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Daydreamer, a Ras effector and GSK-3 substrate, is important for directional sensing and cell motility

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ABSTRACT How independent signaling pathways are integrated to holistically control a biological process is not well understood. We have identified Daydreamer (DydA), a new member of the Mig10/RIAM/lamellipodin (MRL) family of adaptor proteins that localizes to the leading edge of the cell. DydA is a putative Ras effector that is required for cell polarization and directional movement during chemotaxis. dydA− cells exhibit elevated F-actin and assembled myosin II (MyoII), increased and extended phosphoinositide-3-kinase (PI3K) activity, and extended phosphorylation of the activation loop of PKB and PKBR1, suggesting that DydA is involved in the negative regulation of these pathways. DydA is phosphorylated by glycogen synthase kinase-3 (GSK-3), which is required for some, but not all, of DydA’s functions, including the proper regulation of PKB and PKBR1 and MyoII assembly. gskA− cells exhibit very strong chemotactic phenotypes, as previously described, but exhibit an increased rate of random motility. gskA− cells have a reduced MyoII response and a reduced level of phosphatidylinositol (3,4,5)-triphosphate production, but a highly extended recruitment of PI3K to the plasma membrane and highly extended kinetics of PKB and PKBR1 activation. Our results demonstrate that GSK-3 function is essential for chemotaxis, regulating multiple substrates, and that one of these effectors, DydA, plays a key function in the dynamic regulation of chemotaxis.

INTRODUCTION

Chemotaxis, or directed cell movement up a chemoattractant gradient, plays a key role in a range of biological processes, including innate immunity, metastasis of cancer cells, tissue development, food foraging, and the formation of multicellular structures in free-living organisms such as Dictyostelium (Eccles, 2004; Martin and Parkhurst, 2004; Böttcher and Niehrs, 2005; Sasaki and Firtel, 2006). Cells are able to sense extracellular gradients as shallow as a 2% difference in chemoattractant concentration across the cell and are able to amplify that gradient intracellularly to produce a highly polarized cell in which the activity of leading edge– and posterior–specific signaling components are highly restricted to the respective poles of the cell (Van Haastert and Veltman, 2007; Janetopoulos and Parkhurst, 2004; Böttcher and Niehrs, 2005; Sasaki and Firtel, 2006). The Ras family of small GTPases, including Ras and Rap1, are key upstream regulators of directional sensing and chemotaxis. Dictyostelium cells in which Ras function has been abrogated exhibit delayed polarization when placed in a shallow chemoattractant gradient compared to wild-type cells (Klösch et al., 2008). Our results demonstrate that GSK-3 function is essential for chemotaxis, regulating multiple substrates, and that one of these effectors, DydA, plays a key function in the dynamic regulation of chemotaxis.
chemoattractant gradient and, once polarized, move randomly, being unable to sense the direction of the gradient (Sasaki et al., 2004). Proper spatiotemporal regulation of at least two Ras-mediated pathways is required in Dictyostelium for efficient directed migration: the class 1 phosphoinositide-3-kinase (PI3K) pathway, which is activated predominantly by RasG, and the target of rapamycin complex 2 (TORC2) pathway, which is activated predominantly by RasC (Lee et al., 1999; Funamoto et al., 2002; Sasaki et al., 2004, 2007; Sasaki and Firtel, 2006; Bolouarni et al., 2006; Charest et al., 2010). PI3K and TORC2 lead to the local activation of Akt/PKB and the related kinase PKB1 and other downstream effectors, resulting in changes in the cytoskeleton and directed migration (Kamimura et al., 2008; Kamimura and Devreotes, 2010; Charest et al., 2010).

Efficient chemotaxis has also been shown to require additional Ras and Rap1 effectors, signaling from guanylyl cyclase and phospholipase A2, and, more recently, glycogen synthase kinase-3 (GSK-3; Veltman and Van Haastert, 2006; Veltman et al., 2008; van Haastert et al., 2007; Chen et al., 2007; Teo et al., 2010; Kim et al., 2011). GSK-3, identified in mammalian cells as a kinase that phosphorylates and regulates glycogen synthase, controls a wide range of biological processes, including insulin and Wnt signaling, cell survival, neuronal development, and cell migration (Embi et al., 1980; Hemmings et al., 1981; Cross et al., 1995; Pap and Cooper, 1998; Papkoff and Aikawa, 1998; Kozlovska et al., 2002; Manoukian and Woodgett, 2002; reviewed in Jope and Johnson, 2004; Kim et al., 2009). Dictyostelium GSK-3 was discovered in a genetic screen for regulators of cell fate determination (Hanwood et al., 1995; Kawata et al., 1997; Ginger et al., 2000; Grimson et al., 2000) and subsequently demonstrated to be required for chemotaxis (Teo et al., 2010; Kim et al., 2011). GSK-3-null (gskA) cells move more slowly and have a deeply decreased directionality when compared with wild-type cells (Teo et al., 2010; Kim et al., 2011). gskA cells were reported to have reduced production of the PI3K product phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P_3) and reduced phosphorylation of the activation loop (AL) of Akt/PKB and the related kinase PKB1 (Teo et al., 2010).

To obtain more insight into the mechanisms by which Ras and Rap1 control chemotaxis, we performed a bioinformatics screen for proteins with potential Ras/Rap1 binding domains and identified Daydreamer (DydA), a member of the Mig10/RIAM/lamellipodin (MRL) family of adaptor proteins, as a new Ras/Rap1 effector. We show that DydA localizes to the leading edge of chemotaxing cells and plays key roles in regulating both F-actin polymerization and MyoII assembly. On the basis of the chemotactic defects of the DDB_G0287875 null strain described below, we have named the protein Daydreamer, or DydA.

To determine whether DydA plays a role in chemotaxis, we generated dydA− cells by homologous recombination and analyzed the chemotactic properties of these cells. dydA− cells exhibit strong chemotactic defects: when placed in a strong chemoattractant gradient emitted by a micropipette, dydA− cells polarize weakly, move with reduced speed and deeply reduced directionality, and have more lateral filopodia (Figure 1, B–D, and Supplemental Movies S1 and S2). In shallow, linear gradients produced in a Dunn chamber, the cells do not move (as noted in Figure 3C, which appears later in this article). The low, measured speed results from jiggling of the cells, which results in a change in the location of the cell’s centroid. Because of the slow, more random, and lackadaisical “pace” of chemotaxis, we named the gene daydreamer (dydA).

Tagged DydA complements the dydA− cell chemotactic defects and DydA− green fluorescent protein (DydA-GFP) localizes to the leading edge of chemotaxing and randomly moving cells (Figure 1, C and E), suggesting that DydA is involved in leading-edge signaling. GFP-DydA also transiently localizes from the cytosol to the plasma membrane in response to uniform (global) chemoattractant stimulation with peak localization at ~7–9 s (Figure 1F). Pretreatment with latrunculin B, an inhibitor of F-actin polymerization, results in a high basal level of DydA at the cortex with no additional recruitment upon chemoattractant stimulation, suggesting that F-actin plays a role in regulating DydA’s cortical localization (Figure 1G). dydA− cells exhibit a very elevated and extended first and second peak of F-actin polymerization and an extended and highly elevated second peak (Figure 1H). The level of assembled MyoII is elevated ~40% in unstimulated cells and, upon chemoattractant stimulation, the kinetics of MyoII are similar to those of wild-type cells (Figure 1I). These findings indicate that DydA plays a role in negatively regulating F-actin polymerization and MyoII assembly.

dydA− cells exhibit an extended and highly elevated plasma membrane localization of the PI(3,4,5)P_3 reporter PH_Yae1 in response to chemoattractant stimulation (Figure 2A), suggesting that PI3K activity is elevated and extended. As elevated levels of PI3P can result in dominant, gain-of-function phenotypes that result in multiple pseudopod formation, we tested whether addition of the PI3K inhibitor LY294002 suppressed the dydA− chemotactic phenotype and found that it did not (unpublished data). We also examined the regulation and activity of Akt/PKB and the related enzyme PKB1, both of which play key roles in regulating leading-edge function during chemotaxis (Meili et al., 1999, 2000; Kamimura et al., 2008). Akt/PKB and PKB1 are activated at the plasma membrane by two phosphorylations, as are mammalian Akt/PKBs (Sarbassov et al., 2005): the ALs are phosphorylated by the two PKD1 isoforms, PdkA and PdkB (Kamimura et al., 2008; Liao et al., 2010), while TORC2 phosphorylates the conserved hydrophobic motif (HM) of Akt/PKB and PKB1 (Lee et al., 2005; Kamimura et al., 2008; Cai et al., 2010; Liao et al., 2010). Chemoattractant-mediated plasma membrane localization of Akt/PKB is mediated through the binding of the PH domain of Akt/PKB to the PI3K product PI(3,4,5)P_3 (Meili et al., 1999;
**FIGURE 1**: dydA− cells exhibit chemotactic defects. (A) Domain structure of DDB_G0287875/Daydreamer. RA, Ras association domain; PH, pleckstrin homology domain; CH, calponin homology domain; PRM, proline-rich motif; S861 and T865, phosphorylated residues. (B) Live imaging of chemotaxing wild-type and dydA− cells. The source of the chemoattractant is located in the lower left corner of the images; images are at 5-min intervals over a 30-min time frame. (C) DIAS analysis of wild-type cells, dydA− cells, and dydA− cells expressing DydA-HHF chemotaxing toward a...
The specific activity of Akt/PKB kinase activity is similar to that in wild-type cells, the total level of Akt/PKB protein and kinase activity is ∼50% that of wild-type cells. The greatest effect of dydA− cells is on the AL phosphorylation of Akt/PKB, consistent with elevated levels of PI(3,4,5)P3 activity.

Role of the RA domains in controlling DydA function

The small GTPases Ras and Rap1 are major regulators of chemotaxis and lie upstream from PI3K and TORC2 (Lee et al., 1999; Funamoto et al., 2002, 2004, 2007; Sasaki and Firtel, 2006; Bolourani et al., 2006, 2008; Charest et al., 2010). As DydA has two RA domains, we examined the possible role of DydA on the
activation of Ras and Rap1. Ras activation was quantitated using pull-down assays employing two Ras-binding domains (RBDs) with different binding specificities: the Raf1-RBD (RBD Raf1), which preferentially binds RasG, RasD, and RasB, and the Byr2-RBD (RBD Byr2), which preferentially binds RasC and, with a lower affinity, RasG (Kae et al., 2004; Zhang et al., 2008). As previously reported (Kae et al., 2004; Sasaki et al., 2004; Zhang et al., 2008; Charest et al., 2010), using RBD Raf1, we found that wild-type cells exhibit a low basal level of Ras-GTP, which rises rapidly in response to chemoattractant stimulation with a peak at ~5 s and then returns quickly to basal levels. In contrast, the activity measured using RBD Byr2 peaks at ~10 s (Figure 2F). This difference in the observed Ras activation kinetics in wild-type cells using the RBD Raf1 versus the RBD Byr2 probe is consistent with previous findings that RasG activation peaks at ~5 s, whereas RasC peaks at ~10 s (Kae et al., 2004; Sasaki et al., 2004; Zhang et al., 2008). Surprisingly, in dydA− cells, Ras activation using the RBD Raf1 probe is not increased, but is slightly decreased and delayed (Figure 2F), suggesting that the cause for the greatly extended and elevated cortical localization of PH ctsk is independent of the level of Ras activation. Ras activity as measured using RBD Byr2 is unaltered in dydA− cells compared with that in wild-type cells. Similarly, we found chemoattractant-mediated Rap1 activation to be unaltered in these cells (Figure S1B).

To determine whether the DydA RA domains bind Ras and/or Rap1, similar to RA domains of other MRL members (Lafuente et al., 2004), we expressed RA1 and RA2 as GFP fusions in wild-type cells and used the glutathione S-transferase (GST) fusions of the GDP and GTP forms of RasG, RasC, and Rap1 (RapA), and found that GFP-RA1 was only pulled down by RasG-GTP/S from Dictyostelium cell lysates (Figure 3A). We also did not observe binding of the activated form of Rap1 with a DydA fragment containing RA1 and the adjacent PH domain (unpublished data). None of the Ras proteins pulled down GFP-RA2. We also examined the ability of the activated (GppNHp-bound) form of recombinant GST-RasC, -RasG, -RasD, which is closely related to RasG, and -Rap1 to bind recombinant RA1 and RA2 and found that only RasG- GppNHp and RasD-GppNHp showed any detectable binding (Figure S1C). Finally, we examined the ability of recombinant RA1 to stimulate the dissociation of mGppNHp from RasG, an assay of RA1 binding to RasG-GTP. In agreement with our other data indicating RasG-GTP binds RA1, we show that RA1 has GDI activity by demonstrating that addition of RA1 inhibits the dissociation of 2′,3′-O-(N-Methylanthraniloyl)-guanosine-5′-[(β,γ)-imido]triphosphate (mGppNHp) from RasG and that the inhibition depends upon the concentration of RA1 (Figure S1D). As RasG is expressed throughout growth and aggregation, and RasD is preferentially expressed during multicellular development (Reymond et al., 1984; Robbins et al., 1989), we suggest that RasG is the major Ras protein that binds to DydA through RA1. We cannot exclude that another Ras protein we have not tested may interact with either RA domain.

To further understand the role of the two RA domains, we examined the phenotypes of dydA− cells expressing His-hemagglutinin-FLAG (HHF)-tagged DydA lacking both RA domains (DydAΔRA1+2-HHF) and found that the cells exhibit strong chemoattractant phenotypes, indicating that the RA domains are required for DydA function. In a steep gradient emitted by a micropipette, the cells are less polarized and move slightly more slowly than wild-type cells. In a shallow, linear gradient produced in a Dunn chamber (Zicha et al., 1991), the cells are unable to migrate and the phenotype is as severe as that observed for dydA− cells (Figure 3, B and C). When we analyzed the profile of F-actin polymerization and MyoII assembly in dydA− cells expressing DydAΔRA1+2-HHF, we found that both peaks of F-actin polymerization are reduced compared with wild-type cells and that the first peak of actin decreases much faster than in wild-type cells (Figure 3D). This suggests that DydAΔRA1+2 inhibits F-actin polymerization and facilitates actin depolymerization. We also found that MyoII assembly is dramatically reduced in dydA−/DydAΔRA1+2 cells compared with wild-type cells (Figure 3E). These findings were unexpected, as dydA− cells show elevated basal levels of assembled MyoII but normal kinetics and levels of chemoattractant-mediated assembly, suggesting that DydAΔRA1+2, directly or indirectly, inhibits MyoII assembly and F-actin polymerization. In addition, we found that the phosphorylations of the HM and AL sites of Akt/PKB and PKB1 are extended, as is the case for dydA− cells (Figure 3, F and G, and quantitation in Figure S1A). DydAΔRA1+2-GFP localizes to the leading edge of chemotaxing cells (Movie S3). Interestingly, DydAΔRA1+2-GFP exhibits a highly elevated level of cortical localization upon chemoattractant stimulation but with response kinetics similar to those of wild-type cells (Figure 1F). These observations, together with the fact that the activation of Rap1 is not severely altered in dydA− cells, suggest that DydA might act as a downstream effector of Ras that directly or indirectly regulates F-actin polymerization and MyoII assembly. Furthermore, the RA domains are required for modulating the regulation of DydA function but not its localization per se.

The CH domains and the PRM, but not the PH domain, are involved in localizing DydA to the leading edge. The MRL family member lamellipodin is recruited to the leading edge of fibroblasts upon platelet-derived growth factor (PDGF) stimulation through the interaction of its PH domain with phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2; Krause et al., 2004). As DydA localizes to the leading edge of chemotaxing Dictyostelium cells, we tested whether this localization requires the PH domain of DydA. We found that DydAΔPH-GFP still localizes to the leading edge of migrating cells, translocates to the membrane upon global stimulation with kinetics similar to DydA-GFP, and complements the dydA− cells’ chemoattractant phenotypes, indicating the PH domain is not required (Figures 3, H and I, and S1F). We used the PH domain of DydA in a lipid-binding assay and found that it predominantly binds phosphatidylinositol 3-phosphate (PI(3)P), a phospholipid predominantly found in endosomes and phagosomes, but not PI(3,4)P2 or PI(3,4,5)P3, products of class I PI3Ks (Figure S1E). We also found that treatment of dydA− cells expressing GFP-DydA with the PI3K inhibitor LY294002 does not block GFP-DydA recruitment to the plasma membrane, indicating that PI3K is not required for the recruitment to the plasma membrane (unpublished data).

Interestingly, the stimulus-dependent cortical translocation of DydA was delayed and reduced in the absence of both CH domains (dydA−/DydAΔCH1+2-GFP; Figure 3I), indicating a function of the CH domains in DydA cortical localization. The deletion of both CH domains causes defects during chemotaxis: in a steep gradient, the cells move with a slightly higher speed than wild-type cells, but they turn more often than wild-type cells, and we observed an increase in bifurcated pseudopodia (Figure S1C), a phenotype that has been reported for mutants of the SCAR/WAVE complex (Blagg et al., 2003). DydA+/DydAΔCH1+2-HHF cells exhibit an extended F-actin first peak and an elevated second peak (Figure 3D). These cells also exhibit an elevated basal level of assembled MyoII and kinetics similar to those of dydA− cells (Figure 3E), suggesting the CH domains are important in modulating the levels of F-actin and assembled MyoII. In addition to the CH domains, the PRM plays a role in the cortical localization of DydA in response to a chemoattractant stimulus.
DydA ΔPRM-GFP shows reduced localization to the cortex in response to chemoattractant stimulation, and dydAΔ cells expressing DydA ΔPRM-HHF show reduced migration speed (Figures 3I and S1C); a deletion of the CH domains together with the PRM domain further reduces the localization of the protein to the cortex and the leading edge (Figure 3I), indicating that these domains contribute to the localization of DydA to the leading edge but are not solely responsible for DydAs subcellular localization. The PRM from the mammalian MRL member lamellipodin binds VASP, which is required for lamellipodin function. We tested whether the DydA PRM binds to Dictyostelium VASP in a pull-down assay and were unable to detect any binding (unpublished data). Furthermore, unlike the PRM of lamellipodin, which has a sequence context similar to that of other VASP-binding PRM domains, the sequence of the DydA PRM does not. Taken together, our findings indicate that the CH and PRM domains, but not the PH domain, contribute to the localization of DydA to the leading edge of chemotaxing cells and are important for the function of DydA in regulating the cytoskeleton.

GSK-3 phosphorylates DydA and is required for some, but not all, DydA functions

We previously reported using total-cell phosphoproteomic analysis of unstimulated (0 s, basal) cells and cells stimulated for 10 s (the time of maximum activation of many leading-edge pathways) and 60 s (adaptation) to identify proteins that are differentially phosphorylated in response to chemoattractant stimulation (Charest et al., 2010). These phosphoproteomic data indicated that in wild-type cells, DydA is constitutively phosphorylated at Ser860/861 and Thr865, which lie between the PRM and the C-terminal RA2 domain (Figure 1A; unpublished data). Treatment of DydA ΔHHF purified from dydAΔ cells with λ-protein phosphatase results in an increase in DydA ΔHHF mobility on SDS gels, consistent with the protein being phosphorylated (Figure 4A).

The identified phosphorylation site in DydA matches the GSK-3 consensus motif Ser/Thr-(X-X-X)-pSer/pThr, with X being any amino acid (Figure 4B). GSK-3 has a unique substrate specificity whereby, for efficient phosphorylation by GSK-3, many substrates require a Ser/Thr priming phosphorylation at a site located four residues C-terminal to the site of GSK-3 phosphorylation, with both the priming phosphorylation and the phosphorylation by GSK-3 often being preferentially directed (Ser/Thr-Pro-Pro-X)-pSer/Thr-Pro (Frame et al., 2001). As the DydA phosphorylation site conforms to a GSK-3 consensus site with both sites followed by a Pro (Figure 4B), we repeated the whole-cell phosphoproteomic analysis of wild-type cells and, at the same time, cells lacking GSK-3 (gskAΔ cells) before and at 10 and 60 s after chemoattractant stimulation. When compared with wild-type cells, the phosphorylation of DydA at the GSK-3 site in gskAΔ cells is dramatically reduced, which is consistent with a decrease in the mobility of DydA ΔHHF purified from gskAΔ cells compared with that from dydAΔ cells (wild-type for gskA) on SDS gels (Figure 4, C and D).

To verify that DydA is a direct target of GSK-3, we expressed DydA ΔHHF in gskAΔ cells, immunoprecipitated the tagged protein, and used it in an in vitro kinase assay with recombinant human GSK-3β. Figure 4D illustrates that DydA can be directly phosphorylated by GSK-3β in vitro. Phosphorylation data on DydA carrying Ala mutations of Ser860 or Ser861 together with Thr865 indicate that Ser861 is the residue that is preferentially phosphorylated by GSK-3β, which is consistent with the consensus site for GSK-3 (Figure 4D). Unexpectedly, DydA ΔHHF in which three Ser residues (Ser859-861) are mutated to Ala exhibits moderate phosphorylation (Figure 4D). A possible explanation for this observation might be that these mutations lead to changes that make the adjacent Ser highly accessible for GSK-3β. The in vitro kinase assay, together with the phosphoproteomic findings, suggests that DydA is a direct target for GSK-3 in vivo. Interestingly, we observed some phosphorylation of DydA in gskAΔ cells (Figure 4, C and D). The phosphorylation occurs to a very small extent in the GSK-3 consensus motif, but mainly on adjacent Ser/Thr residues (see Discussion).

To examine the importance of phosphorylation by GSK-3 for the function of DydA, we expressed the nonphosphorylatable form of Daydreamer (DydA861A/865A-HHF) in dydAΔ cells. In a steep chemotactic gradient, these cells migrate more slowly than wild-type cells, but the defects are less severe than in the dydAΔ cells (Figure 4E). However, in a shallow, linear gradient, these cells do not migrate at all and the defects are as strong as those of dydAΔ cells (Figure 4F), indicating that phosphorylation of DydA by GSK-3 plays an important role for the function of DydA during chemotaxis. Like dydAΔ cells, dydAΔ cells expressing DydA861A/865A-HHF exhibit increased MyoII assembly upon chemoattractant stimulation (Figure 3E). The elevated and extended phosphorylation of the AL and HM motifs of Akt/PKB and PKBR1 exhibited by dydAΔ cells is only partially suppressed by expressing DydA861A/865A-HHF, while the elevated F-actin response is fully suppressed (Figures 3, D, F, and G, and S1A). These observations suggest that some, but not all, functions of Daydreamer require phosphorylation by GSK-3. We tried to generate a phosphomimetic version of DydA by mutating Ser861 to Glu and Thr865 to Asp. Expression of DydA861E/865D-HHF in dydAΔ cells does not complement dydAΔ phenotypes but produces dominant effects: wild-type as well as dydAΔ cells expressing DydA861E/865D exhibit deeply reduced speed and lower directionality (unpublished data).

gskAΔ cells exhibit strong chemotactic defects

As DydA requires phosphorylation by GSK-3 for full function, we wanted to understand the role of GSK-3 in context with GSK-3’s overall role in chemotaxis. To this end, we conducted an analysis of the phenotype of gskAΔ cells similar to that which we performed on dydAΔ cells. Consistent with previously reported observations, we found that gskAΔ cells exhibit strong chemotactic defects (Teo et al., 2010; Kim et al., 2011; Figure 5, A and B, and Movies S4 and S5). The cells are less polarized, show a reduced speed compared with wild-type cells, lack directionality in their migration, and move almost randomly in a chemoattractant gradient emitted by a micropette. These defects are more severe than those of dydAΔ cells. During development, gskAΔ cells form small aggregates through random collision and adhesion rather than directed migration toward each other (Figure S2A). The migration parameters of developed, “aggregation-competent” gskAΔ cells migrating in a chemoattractant gradient are similar to those of cells moving randomly in the absence of a chemoattractant gradient (Figure 5A), suggesting that GSK-3 has an important role in gradient sensing rather than cell motility and/or linking directional sensing with persistent, localized F-actin polymerization at the site on the cortex closest to the chemoattractant source. In addition, the analysis of random movement of vegetative cells demonstrates that gskAΔ vegetative cells move faster than wild-type cells (Figure 5C), suggesting that the lack of efficient chemotaxis of gskAΔ cells is not due to defects in motility.

The level of F-actin polymerization in gskAΔ cells is similar to that of wild-type cells, but the first peak decreases faster than in wild-type cells (Figure 5D). However, the level of MyoII assembly upon chemoattractant stimulation is reduced dramatically in gskAΔ cells (Figure 5E). Interestingly, the rapid decrease of the first peak of F-actin and the dramatic reduction in MyoII assembly are also observed in dydAΔ cells expressing DydA ΔRA1+2-HHF. Because MyoII assembly is
FIGURE 3: Role of the DydA domains in controlling DydA function. (A) Pull down in Dictyostelium lysate with the indicated inactive (GDP) or active (GTP) bound GST-GTPase as bait and GFP-DydRA1 as prey. The amount of prey and bait (bottom panel) were detected by Western blotting using antibody specific for GFP or GST, respectively. (B and C) DIAS analysis of chemotaxis of dydA− and wild-type cells in response to a chemoattractant gradient produced by a micropipette (B) or in a Dunn chamber (C). Data represent mean ± SD; speed indicates the speed of the cells’ centroid movement along the total path; directionality indicates the linearity of the migration paths; direction change is...
regulated by Rap1 (Kortholt et al., 2006; Jeon et al., 2007), we analyzed the activation of Rap1 using RBDRasGDP in pull-down assays and observed a slight increase in the basal level of activated Rap1 and a slightly lower level of Rap1 activation on stimulation in gskA− cells compared with wild-type cells (Figure S2B). But these slight differences cannot account for the dramatic reduction of Myosin II observed in the gskA− cells. This indicates that GSK-3 affects Myosin assembly mainly downstream from or independent of Rap1, possibly through DydA, which also regulated Myosin assembly.

Because of the strong directionality defects exhibited by gskA− cells, we examined the activity of Ras using the two RBDs with different binding specificities, RBDRasR1 and RBDRasR2, as described for DydA. Compared with wild-type cells, the kinetics of Ras activation using RBDRasR1 are delayed and extended in gskA− cells, with a broader peak centering at 10–20 s and with high levels of Ras-GTP still present at 30 s (Figure 6A). In contrast, when using RBDRasR2, we found that the basal level of Ras-GTP was highly elevated and increased only slightly upon stimulation to the wild-type level and then decreased slowly to the high basal level observed in unstimulated gskA− cells, indicating a misregulation of Ras levels and kinetics in gskA− cells. This is in sharp contrast to dydA− cells, which show almost normal Ras regulation.

As discussed in the DydA Ras analysis, of the tested Ras proteins, RasC-GTP preferentially binds to RBDRasR2, suggesting the elevated Ras-GTP levels assayed using the RBDRasR2 probe are due to elevated RasC-GTP. We tested this directly by expressing epitope-tagged RasC in wild-type and gskA− cells and assaying the kinetics and relative levels of RasC-GTP. Unexpectedly, we found that the normalized level of RasC activation was slightly lower in gskA− compared with that in wild-type cells. We suggest that the elevated Ras activation assayed by the RBDRasR2 probe is a different Ras and one that has not been previously characterized.

Because the kinetics of Ras activation in gskA− cells are highly aberrant, we investigated two known Ras effectors, TORC2 and PI3K, by analyzing the phosphorylation of the HM and AL of Akt/PKB and PKBR1. Compared with our observations in wild-type cells, both Akt/PKB and PKBR1 have a delayed peak and extended kinetics of HM phosphorylation in gskA− cells (Figures 6B and S2C). The phosphorylation of the AL is extended in Akt/PKB and PKBR1, with the effect being most pronounced on the Akt/PKB AL (Figures 6B and S2C). Akt/PKB shows a highly increased basal phosphorylation of the AL. We see a corresponding higher basal activity for the Akt/PKB kinase and greatly extended chemotactant-mediated activation of Akt/PKB and PKBR1 kinase activity (Figure 6, C and D). These findings are in conflict with the results of Teo et al. (2010), who observed no phosphorylation of the AL of Akt/PKB and PKBR1 in gskA− cells. Our observations suggest that the regulation of Ras, Akt/PKB, and PKBR1 is highly aberrant in gskA− cells.

To determine whether the highly extended phosphorylation of the Akt/PKB AL is the result of elevated PI3K activity, which could result in an extended localization of Akt/PKB at the plasma membrane, we examined the kinetics and extent of plasma membrane localization of the PI(3,4,5)P3-responsive reporter PHfluo-GFP. Unexpectedly, we found that PHfluo-GFP has a deeply reduced level of chemotactant-stimulated membrane localization in gskA− compared with wild-type cells, but with the peak level remaining for a more extended time than that in wild-type cells (Figure 6E; Teo et al., 2010). When we examined the kinetics of extent of chemotactant-stimulated PI3K cortical localization using GFP-PI3Kloc (a PI3K localization reporter, rather than full-length PI3K, which leads to elevated PI(3,4,5)P3 levels and aberrant phenotypes [Funamoto et al., 2002]), we found that, like the PI(3,4,5)P3 levels assayed using PHfluo-GFP, the proportion of PI3K that localized to the cortex was reduced, and the kinetics were extended (Figure 6F). These results indicate that changes in the phosphorylation of Akt/PKB in gskA− cannot be explained by increases in PI(3,4,5)P3 levels or PI3K activity.

**DISCUSSION**

**Role of DydA in regulating chemotaxis**

We have identified DydA, a new member of the MRL family of adaptor proteins, and show that it is required for multiple regulatory pathways that control directional sensing and cell motility. dydA− cells move more slowly than wild-type cells in a steep chemotactic gradient and with a significant reduction in directionality compared with that of wild-type cells. Thus DydA is required for both cell motility and directional sensing. In response to chemotactant stimulation, a number of signaling pathways are activated normally in dydA− cells, indicating that the cells are not generally deficient in responding to chemotactant. We suggest that DydA is required to efficiently amplify the chemotactant gradient, as the cells exhibit significantly more severe defects in shallow than steep gradients. Because DydA is required for multiple chemotactic pathways, binds RasG-GTP via a domain required for its functions, and requires phosphorylation by GSK-3 for full function, we propose that DydA helps integrate the function of several signaling pathways to regulate cell polarity and chemotaxis.

The DydA RA1 domain binds RasG-GTP, but not RasC-GTP (another Ras required for chemotaxis in Dictyostelium), and DydA lacking RA1/2 is unable to complement dydA− phenotypes and results in dominant-negative phenotypes when expressed in wild-type cells. We are unable to test, and therefore directly demonstrate, that RasG activation is required for DydA, because of the strong phenotypes of rasG− and RasG-gain-of-function strains. However, as Ras activation is not greatly altered in dydA− cells, we suggest that DydA lies downstream of RasG-GTP. This result, combined with the requirement of the RA domains, is consistent with a model that DydA plays a key role in regulating RasG-mediated polarity and directionality. Furthermore, consistent with DydA's role in directional sensing, DydA localizes to the leading edge of migrating cells and transiently to the cell cortex in response to chemotactant stimulation in a process that requires, in part, the CH domains and the PRM. Although the RA domains are required for DydA function, they are not required to localize DydA to the cortex but may negatively modulate DydA cortical binding as DydA lacking these
FIGURE 4: Phosphorylation of DydA by GSK-3. (A) Phosphatase treatment of DydA-HHF. Phosphorylation of DydA from dydA− cells was determined in the presence or absence of λ-protein-phosphatase. (B) Phosphorylation site of DydA and the GSK3 consensus motif. (C) Phosphoproteomic data of DydA in wild-type and gskA− cells. Phosphorylation occurs in three different clusters of Ser/Thr. (D) In vitro kinase assay with DydA-HHF. Phosphorylation of DydA-HHF and DydA-HHF carrying Ala mutations in the GSK-3 consensus motif purified from gskA− cells was examined in the presence and absence of GSK-3β. (E and F) DIAS analysis of chemotaxis of dydA−/DydA S861A/T865A-HHF and control cells in response to a chemoattractant gradient produced by a micropipette (E) or in a Dunn chamber (F). Data represent mean ± SD; speed indicates the speed of the cells’ centroid movement along the total path; directionality indicates the linearity of the migration paths; direction change is a relative measure of the number and frequency of turns of the cells; roundness is a measure of the polarization of the cells.
domains exhibits a highly elevated cortical binding. We also observed an extended phosphorylation of the ALs of AKT/PKB and PKBR1, which is consistent with the extended activity of PI3K based on PH-domain localization studies. However, the relative levels and kinetics of chemoattractant-mediated kinase activity are only slightly extended compared with wild-type cells.

Our analyses indicate that DydA is required for the regulation of the cytoskeleton. dydA− cells exhibit a highly elevated first and second peak of F-actin assembly. As the basal level of F-actin is similar to that seen in wild-type cells, we suggest that DydA functions to negatively regulate the extent of chemoattractant-mediated F-actin polymerization, although we have not demonstrated that this is a direct effect. This is in contrast to the MRL protein lamellipodin, which stimulates F-actin polymerization at the leading edge through the recruitment of VASP. We also found that the basal levels of MyoII are elevated in dydA− cells. A direct role for DydA in F-actin polymerization and MyoII assembly downstream of Ras family GTPases is also supported by the considerable inhibition of both responses in dydAΔRA1+2 cells expressing DydAΔRA1+2. We suggest that DydAΔRA1+2 acts as an active form of DydA, binds to and blocks the function of DydA effectors, and inhibits the regulation of F-actin assembly and/or promotes faster F-actin depolymerization.

FIGURE 5: gskA− cells exhibit severe chemotactic defects. (A) DIAS analysis of chemotaxis and randomly moving aggregation-competent wild-type and gskA− cells. Data represent mean ± SD; speed indicates the speed of the cells’ centroid movement along the total path; directionality indicates the linearity of the migration paths; direction change is a relative measure of the number and frequency of turns of the cells; roundness is a measure of the polarization of the cells. (B) Trajectories of cells moving toward CAMP. The plot represents the angle between the start and the end point of a cell relative to the chemoattractant gradient, represented by the vertical line. (C) DIAS analysis of randomly moving vegetative cells. Data represent mean ± SD; speed indicates the speed of the cells’ centroid movement along the total path; directionality indicates the linearity of the migration paths; direction change is a relative measure of the number and frequency of turns of the cells; roundness is a measure of the polarization of the cells. (D and E) cAMP-induced F-actin polymerization and MyoII assembly, respectively, in gskA− cells. Data represent the mean ± SE of at least three independent experiments, with the starting condition in wild-type cells taken as 1.0.
FIGURE 6: Regulation of effector pathways by GSK-3. (A) Ras activation in gskA<sup>−</sup> cells. cAMP-induced Ras activation was assessed using RBD<sub>Raf1</sub> and RBD<sub>Byr2</sub> in pull-down assays. Levels of total Ras and Ras-GTP were detected by immunoblotting with an anti–pan-Ras antibody and quantified, with the maximum value of wild-type cells taken as 1.0; data are mean ± SD of at least three independent experiments. (B) Phosphorylation of the HM and AL of Akt/PKB and PKBR1 in wild-type and gskA<sup>−</sup> cells in response to cAMP. Coomassie Blue staining was used as loading control. (C and D) cAMP-induced Akt/PKB and PKBR1 kinase activity, respectively. H2B was used as substrate; Akt/PKB and PKBR1 protein levels were determined by immunoblotting; for quantification, the maximum value of wild-type cells was taken as 1.0; data are mean ± SE of at least three independent experiments. (E and F) Translocation kinetics of PH<sub>crac</sub>-GFP (E) and N-termPI3K-GFP (a reporter for PI3K localization) (F) in wild-type and gskA<sup>−</sup> cells in response to cAMP stimulation. The data represent the mean ± SE of relative fluorescence intensity of membrane-localized PH<sub>crac</sub>-GFP and PI3K-GFP as a function of time after cAMP stimulation with the starting condition taken as 1.0.
DydA function requires GSK-3

Through in vitro and in vivo studies, we verified DydA as a direct target of GSK-3 and show that this phosphorylation is required for many of DydA functions. As the nonphosphorylatable form of DydA complements some DydA functions and localizes to the cell cortex and appears stable, we do not believe the mutant protein is misfolded. Our data indicate that DydA affects multiple functions required for chemotaxis. On the one hand, it is regulating F-actin polymerization, which seems to be independent of the GSK-3 phosphorylation. On the other hand, DydA is negatively regulating MyoII assembly, a function that is dependent of the phosphorylation by GSK-3.

We observed a small amount of phosphorylation of the GSK-3 site of DydA, even in the absence of GSK-3, and at Ser/Thr residues adjacent to the GSK-3 consensus site. We do not know whether this phosphorylation of DydA in the absence of GSK-3 has any biological relevance, because this phosphorylation appears only in the absence of GSK-3 and our data indicate that under normal conditions, DydA is constitutively phosphorylated, presumably by GSK-3 and a priming kinase. The absence of GSK-3 might make this site accessible to other kinases, resulting in some phosphorylation at sites independent of the GSK-3 consensus site. In addition, there is a GSK-3-like kinase in Dictyostelium encoded by the gka gene (DDB_G0270218; Goldberg et al., 2006) that might be responsible for the residual phosphorylation in the absence of GSK-3. We do not know whether Gka is expressed at aggregation stage or whether it acts as a functional kinase recognizing the same consensus site as GSK-3. The stimulus-independent, constitutive phosphorylation of DydA supports the role of GSK-3 as a permissive signal as proposed by Teo et al. (2010). However, we cannot exclude the possibility that DydA phosphorylation is dynamically regulated with a rapid cycle of phosphorylation/dephosphorylation.

GSK-3 is a major regulator of chemotaxis in Dictyostelium

Little was understood about the function of GSK-3 in chemotaxis in Dictyostelium. Our study uncovered a new component of the GSK-3 regulatory network that helps mediate chemotaxis. Previous studies had demonstrated that gskA cells have highly aberrant chemotaxis, and one suggested this was due to a loss of PI3K regulation and could be suppressed by overexpression of inositol monophosphate kinase (IMP-A), which increases PI(3,4,5)P3 by increasing the levels of the PI3K substrate PI(4,5)P2 (Teo et al., 2010). As increased PI(3,4,5)P3 can cause gain-of-function phenotypes, it is possible that the suppression of the gskA chemotactic phenotype by IMP-A may be indirect. Others’ findings and our own suggest that PI(3,4,5)P3 levels are reduced two- to threefold based on PH-domain localization studies (Teo et al., 2010). However, as loss of PI3K has only modest effects on chemotaxis in step gradients (Funamoto et al., 2002; Hoeller and Kay, 2007; Takeda et al., 2007; Veltman et al., 2008), it is unlikely that decreased PI(3,4,5)P3 results in the severe gskA phenotypes.

We provide evidence that Ras activity is highly aberrant in gskA cells, suggesting a basis for the severe defects in directional sensing observed in the gskA cells. The high basal Ras activity measured using the RBD-GFP might be due to an up-regulation of basal RasC activity. The RasC effector TORC2 was thought to be inactive in the gskA cells (Teo et al., 2010), but in our hands, TORC2 was activated in the absence of GSK-3 and strongly phosphorylated the HM of Akt/PKB and PKBR1. This is thought to be a prerequisite for the AL phosphorylation mediated by the two PKD1 isoforms, PkdA and PkdB (Kamimura et al., 2008; Liao et al., 2010). PkdA mainly phosphorylates the AL of Akt/PKB and only partially affects PKBR1 phosphorylation, whereas PkdB exclusively phosphorylates the AL of PKBR1. Kamimura and Devreotes (2010) demonstrated that transient PkdA activation is independent of PI(3,4,5)P3 and TORC2, but that RasC may directly affect PkdA activity (Kamimura and Devreotes, 2010). Therefore increased RasC activity in the gskA cells might lead to increased PkdA activity and higher phosphorylation of the AL of Akt/PKB and higher Akt/PKB activity. Because the AL of PKBR1 is also phosphorylated by PkdB, effects are less prominent for PKBR1 AL phosphorylation. At this point, we do not know how GSK-3 might affect RasC activity. It was unexpected that the highly increased and extended activity of Akt/PKB and PKBR1 in gskA cells did not lead to severe changes in F-actin polymerization. It might be possible that the dramatic decrease in PI(3,4,5)P3 counterbalances the effects of increased Akt/PKB and PKBR1 activity on F-actin polymerization.

Our findings that cells expressing the nonphosphorylatable form of DydA exhibit extended AL and HM phosphorylation suggest that DydA might mediate, in part, GSK-3’s role in the regulation of the phosphorylation of PKB and PKBR1. In addition, the effect of GSK-3 on MyoII assembly might be mediated through DydA, since DydA lacking both RA domains exhibits a similar defect in MyoII assembly. It is clear that the phenotype of gskA cells is complex, and GSK-3 most likely functions through a number of substrates, including DydA, as part of a larger network that controls chemotaxis. Our phosphoproteomic analysis suggests additional substrates for GSK-3. However, additional biochemical and genetic analyses will be required to demonstrate these are bona fide GSK-3 substrates and that phosphorylation by GSK-3 is required for their function.

MATERIALS AND METHODS

Cell culture

Cells were grown in axenic HL5 medium at 22°C. For the expression of GFP- or epitope-tagged proteins, 20 μg/ml Geneticin (Gibco, Grand Island, NY) was added. Knockout cell lines were selected with 7.5 μg/ml blasticidin S. For obtaining aggregation-competent cells, log-phase vegetative cells were washed with 12 mM Na/K phosphate buffer, resuspended at 5 × 10⁶ cells/ml in 12 mM Na/K phosphate buffer, and pulsed with 30 nM cAMP at 6-min intervals for 5.5 h. Aggregation-competent cells were used for the assays unless otherwise indicated.

Strains and constructs

Ax-2 or KAx-3 were used as wild-type. gskA cells were obtained from the Dictyostelium Stock Center. We confirmed that these were gskA cells by Southern analysis. We also did not identify any GskA peptides identified in wild-type cells in the gskA strain using mass spectrometry, confirming that the protein was not present at detectable levels (unpublished data). We also tested the gskA strain for the expression of two aggregation genes (encoding the phosphodiesterase [PDE] psdA and the adenyl cyclase AcaA) under our conditions used to study gskA cells (5.5 h cAMP pulsing), which were found previously to be poorly expressed in gskA cells plated for development compared with wild-type cells (Teo et al., 2010). We found that AcaA exhibited normal expression, while psdA expression was not normal (Figure S2D). We assume the difference between our results and those of Teo et al. (2010) are due to different conditions of development between the experiments reported here and those of Teo et al. (2010).

Gene disruption for DydA was performed by insertion of a blastidin-S resistance cassette at base pair 561. Clones were selected in the presence of blastidin S, and gene disruption was confirmed by PCR and Southern blotting. DydA was cloned from cDNA
(3636 base pairs). Deletion constructs and constructs carrying point mutations for DydA were generated using QuikChange site-directed mutagenesis (Stratagene, Agilent, Santa Clara, CA). For DydA, the following deletions were generated:

DydΔAR1 + 2: deletion of base pairs 13–270 + 3334–3558 (aa 5–90 + 1112–1196)
DydΔPH: deletion of base pairs 397–675 (aa 133–225)
DydΔACH1 + 2: deletion of base pairs 835–1500 (aa 279–500)
DydΔAPRM: deletion of base pairs 1756–2106 (aa 586–702)

GFP- or His-HA-FLAG (HHF) epitope-tagged versions of Daydreamer were generated in Exp-4 (obtained from the Dictyostelium Stock Center) carrying 3' tags for GFP or HHF. GST-DydA1,2,798 (aa 1–266) and the constructs for the yeast two-hybrid system were generated using Gateway Cloning (Invitrogen, Carlsbad, CA), with pDEST21 as the entry vector and pDEST15 or pDEST22 and pDEST32 as the expression vector(s).

Chemotaxis and cell motility
Chemotactic analysis, the analysis of the global response to a chemoattractant, and vegetative cell motility analysis were performed as previously described (Chung and Firtel, 1999; Sasaki et al., 2007; Wessels et al., 1998; Zhang et al., 2008).

Communoprecipitation
Log-phase vegetative cells (1 × 10⁹) were washed with 12 mM Na/K phosphate buffer and lysed in lysis buffer (50 mM HEPES, pH 8, 150 mM NaCl, 0.5% Triton-X100, 10% glycerol, protease inhibitors, phosphatase inhibitors) for 1 h at 4°C. For communoprecipitation after stimulation, aggregation-competent cells were stimulated with 1 μM cAMP, different time points were collected, and the cells were lysed in buffer for 1 h at 4°C on a rotator. After centrifugation, the supernatant was incubated with 6 μl of 50% FLAG-agarose beads (Sigma-Aldrich, St. Louis, MO) for 1 h at 4°C on a rotator. The beads were washed three times with lysis buffer and used for kinase assays or phosphatase treatment.

Phosphatase treatment
After communoprecipitation, the beads were washed once in lysis buffer without phosphatase inhibitors and once in phosphatase buffer and then incubated with 800 U α-λ-protein phosphatase (NEB, Ipswich, MA) for 30 min at 37°C. The beads were resuspended in SDS-sample buffer and separated on SDS–PAGE, and the protein was detected with α-FLAG M2 antibody (Sigma-Aldrich).

In vitro kinase assay
After communoprecipitation, the beads were washed once in kinase buffer (5 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.4 mM EDTA, 4 mM MgCl₂, 0.05 mM dithiothreitol [DTT], 40 ng/μl bovine serum albumin) and then incubated with 100 ng human recombinant GSK-3β (Cell Signaling Technology, Danvers, MA) in the presence of radiactively labeled ATP (250 μM, 2 μCi/ μl) for 20 min at room temperature. All liquid was removed, the beads were resuspended in SDS-sample buffer, and the proteins were separated on SDS–PAGE. The signals were detected by autoradiography.

Ras and Rap1 activity assays
Ras activity assays were performed as previously described (Sasaki et al., 2004; Zhang et al., 2008). Rap1 activity assays were performed as described previously (Jeon et al., 2007). The experiment was repeated at least three times on separate days with internal wild-type controls. The Western blots were quantified using ImageJ.

F-actin polymerization and MyoII assembly
Log-phase vegetative cells were washed with 12 mM Na/K phosphate buffer and resuspended at 5 × 10⁶ cells/ml in 12 mM Na/K phosphate buffer. The cells were starved for 1 h and then pulsed with 30 nM cAMP at 6-min intervals for 4.5 h. The cells were washed once with 12 mM Na/K phosphate buffer and incubated for 30 min before stimulation. After cells were stimulated with 1 μM cAMP, different time points were collected, and cytoskeletal proteins were isolated as described previously (Steimle et al., 2001). The samples were separated on SDS–PAGE and stained with Coomassie Blue. Protein amounts were quantified using ImageJ. The experiment was repeated at least three times on separate days with internal wild-type controls.

Phalloidin staining
Phalloidin staining was performed as described previously (Chung et al., 2000).

Akt/PKB kinase activity assay
Akt/PKB and PKBR1 kinase activities were performed and analyzed as described previously (Meili et al., 1999). H2B was used as a substrate. The experiment was repeated at least 3 times on separate days with internal wild-type controls. The Western blots were quantified using ImageJ.

PKB/PKB1 phosphorylation
Aggregation-competent cells were stimulated with 1 μM cAMP. Different time points after stimulation were lysed in 2× SDS-sample buffer, and equal amounts of total protein were separated on SDS–PAGE. Phosphorylation of Akt/PKB and PKBR1 at the HM was detected using α-phospho-p70 S6 kinase antibody (Cell Signaling Technology, Danvers, MA), and phosphorylation at the AL was detected using α-phospho-protein kinase C (pan) antibody (Cell Signaling Technology; Kamimura et al., 2008). Coomassie Blue staining of total proteins was used as loading control. The experiment was repeated at least three times on separate days with internal wild-type controls. The Western blots were quantified using ImageJ.

Lipid-binding assay
GST-DydA1,2,798 and GST alone were expressed in bacteria and purified using glutathione-Sepharose 4B beads (GE Healthcare, Waukesha, WI) according to the manufacturer’s protocol. PIP strips from Echelon Biosciences (Salt Lake City, UT) were incubated with 0.5 μg/ml protein following the provided protocol. The GST proteins were detected using α-GST antibody (Cell Signaling Technology).

Pull-down experiments
N-terminal GFP-tagged DydRa1 and DydRA2 were expressed in Dictyostelium from the previously published pDM317 plasmid (Veltman et al., 2009). Cells were lysed by incubation in lysis buffer (10 mM Na₂HPO₄, pH 7.2, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM Na₂VO₄, 5 mM NaF, and protease inhibitor cocktail [Roche, Indianapolis, IN]) for 10 min on ice. Unsoluble proteins were spun down, and precleared lysate was used in pull down with different small G-protein samples. GST fusion of c-truncated small G proteins (RapA, RasC, and RasG) were
purified as described before (Kortholt et al., 2006). Proteins were loaded with nucleotides (GDP or GTPS) by incubation for 1 h at room temperature with 100x excess of the given nucleotide (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 10 mM EDTA, 5 mM DTT), and the reaction was stopped by adding MgCl2 to a final concentration of 20 mM. Proteins were prebound to GSH beads (GE Healthcare) for 1 h at 4°C, and unbound fraction was washed away with PBS. Beads were subsequently mixed with Dictyostelium cell lysates and incubated 4°C overnight with rotation. PBS washing steps were performed to wash away the unbound proteins, and beads were boiled in 1x SDS loading buffer. Prey and bait proteins were detected by means of Western blotting with GFP- or GST-specific antibodies (SC9996 [Santa Cruz Biotechnology, Santa Cruz, CA] or 27-4577-01 [GE Healthcare]).

Protein purification and GDI assays

DyDR1A and DyDR2A were expressed from a pGEX4T1 plasmid containing an N-terminal GST and Tobacco Etch Virus (TEV) cleavage side. Proteins were isolated by glutathione affinity purification, proteolytic cleavage, and size exclusion chromatography as previously described (Kortholt et al., 2006). The purified proteins were analyzed by SDS–PAGE, and the concentration was determined by Bradford's method (Bio-Rad, Hercules, CA). The purified protein was used in a GDI assay as previously described (Kortholt et al., 2006).

Mass spectrometry and phosphopeptide analysis

For the phosphoproteomics assay, samples were prepared as previously described (Charest et al., 2010). Phosphopeptide analysis was performed as described previously (Para et al., 2008). Briefly, we lysed aggregation-competent Ax-2 and gskA- cells before and after stimulation with cAMP. After tryptic digestion, the phosphopeptides were enriched, and the samples were analyzed with liquid chromatography–tandem mass spectrometry. The raw data were searched against the sequences from dictyBase (Eichinger et al., 2005) using Spectrum Mill software (Agilent Technologies, Inc. Santa Clara, CA).

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