho*I and ligated into pBlueScript II SK(-) and into a modified EXP-4(+)-T7 tag vector, both plasmid digested with the same enzymes, to generate plasmids pJL1 and pJL2 respectively. We used pJL1 to generate the single (*glkA*$^-$) and double knockouts (*glkA*$^-$/*gskA*$^-$) in wild-type (Ax2) and *gskA*$^-$ cells with a blasticidin and a hygromycin resistance cassette, respectively. pJL2 overexpresses the GlkA protein with a T7 tag (MASMTGGQQMG) at the N-terminus of the protein. Using primers JL5 and JL4, a *Bgl*II/*Xho*I PCR product containing the *glkA* gene was amplified from wild-type genomic DNA, cleaned, and ligated into a modified EXP-4(+)-GFP