ABSTRACT We have previously shown that the Dictyostelium protein phosphatase 2A regulatory subunit B56, encoded by psrA, modulates Dictyostelium cell differentiation through negatively affecting glycogen synthase kinase 3 (GSK3) function. Our follow-up research uncovered that B56 preferentially associated with GDP forms of RasC and RasD, but not with RasG in vitro, and psrA cells displayed inefficient activation of multiple Ras species, decreased random motility, and inefficient chemotaxis toward cAMP and folic acid gradient. Surprisingly, psrA cells displayed aberrantly high basal and poststimulus phosphorylation of Dictyostelium protein kinase B (PKB) kinase family member PKBR1 and PKB substrates. Expression of constitutively active Ras mutants or inhibition of GSK3 in psrA cells increased activities of both PKBR1 and PKBA, but only the PKBR1 activity was increased in wild-type cells under the equivalent conditions, indicating that either B56- or GSK3-mediated suppressive mechanism is sufficient to maintain low PKBA activity, but both mechanisms are necessary for suppressing PKBR1. Finally, cells lacking RasD or RasC displayed normal PKBR1 regulation under GSK3-inhibiting conditions, indicating that RasC or RasD proteins are essential for GSK3-mediated PKBR1 inhibition. In summary, B56 constitutes inhibitory circuits for PKBA and PKBR1 and thus heavily affects Dictyostelium chemotaxis.

INTRODUCTION Motility is a process that involves multiple signaling pathways designed to allow cells to receive extracellular signals and orchestrate intracellular signaling network, which in turn affects cytoskeleton-based cell motility machinery. During the aggregation stage of Dictyostelium, the extracellular cAMP activates G protein–coupled receptors, resulting in the dissociation of heterotrimeric G proteins into Gα and Gβγ subunits (Janetopoulos et al., 2001; Xu et al., 2005; reviewed in Swaney et al., 2010). The Gβγ subunits have been suggested to activate multiple downstream signaling components, including small GTPases such as RasC, RasG, RasB, and Rap1.

Activation of RasG is critical for local accumulation of phosphatidylinositol-3,4,5-triphosphate (PI(3)P) and, consequently, localizing pleckstrin homology (PH) domain–containing proteins such as PKBA, CRAC, and PhdA from the cytosol to the leading edge, where extensive remodeling of cytoskeleton is mediated by phosphorylation of protein kinase B (PKB) substrates (Funamoto et al., 2002; Iijima and Devreotes, 2002). RasC, in parallel with RasG, mediates activation of TorC2 and subsequent activation of PKBA and PKBR1 at the leading edge (Insall et al., 1996; Lee et al., 2005; Kae et al., 2007; Kamimura et al., 2008). RasB and Rap1 proteins are known to mediate chemoattractant-mediated suppression of myosin II through activating myosin heavy chain kinase A (MHCKA) (Kortholt et al., 2006; Jeon et al., 2007a,b; Mondal et al., 2008). Rear contraction and adhesion to the matrix are shown to be dependent on the cGAMP-mediated activation of myosin II.

An important family of kinases that modulates cytoskeletal remodeling in Dictyostelium is the AGC family of kinases, Akt/PKBA and protein kinase B–related 1 (PKBR1). In response to cAMP stimulation, RasG-dependent phosphatidylinositol 3 kinase (PI3K) activation transiently produces PI(3)P, to which PKBA translocalizes with its PH domain. Once at the plasma membrane, PKBA becomes phosphorylated by phosphoinositide-dependent kinase A (PdkA) and the Tor complex 2 (TorC2) in the activation loop (AL) site and

PP2A/B56 and GSK3/Ras suppress PKB activity during Dictyostelium chemotaxis

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the hydrophobic motif (HM) site, respectively (Meili et al., 1999; Kamimura et al., 2008; Kamimura and Devreotes, 2010; Cai et al., 2010; Liao et al., 2010). In contrast to PKBA, PKBR1 does not have the PH domain; instead, PKBR1 is permanently anchored at the membrane through a myristoylation site present on its N-terminus (Meili et al., 2000). PKBR1, however, also becomes activated upon cAMP stimulation by phosphorylation in both the AL site by PdkA and in the HM site by TorC2 (Kamimura et al., 2008, 2010; Cai et al., 2010; Liao et al., 2010). Despite this difference in their subcellular localization mechanisms, both PKBA and PKBR1 activation involves Pdk1- and TorC2-mediated phosphorylation. The mechanism of cAMP-mediated activation of TorC2 is well characterized, but that of Pdk1 is still to be fully uncovered.

Previous studies also showed that Ras proteins have both distinct and overlapping functions (Khosla et al., 2000), and cells lacking RasG, but not RasC, express higher level of RasD (Bolourani et al., 2010). Considering that rasG−/− cells express lower level of RasD compared with the rasG+ cells, RasC is likely contributing to the up-regulation of RasD expression (Bolourani et al., 2010).

Cells overexpressing constitutively active RasD(G12T) mutant displayed exaggerated expression of the prestalk A cell marker ecma. Interestingly, cells expressing dominant-negative glycogen synthase kinase 3 (dnGSK3 [K84M, K85M]; Kim et al., 2002; Lee et al., 2008) or treated with the GSK3 inhibitor LiCl also showed similarly enhanced ecma expression. It was thus suggested that Ras and GSK3 may interact in the context of prestalk cell differentiation (Weeks, 2000). Previous studies showed that gsk3−/− cells are not only defective in cell differentiation but also highly compromised in chemotaxis (Teo et al., 2010; Kim et al., 2011). Potential mechanisms behind the gsk3−/− chemotaxis phenotype include biased localization of PI3K toward the plasma membrane, high poststimulus Ras activity, and compromised PKB1-inhibiting signal through Ras effector Daydreamer (Teo et al., 2010; Kölsch et al., 2012; Sun et al., 2013). There exist, however, contrasting data for the effect of GSK3 ablation on PKB1 and PKBA activity (Teo et al., 2010; Kölsch et al., 2012; Sun et al., 2013). Thus, to provide additional insight into the role of GSK3 for the PKB regulation, we analyzed the effects of GSK3 on PKBs by expressing dnGSK3 or treating cells with LiCl.

Previous studies demonstrated that a B56 subunit inhibits Akt in Caenorhabditis elegans and in differentiated 3T3-L1 adipocyte cells (Padmanabhan et al., 2009). Another line of studies also showed that protein phosphatase 2A (PP2A) could dephosphorylate phospho-ERK2 and phospho-Akt through scaffolding protein IEX-1, which was induced by active Erk2. IEX-1 functions as a scaffolding platform for ERK2 and PP2A, and thus facilitates Erk2-mediated phosphorylation of B56 that stimulates B56 dissociation from the PP2A core dimer, which then no longer inhibits Erk2 and Akt (Leteumeux et al., 2006; Rocher et al., 2007). Furthermore, studies using mammalian cells demonstrated that insulin-mediated activation of PDK and mTorC2 not only activates Akt, but also stimulates the formation of the PP2A/B56 holoenzyme complex, which in turn dephosphorylates phospho-Akt (Rogers et al., 2011). These studies indicate that B56-mediated Akt inhibition is widely conserved among eukaryotes. In this report, we show the PP2A regulatory subunit, B56, and the glycogen synthase kinase 3 (GSK3) negatively regulate PKB activation in Dictyostelium cells, providing a novel insight into PKB regulation.

RESULTS

B56 preferentially associated with inactive RasC and RasD in vitro

As B56 is a known regulatory subunit for PP2A, the recombinant Dictyostelium B56 protein associated with PP2A catalytic subunit. In addition, the GST-B56 pull-down complex also included a small GTPase Ras (Figure 1A). To uncover the identity of Ras species that can associate with GST-B56, the whole-cell lysates from cells expressing Flag-tagged RasG, RasD, and RasC were incubated with GST-B56 and the Ras proteins associated with B56 were analyzed by Western blot. GST-B56 associated with Flag-RasD and Flag-RasC, but not with Flag-RasG (Figure 1, B and C).

Given that the GST pull-down assays were performed with whole-cell lysates from unstimulated cells, in which the majority of the Ras proteins are inactive, it is likely that GDP-Ras proteins were included in the GST-B56 pull-down complex. To determine whether activated GTP-Ras proteins can also associate with B56, the Flag-Ras-containing lysates were treated with GTP-γ-S and then incubated with GST-B56, GST-Raf1–Ras binding domain (RBD), or GST-Byr2-RBD proteins. On incubation with GTP-γ-S, more Ras proteins associated with GST-RBD, but significantly fewer Ras proteins were included in the GST-B56 pull-down complex (Figure 2A). In addition, the recombinant proteins of constitutively active or dominant-negative mutant Flag-RasD and Flag-RasC were generated in Escherichia coli and were incubated with either GST-B56 or GST-RBD proteins. Consistent with GTP-γ-S experiments, the constitutively active Ras mutants displayed less binding to GST-B56, and the dominant-negative Ras mutants exhibited enhanced association with GST-B56 (Figure 2B).

The discovery that B56 can associate not only with RasD but also with RasC suggests that B56 is likely to be involved in the regulation of cell motility in addition to cell differentiation, which was previously reported (Lee et al., 2008). Considering that GDP-Ras bound more tightly to B56 and that certain RasGefs such as RasGefQ prefer GDP-Ras (Mondal et al., 2008), it is plausible that B56 may facilitate recruiting GDP-Ras to specific RasGef proteins.
**psrA**− cells exhibited compromised CAMP-induced Ras activation

Dictyostelium cells possess 11 different Ras species and two Rap proteins (http://dictybase.org; Chattwood et al., 2014); among those, RasG and RasC have been shown to be critical regulators of cellular migration (Kortholt and van Haastert, 2008). RasG and RasC regulate recruitment and activation of several downstream effectors involved in cellular motility, including PI3K and TorC2. Axenically grown cells lacking both RasG and RasC exhibited severely compromised motility and cAMP-mediated signaling (Kortholt and van Haastert, 2008; Charest et al., 2010).

To determine whether B56 also affects Ras activity, active Ras levels were measured using the RBD of mammalian Raf1 or the RBD of *Schizosaccharomyces pombe* Byr2. The peak Ras activities were detected in wild-type cells at 5 s after cAMP stimulation, which decreased thereafter, but *psrA*− cells exhibited significantly reduced Ras activation in response to cAMP stimulation (Figure 3, A and B).

*psrA*− cells were defective in random and directional motility

*psrA*− cells stimulated with pulsatile CAMP exhibited an average velocity of random motility that is 50% reduced compared with *Wt* cells (Figure 4A). When the cell motility of *psrA*− cells was examined in the presence of an external CAMP gradient (10 μM CAMP), *psrA*− cells moved more slowly compared with *Wt* cells, exhibiting an average velocity reduced by 60% compared with *Wt* cells (Figure 4B). Furthermore, *psrA*− cells displayed significantly compromised chemotaxis; *psrA*− cells exhibited an ~50% reduced chemotaxis index compared with that of wild-type cells. These results suggest that B56 is part of a regulatory network controlling both random and directional motility of aggregation-competent Dictyostelium. To further corroborate that PP2A/B56 is an essential part of the cell motility regulatory network, axenically grown vegetative-stage wild-type and *psrA*− cells were challenged with a micropipette filled with folic acid (0.1 mM). As shown in Figure 4C, *psrA*− cells exhibited statistically significant compromise in directional migration toward the folic acid gradient, indicating that B56 is a critical factor for chemotaxis machinery. *psrA*− cells also exhibited an ~50% reduction in speed.

**PKBR1 activity and PKBs substrate phosphorylation were aberrantly high in *psrA*− cells**

Considering that *psrA*− cells have generally low Ras activity, we hypothesized that PKBA and PKBR1 activation may also be compromised in *psrA*− cells. For examination of PKBA and PKBR1 activation, aggregation-competent cells were stimulated with CAMP and the phosphorylation levels of PKBA and PKBR1 at the activation loop (AL) site were examined as previously described (Cai et al., 2010). On cAMP stimulation, wild-type cells exhibited phosphorylation at the AL site in PKBR1 and PKBA between 15 and 30 s, which decreased back to the basal level at 60 s after cAMP stimulation. In contrast, a higher basal level of phosphorylated PKBR1, but not PKBA, was observed in *psrA*− cells compared with *Wt* cells (Figure 5A). Furthermore, *psrA*− cells displayed higher basal phosphorylation of T470 of the HM of PKBR1 (*p < 0.05*; Figure 5B). Total protein levels stained with Coomassie were used as a loading control.

To further investigate the effect of B56 in PKB regulation, we examined the phosphorylation of putative PKB substrates containing the motif R-x-R-x-x-S/T-x-x (x denotes a random amino acid) in *psrA*− as previously described (Kamimura et al., 2008; Cai et al., 2010). As expected, there were higher levels of phosphorylation of previously identified PKB substrates in *psrA*− cells compared with *Wt* cells (Figure 5C). In addition, there were several novel phospho-PKB substrates (marked with asterisks) that seemed to be unique to *psrA*− cells, the identification of which requires further investigation.

To determine whether *psrA*− cells overproduce PKBR1, thus permitting Pdk1 and TorC2 to more easily phosphorylate PKBR1, we compared the levels of PKBR1 messages in aggregation-competent wild-type and *psrA*− cells by reverse transcription PCR (RT-PCR) (Figure 5D). The levels of PKBR1 messages in wild-type and *psrA*− cells were comparable, as were the Ig7 messages, the internal control. Thus B56 affects PKBR1 activity without altering its expression level.

**Introducing constitutively active RasD increased PKBA and PKBR1 activities in *psrA*− cells**

Considering that RasD exerted a negative effect on chemotaxis toward CAMP (Khosla et al., 2000), we hypothesized that RasD may mediate PKBR1 adaptation and that compromised RasD activity in *psrA*− cells might therefore have caused increased PKBR1 phosphorylation. To that end, RasD activity in *psrA*− cells was determined as shown in Figure 6A: the Flag-RasD activities were significantly lower in *psrA*− cells compared with *Wt* cells and the phosphorylation levels of PKBA and PKBR1 activation may also be compromised in *psrA*− cells.

To determine whether the low RasD activity in *psrA*− cells caused a high level of PKB phosphorylation, we expressed constitutively active RasD mutant in wild-type and *psrA*− cells (Figure 6, B and C). Contrary to expectations, RasD(G12T) expressing wild-type and *psrA*− cells displayed even higher levels of active PKBR1 compared with their control cells (*p < 0.05; *psrA*− cells, **p < 0.01), while RasC(G13T)-expressing *psrA*− cells displayed enhanced levels...
Consistent with the phenotype of dnGSK3-expressing cells, LiCl-treated wild-type cells displayed a statistically significant increase in the basal PKBR1 level (\( \ast \ast p < 0.01 \)) as well as cAMP-induced PKBR1 activation (Figure 8A). \( \text{psrA}^- \) cells pretreated with LiCl also exhibited higher basal and cAMP-induced PKBR1 phosphorylation (Figure 8B). \( \text{gsk3}^- \) cells, in contrast to the cells expressing dnGSK3 or treated with LiCl, showed no PKBR1 phosphorylation regardless of LiCl treatment (Figure 8C), indicating that the effect of LiCl is GSK3 dependent. Thus the high GSK3 activity in \( \text{psrA}^- \) cells (Lee et al., 2008) is unlikely to be the main cause for the high PKBR1 activity in \( \text{psrA}^- \) cells. We also noticed that, unlike dnGSK3-expressing \( \text{psrA}^- \) cells, \( \text{psrA}^- \) cells treated with LiCl demonstrated no increase in PKBA. Although LiCl can clearly inhibit GSK3, it can also block cAMP-dependent PI3 generation and thus insulate PKBA activation (King et al., 2009). The molecular nature of LiCl-mediated inhibition of cAMP-induced PKBA activation is currently unclear, but in any case, PP2A/B56 is not necessary for the LiCl effect.

**Ras is essential for dnGSK3- and LiCl-mediated regulation of PKBR1 activation**

Recent reports suggested that GSK3 affects Ras adaptation (Kölsh et al., 2012; Sun et al., 2013). Considering that Ras proteins are abnormally regulated in \( \text{gsk3}^- \) cells and they are PKBR1 activators, it is plausible that GSK3 inhibits PKBR1 through Ras proteins. To determine whether RasD is necessary for GSK3-mediated PKBR1 regulation, we overexpressed dnGSK3 in \( \text{rasD}^- \) cells. When the PKBR1 activation was examined in \( \text{rasD}^- \) cells overexpressing dnGSK3, no such drastic increase in PKBR1 phosphorylation as observed in

### FIGURE 3: The chemoattractant cAMP-induced Ras activation was compromised in \( \text{psrA}^- \) cells. (A) \( \text{psrA}^- \) cells exhibited reduced amplitude of Raf1-RBD binding activity (presumably GTP-RasD and GTP-RasG) in response to cAMP stimulation compared with Wt cells. The Raf1-RBD binding activity of \( \text{psrA}^- \) cells at 5 s after cAMP stimulation is only ~60% compared with that of wild-type cells (three independent experiments; *, \( p < 0.05 \)). (B) \( \text{psrA}^- \) cells also showed similar reduction (~30%) in Byr2-RBD binding activity (presumably GTP-RasC) in response to cAMP stimulation compared with Wt cells (three independent experiments, \( t \) test; *, \( p < 0.05 \)). Error bars represent SD.
FIGURE 4: 

**psrA**− cells displayed compromised random motility and chemotaxis. (A and B) Aggregation-competent wild-type cells after 4 h of pulsatile cAMP stimulation displayed robust random (∼10 μm/min) and directional (8 μm/min) motility toward cAMP gradient (10 μM cAMP). In contrast, **psrA**− cells exhibited only ∼50% of random and ∼40% of directional motility compared with wild-type cells. The chemotaxis index of **psrA**− cells toward cAMP gradient was ∼50% of that for wild type. Error bars represent SD. All three p values were < 0.01. (C) Axenically grown vegetative wild-type and **psrA**− cells were challenged with 0.1 mM folic acid, and their movements were analyzed for 20 min. The chemotaxis index and speed of movement of **psrA**− cells under folic acid gradient were reduced to ∼60% and ∼50% of the wild-type level, respectively. Error bars represent SE of the mean. All p values were < 0.01.
A

| Wt | psrA- |
|----|-------|
| 0  | 15    | 30 | 60 | 0 | 15 | 30 | 60 |

sec cAMP
PKBR1(Tp309) (AL site)
PKBA(Tp278) (AL site)

Total Protein

![Image of Western Blot](image1)

![Graph of Relative Band Intensity](graph1)

B

| Wt | psrA- |
|----|-------|
| 0  | 15 | 30 | 180 | 0 | 15 | 30 | 180 |

sec cAMP
PKBR1(Tp470) (HM site)

Total protein

![Image of Western Blot](image2)

![Graph of Relative Band Intensity](graph2)

C

| Wt | psrA- |
|----|-------|
| 0  | 15'' | 30'' | 3' | 0 | 15'' | 30'' | 3' |

cAMP

![Image of Western Blot](image3)

(shorter exposure)

(longer exposure)

D

| Ig7 | Wt | psrA- |
|-----|----|-------|
| PKBR1 |

RT-PCR
wild-type or psrA− cells expressing dnGSK3 was observed: no detectable level of active PKBR1 was observed from cells without stimulation, but no statistically significant difference in PKBR1 phosphorylation was observed in response to cAMP stimulation (*p > 0.05; Figure 9A). Increased total GSK3 levels of dnGSK3-expressing rasD− cells compared with the control cells are shown (Figure 9A). Furthermore, when rasD− or rasC− cells were treated with LiCl, no significant increase in PKBR1 phosphorylation was observed compared with nontreated cells (*p > 0.05; Figure 9, B and C) in contrast to LiCl-treated wild-type and psrA− cells, which exhibited higher levels of PKBR1 activation (Figure 8, A and B). Altogether these results suggest that GSK3 affects PKBR1 activity via a RasD− or RasC-dependent mechanism.

**DISCUSSION**

We report here that B56/PP2A associates with GDP-RasC and GDP-RasD and significantly affects PKB activities in the context of chemotaxis. PP2A function was reported to be essential for chemotaxis by mediating proper regulation of the scaffolding protein Sca1 and Ras proteins (Charest et al., 2010). Currently it is unclear whether B56 or another regulatory B subunit has a role in Sca1-mediated Ras regulation, because no regulatory subunit was identified from the Sca1 protein complex from either unstimulated or stimulated cells (Charest et al., 2010). On receiving a cAMP signal, the PP2A/Sca1/Aimless/Gef4 complex relocalized to the plasma membrane from the cytosol and thus activated GDP-RasC (Charest et al., 2010). GDP-Ras/B56 may associate with the PP2A/Sca1/Aimless/Gef4 complex in response to cAMP, which in turn would facilitate GDP-RasC interaction with Aimless/RasGef. On activation of RasC and subsequently PKBs, the Sca1 complex will be phosphorylated and become inactivated by PKBs. For chemotaxing cells to respond to another round of signal, dephosphorylation of both PKBs and Sca1 complex must occur.

Cells lacking both PKBR1 and PKBA display lower Sca1 phosphorylation and high basal and poststimulus RasC activity. Active RasC will then activate PKBs, which in turn will inhibit Sca1 complex, and thus a negative-feedback loop will be formed (Charest et al., 2010). However, given that activation of PKBR1 and PKBA require cAMP signaling, it is puzzling that basal RasC activity is also aberrantly high in pkbr1−/pkba− double-knockout cells. One possibility is that PKBR1 is not only modulating Sca1/RasC signaling but is also involved in the proper expression of certain genes involved in the regulation of RasC (such as RasGAP), considering that PKBR1 can also affect cell differentiation (Meili et al., 2000).

Chemoattractant-mediated activation of specific Ras proteins led to specific downstream events orchestrating directional cell migration. However, previous studies also showed a compensation mechanism between Ras proteins: ablation of RasG increased the level of RasD, but that of RasC showed no such compensation (Bolourani et al., 2010). Thus, expressing high levels of RasD, rasG− cells are able to stimulate PKBs. In contrast, lacking RasD, rasC− cells are unable to activate PKBs. rasG−/rasC− cells display a significantly lower RasD level compared with that of rasG− cells, and thus phosphorylation of PKBs and PKB substrates in rasG−/rasC− is lower than in rasG− cells, which is consistent with an earlier study (Srinivasan et al., 2013). In addition, a previous study showed that RasD can form a protein complex including ERK2, PKA, GRP125, and Filamin (Bandala-Sanchez et al., 2006), indicating a possibility that RasD may mediate association of PP2A/B56 with PKBA. As GTP-RasD/ PKBA complex adapts to GDP-RasD/PKBA as a post-cAMP stimulus response, B56/PK2A may join the newly emerging GDP-RasD-PKBA complex and thus inactivate PKBA. Thus, PP2A/B56 could function as a PKB phosphatase similarly to the mammalian PP2A/B56 that dephosphorylates Akt (Rodgers et al., 2011).

The current study also demonstrated that GSK3/RasD is another significant component of PKB inhibitory circuits. Inhibition of GSK3 either with LiCl or expressing dnGSK3 resulted in a significant increase in the level of PKBR1 phosphorylation in wild-type and psrA− cells but not in cells lacking RasD or RasC. Interestingly, the dnGSK3-expressing psrA− cells but not the wild-type cells expressing dnGSK3 exhibited exaggerated cAMP-mediated PKBA activation in addition to the high PKBR1 activity. Thus either a B56− or a GSK3-mediated suppressive mechanism is sufficient to maintain low PKBA activity, but both mechanisms are necessary for suppressing PKBR1 activity. In addition, we speculate that both PP2A/B56 and GSK3/RasD are likely functioning at the upstream components of PKBs such as Pdk1 or Torc2, but B56/PP2A could directly dephosphorylate PKBA in a RasD-dependent manner.

Consistent with the high PKB activities, the levels of phosphorylation of PKB substrates are also high in psrA− cells. Misregulation of Talin and p21-activated kinase A (PAKa) (Chung et al., 2001) would have immediate impact on the cell migration, but other proteins—either previously identified PKB substrates (pp350, pp200/pp180 for GefN, pp140 for GacG/PakA, pp110 for GefS/Pi5K, pp65/67 for GacQ) or several novel targets reported here—may also participate in the orchestration of the cell migration control.

Regulation of PKBs is a complex event. There seem to exist multiple mechanisms of activation and adaptation that involve multiple Ras proteins, PP2A/B56, and GSK3, either functioning in parallel or cooperatively. Furthermore, feedback inhibition and gene expression circuits are likely implicated. Further investigation is necessary to fully uncover the mechanism of PKB regulation, which will provide novel mechanistic insight on the regulation of PKBs that not only
regulate chemotaxis but other F-actin remodeling–mediated events such as phagocytosis and macropinocytosis.

MATERIALS AND METHODS

Cell culture

Ax3, psrA−, gsk3−, rasC−, rasD−, psrA*/FlagRasD(G12T), psrA*/FlagRasCG13T, psrA*/dnGSK3, Wt/dnGSK3, and cells were grown with axenic medium (14.3 g peptone [Difco, Detroit, MI], 7.15 g yeast extract [Oxoid, Hampshire, United Kingdom], 15.4 g glucose [Fisher Scientific, Waltham, MA], 0.525 g Na2HPO4⋅7H2O, 0.48 g KH2PO4, 0.53 g Na2HPO4⋅7H2O in 1 l of water, pH 6.5–6.9). The medium was complemented with 5 mg/ml of blasticidin (InvivoGene, San Diego, CA) for psrA−, rasC−, rasD−, and gsk3− strains. In addition, for gsk3− cells, 25 mg/ml of thymidine (Arcos Organics/Fisher Scientific, Waltham, MA) was added to the medium. For strains overexpressing FlagRasC, FlagRasD, FlagRasCG13T, FlagRasD(G12T), or dnGSK3, 20 μg/ml of G418 (Gibco) was used as a selective agent for the medium.

Recombinant ras proteins

Ras mutant constructs were generated by using the QuikChange Site-Directed Mutagenesis kit (Stratagene/NEB, Ipswich, MA). Each construct was fully sequenced and subcloned into an E. coli expression vector (pGEX-4T-2) in which the GST-encoding sequence was substituted with a Flag-tag sequence, and the recombinant proteins were immunopurified by anti-Flag antibody and eluted with Flag peptide for various binding assays.

Pull-down assays

To examine Ras activation in response to cAMP stimulation, log-phase vegetative cells, either Wt, psrA−, or various Ras mutant–expressing cells, were starved for 1 h and stimulated with 50 nM cAMP at 6 min intervals for 4 h at room temperature at a concentration of 20×10^6 cells/ml in DB buffer (2 mM MgCl2, 0.2 mM CaCl2, 7.4 mM NaH2PO4, 4mM Na2HPO4). Aggregation-competent cells were washed once with ice-cold DB buffer and treated with 5 mM caffeine at room temperature for 20 min at a concentration of 20×10^6 cells/ml. Following treatment, cells were washed with ice-cold DB buffer once, resuspended with 5 ml of DB buffer, and shaken

normalizing the total Flag-RasD amount in the input. (B) Wt cells expressing Flag-RasD(G12T) also exhibited augmented basal and poststimulus phosphorylation of PKBR1 (three independent experiments, 2.5 times higher 15-s poststimulus level; *, p < 0.05). Wt cells expressing Flag-RasD(G12T) showed no significant changes in the levels of PKBA phosphorylation (*, p > 0.05). The phospho-PKBR1 levels were normalized to Coomassie-stained total proteins. (C) Introducing the constitutively active Flag-RasD(G12T) in psrA− cells resulted in even higher basal and poststimulus phosphorylation of PKBA (three independent experiments, approximately twofold higher at 15-s poststimulation; **, p < 0.01). Significantly higher levels of poststimulus phosphorylation of PKBA were observed in Flag-RasD(G12T)-expressing psrA− cells (**, p < 0.01). The phospho-PKBR1 levels were normalized to Coomassie-stained total proteins. (D) psrA− cells expressing Flag-RasC(G13T), as previously reported (Cai et al., 2010), showed increases in basal and poststimulus phosphorylation of PKBR1 (three independent experiments, -2.5-fold higher basal level; **, p < 0.01; 35% higher 15-s poststimulation; *, p < 0.05), but no such change was observed for PKBA. The phospho-PKBR1 levels were normalized to Coomassie-stained total proteins. Error bars represent SD.

FIGURE 6: Introducing constitutively active RasD increased PKBR1 and PKBA activities in psrA− cells. (A) Wild-type and psrA− cells expressing Flag-RasD proteins were stimulated with cAMP, and the total amount of Flag-RasD was normalized. Then the active Flag-RasD protein levels were determined using GST-RBD followed by anti-Flag Western blotting. Relative active RasD levels were quantitated by

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Byr2 and incubated at 4°C for 90 min. Samples were then washed three times using TTG buffer. SDS-containing sample buffer was then added to the pull-down complexes; samples were analyzed using a Tris-glycine–SDS–polyacrylamide gel, and the levels of active Ras were detected by Western blot using anti-Pan-Ras antibody (Calbiochem).

The activated forms of RasD and RasG were purified using recombinant GST-RBD from mammalian Raf1 (GST-RBD-Raf1) and the activated form of RasC was purified using recombinant GST-RBD from S. pombe Byr2 (GST-RBDByr2). Cell extracts were mixed with 4 μg of either purified GST-RBD-Raf1 or purified GST-RBD-Byr2 and incubated at 4°C for 90 min. Samples were then washed three times using TTG buffer. SDS-containing sample buffer was then added to the pull-down complexes; samples were analyzed using a Tris-glycine–SDS–polyacrylamide gel, and the levels of active Ras were detected by Western blot using anti-Pan-Ras antibody (Calbiochem).
cAMP every 6 min for a total of 4 h at room temperature. Aggregation-competent cells were plated at a density of $3 \times 10^4$ cells/cm² on a 35-mm tissue culture dish cover (Falcon 353001; Becton Dickinson) and left to settle down for 5–10 min at room temperature. For examination of the chemotactic response, cells were exposed with a Schmazu micromanipulator to a glass capillary needle filled with either 10 μM cAMP or 0.1 mM folic acid solution. For maintaining the external chemotactic gradient, the capillary needle containing chemoattractant was connected to an Eppendorf FemtoJet at an injection pressure of 20 hPa. A time-lapse video recording using OpenLab Software was used to follow the cell movement at 1-min intervals. For a random motility analysis, aggregation-competent cells were plated as previously described, and no source of external cAMP was used. Chemotactic indices and the speed were analyzed as previously described (Veeranki et al., 2008).

**Determining PKBR1 activation in response to cAMP stimulation**

For examination of PKBR1 activation, aggregation-competent cells were washed once with ice-cold DB buffer and treated with 5 mM caffeine at room temperature for 20 min. After the treatment, cells were washed twice with ice-cold PM buffer (5 mM Na$_3$HPO$_4$, 5 mM KH$_2$PO$_4$, 2 mM MgSO$_4$) and resuspended at $20 \times 10^6$ cells/ml in PM buffer; this was followed by shaking for 2 min at room temperature, and then cells were stimulated with 1 μM cAMP, which was followed by lysis with TTG buffer at 15, 30, and 180 s. PKBR1 phosphorylation was detected with Western blotting using phospho-PKC antibody (Cell Signaling) as previously described by Cai and colleagues (2010). To detect the phosphorylation of PKBR1 substrates containing the motif R-x-R-x-x-S/T-x-x, Western blot using anti–phospho Akt Substrate (Cell Signaling) was done as previously described by Kamimura et al. (2008).

To analyze PKBR1 activation in the presence of LiCl, aggregation-competent cells were treated with 50 mM LiCl for 1 h at room temperature with shaking. Cells were then washed once with ice-cold DB; this was followed by caffeine treatment and stimulation with 1 μM cAMP as previously described. The IC$_{50}$ of LiCl on GSK3 activity is 4 mM by using GSK3-specific peptide substrate (L. W. Kim, unpublished data) as reported previously (Ryves et al., 1998). A measure of 50 mM LiCl is known to be sufficient for the near-complete suppression of GSK3 activity (L. W. Kim, unpublished data, Ryves et al., 1998).

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**Chemotaxis and random motility assays**

Log-phase cells were starved at a concentration of $20 \times 10^4$ cells/ml in DB buffer for 1 h; this was followed by stimulation with 50 nM B56-bound Ras proteins were similarly detected by GST-B56 pull-down assay as previously described (Lee et al., 2008).

**FIGURE 9:** dnGSK3- and LiCl-mediated regulation of phosphorylation of PKBR1 is Ras dependent. (A) rasD− cells showed normal basal and comparable poststimulus phosphorylation of PKBR1. rasD− cells expressing dnGSK3 displayed no significant increase in the basal phosphorylation levels of PKBR1 and PKBA. In response to cAMP stimulation, rasD− cells expressing dnGSK3 exhibited comparable poststimulus phosphorylation of PKBR1. rasD− cells expressing dnGSK3 displayed no significant increase in the basal phosphorylation levels of PKBR1 compared with rasD− cells (*, $p > 0.05$). The phospho-PKB levels were normalized to Coomassie-stained total proteins. Error bars represent SD.
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