A novel Akt/PKB-related kinase is essential for morphogenesis in *Dictyostelium*

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Background: *Dictyostelium* Akt/PKB is homologous to mammalian Akt/PKB and is required for cell polarity and proper chemotaxis during early development. The kinase activity of Akt/PKB kinase is activated in response to chemoattractants in neutrophils and in *Dictyostelium* by the chemoattractant cAMP functioning via a pathway involving a heterotrimeric G protein and PI3-kinase. *Dictyostelium* contains several kinases structurally related to Akt/PKB, one of which, PKBR-1, is investigated here for its role in cell polarity, movement and cellular morphogenesis during development.

Results: PKBR-1 has a kinase and a carboxy-terminal domain related to those of Akt/PKB, but no PH domain. Instead, it has an amino-terminal myristoylation site, which is required for its constitutive membrane localization. Like Akt/PKB, PKBR-1 is activated by cAMP through a G-protein-dependent pathway, but does not require PI3-kinase, probably because of the constitutive membrane localization of PKBR-1. This is supported by experiments demonstrating the requirement for membrane association for activation and *in vivo* function of PKBR-1. PKBR-1 protein is found in all cells throughout early development but is then restricted to the apical cells in developing aggregates, which are thought to control morphogenesis. PKBR-1 null cells arrest development at the mound stage and are defective in morphogenesis and multicellular development. These phenotypes are complemented by Akt/PKB, suggesting functional overlap between PKBR-1 and Akt/PKB. Akt/PKB PKBR-1 double knockout cells exhibit growth defects and show stronger chemotaxis and cell-polarity defects than Akt/PKB null cells.

Conclusions: Our results expand the previously known functions of Akt/PKB family members in cell movement and morphogenesis during *Dictyostelium* multicellular development. The results suggest that Akt/PKB and PKBR-1 have overlapping effectors and biological function: Akt/PKB functions predominantly during aggregation to control cell polarity and chemotaxis, whereas PKBR-1 is required for morphogenesis during multicellular development.

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Background

Akt/PKB is a serine/threonine protein kinase whose activity is regulated by the phosphorylation of conserved residues in the activation loop and carboxy-terminal tail [1,2]. Akt/PKB activation is induced through the stimulation of G-protein-coupled receptors and tyrosine kinases and requires the function of phosphatidylinositol-3 kinase (PI3-kinase) [2–6]. Binding of the amino-terminal PH domain of Akt/PKB to phosphoinositide products of PI3-kinase (such as $PI(3,4,5)P_3$) is thought to help localize Akt/PKB to the plasma membrane [7–9], thereby facilitating its phosphorylation by PDK1, the kinase that phosphorylates Akt/PKB on the activation loop. Akt/PKB when constitutively localized to the plasma membrane via an amino-terminal myristoylation site is constitutively active [1,10]. *Dictyostelium* Akt/PKB lacking the PH domain is only inefficiently activated by upstream pathways (R.M. and R.A.F., unpublished observations). In metazoans, Akt/PKB is involved in regulating cell size, cell and organism survival, cell differentiation, apoptosis and metabolic pathways [11–18].

When *Dictyostelium* amoebae are starved, they aggregate to form a mound of up to 10^5 cells, which subsequently develops into a spore-containing fruiting body. Aggregation is mediated by chemotaxis in response to cAMP, and is activated through pathways activated by G-protein-coupled cAMP receptors [19,20]. We previously demonstrated that *Dictyostelium* Akt/PKB is required for cell polarization and proper chemotaxis in response to cAMP during aggregation of cells to form a multicellular organism [1]. *Dictyostelium* Akt/PKB kinase activity is transiently

stimulated by cAMP via a signaling pathway mediated by a G-protein-coupled receptor and involving PI3-kinase. The Akt/PKB PH domain fused to green fluorescent protein (GFP) localizes to the plasma membrane in response to cAMP stimulation and to the leading edge during chemotaxis. These results suggest that Akt/PKB is an important component of the signaling pathways that control chemotaxis in *Dictyostelium*. Mammalian Akt/PKB is also activated in response to fMet-Leu-Phe (fMLP) and chemokines [21], and a GFP fusion with the Akt/PKB PH domain localizes to the plasma membrane in neutrophils in response to fMLP [22], suggesting a possible conserved role for Akt/PKB in responding to chemoattractants [23].

Here, we describe the regulation and function of an Akt/PKB-related kinase, PKBR-1, in *Dictyostelium*. PKBR-1 is highly homologous to Akt/PKB in the kinase and carboxy-terminal extension but lacks the amino-terminal PH domain. Instead, PKBR-1 is localized to the membrane by myristoylation. PKBR-1 is not essential for aggregation but is required during multicellular development for morphogenesis beyond the mound stage. Like Akt/PKB, PKBR-1 is activated via a cAMP-mediated, G-protein-coupled pathway, which, in contrast to Akt/PKB activation, does not require PI3-kinase; however, like Akt/PKB, membrane localization of PKBR-1 is essential. We present results that suggest Akt/PKB and PKBR-1 serve similar biological roles, with Akt/PKB functioning during aggregation and PKBR-1 acting during mound formation and morphogenesis.

Results

PKBR-1 structure and subcellular localization

A *Dictyostelium* PKBR-1 cDNA was cloned using sequence information from previous PCR screens [24] and GenBank (accession number M59744). A comparison of the deduced PKBR-1 amino-acid sequence with *Dictyostelium* (GenBank U15210) and human Akt/PKB (GenBank M63167) revealed a high degree of homology in the kinase domain and the carboxy-terminal extension (Figure 1a). PKBR-1 lacks the amino-terminal PH domain, however. Instead, it has a consensus myristoylation site (MGXXX(S/T)(K/R); single-letter amino-acid notation, where X is any amino acid) followed by a cluster of basic amino acids, a sequence motif that embodies a putative membrane anchor [25,26]. The two regulatory Akt/PKB phosphorylation sites [1,2] in the activation loop and carboxyl terminus (Ser473/Thr435) are conserved in PKBR-1 (Thr309 and Thr470) and lie in a conserved sequence context.

The presence of a consensus myristoylation site followed by a cluster of basic amino acids is normally sufficient for stable membrane association [25,26]. Using indirect immunofluorescence, we examined the subcellular localization of PKBR-1 tagged at its carboxyl terminus with the hemagglutinin (HA) epitope (PKBR-1–HA). As shown in Figure 1b, PKBR-1–HA is associated with the plasma membrane. When the myristoylation site is inactivated by placing a Myc epitope at the amino terminus of the protein (Myc–PKBR 1), membrane localization is lost and the kinase appears to be found in small clusters or particles within the cytoplasm (Figure 1b). Membrane localization is restored when the c-Src myristoylation site with its basic cluster [25,26] is fused to the PKBR-1 amino terminus (Myr–PKBR-1).

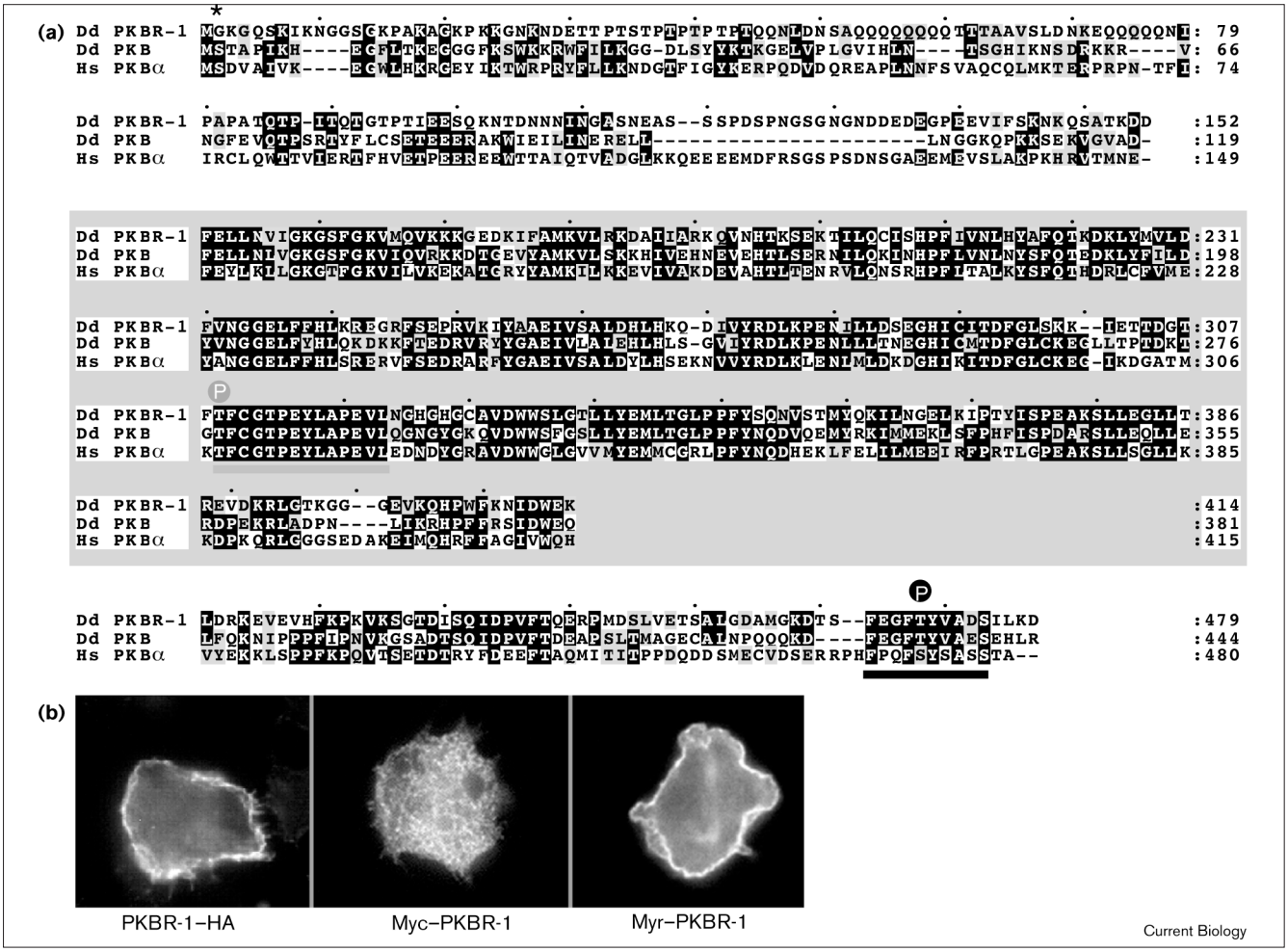
The chemoattractant cAMP activates PKBR-1 via a G-protein-dependent, PI3-kinase-independent pathway

Dictyostelium Akt/PKB is activated in response to the chemoattractant cAMP via a G-protein-dependent pathway from the cAMP receptor that requires the function of PI3-kinase [1]. To investigate whether PKBR-1 is similarly activated, we stimulated aggregation-stage cells with cAMP and PKBR-1 activity was assayed. Cells were lysed at the times shown and PKBR-1 was isolated from these lysates by immunoprecipitation with a polyclonal antibody raised against a carboxy-terminal PKBR-1 peptide (see Materials and methods). PKBR-1 kinase activity was assayed using histone 2B as a substrate. For comparison, we isolated Akt/PKB from the same lysates using a *Dictyostelium* Akt/PKB-specific antibody and assayed Akt/PKB kinase activity as previously described [1]. As we show in Figure 2a, the activation kinetics of the two kinases are almost indistinguishable. There is rapid activation within 10–20 seconds and fast adaptation to basal levels within 120 seconds. To confirm the specificity of the assay for the respective kinases, we performed control experiments using null strains for each kinase. As expected, no Akt/PKB activity was observed in *pkbA* null cells and no PKBR-1 activity was seen in *pkbR1* null cells, whereas *pkbA* null cells had cAMP-activated PKBR-1 activity and *pkbR1* null cells had cAMP-activated Akt/PKB activity (Figure 2a).

Akt/PKB activation in higher eukaryotes as well as in *Dictyostelium* is mediated by PI3-kinases and their phosphoinositide products (see Introduction). We therefore wanted to determine whether PI3-kinases are involved in PKBR-1 activation. Maximal activation of *Dictyostelium* Akt/PKB requires either of the genetically redundant PI3-kinases [27] PIK1 and PIK2, which are most closely related to the subfamily containing mammalian p110α [27], but not PIK3, which is most closely related to the PI3-kinase isoforms activated by Gβγ subunits [1].

To assess the role of PI3-kinase in PKBR-1 activation, we used the PIK1/PIK2 double knockout cells (*pik1*$^-$/*pik2*$^-$ cells) and the PI3-kinase inhibitor LY294002 [28], which inhibits cAMP-mediated activation of Akt/PKB [1]. As previously shown, Akt/PKB activity is significantly reduced in *pik1*$^-$/*pik2*$^-$ cells (compare 'WT' lanes with

Figure 1



Amino-acid sequence, domain structure and sequence features of *Dictyostelium* PKBR-1 and its subcellular localization. **(a)** The derived PKBR-1 sequence is aligned with Akt/PKB from *Dictyostelium* and human cells. The kinase domain (gray background) is highly conserved, followed by a well-conserved 'extension'. There are two potential targets for regulatory phosphorylation: Thr309 (gray bar in the activation loop of the kinase domain) and Thr470 (black bar in the kinase extension). **(b)** Indirect immunofluorescence showing subcellular localization of HA-tagged wild-type PKBR-1 (PKBR-1–HA), a non-myristoylatable PKBR-1 (Myc–PKBR-1), and PKBR-1 fused with the c-Src membrane anchor (Myr–PKBR-1).

'PI3K 1/2 null' lanes in Figure 2b) or when cells are pretreated for 60 seconds with 12.5 μM LY294002 (Figure 2b; the western blot shows levels of Akt/PKB and PKBR-1 protein). In some experiments, the level of inhibition is greater when *pik1*$^{-}$/*pik2*$^{-}$ cells are pretreated with the inhibitor (Figure 2b). This greater inhibition may be due to the inhibition of residual PI3-kinase activity derived from PIK3 or an unidentified PI3-kinase. In contrast, PKBR-1 activation is not reduced in either of the double knockout cells and/or in the presence of the inhibitor (Figure 2b), indicating that PKBR-1 activation is independent of PI3-kinase (the Akt/PKB and PKBR-1 assays were done on the same samples). To examine whether cAMP-mediated PKBR-1 activation functions through a G-protein-coupled pathway, as does cAMP activation of Akt/PKB, we used a cell line in which the gene encoding the sole Gβ subunit was disrupted but which constitutively expressed the cAMP receptor cAR1 [1,29]. As depicted in Figure 2c, cAR1-expressing, *gβ* null cells exhibit no cAMP-mediated activation of PKBR-1 activity.

To determine whether the conserved Akt/PKB activation sites in the activation loop and carboxy-terminal extension are important for PKBR-1 activation, we mutated Thr309 and Thr470 to alanine (PKBR-1^{T309A,T470A}). PKBR-1^{T309A,T470A} was expressed in *pkbR1* null cells and cAMP-mediated activation was assayed. We observed no activity above the basal level (data not shown). Moreover, PKBR-1^{T309A,T470A} expressed in *pkbR1* null cells is unable to complement the null phenotype (see below). These

data are consistent with phosphorylation at these sites being required for PKBR-1 activation.

PKBR-1 exhibits a temporally and spatially regulated pattern of expression

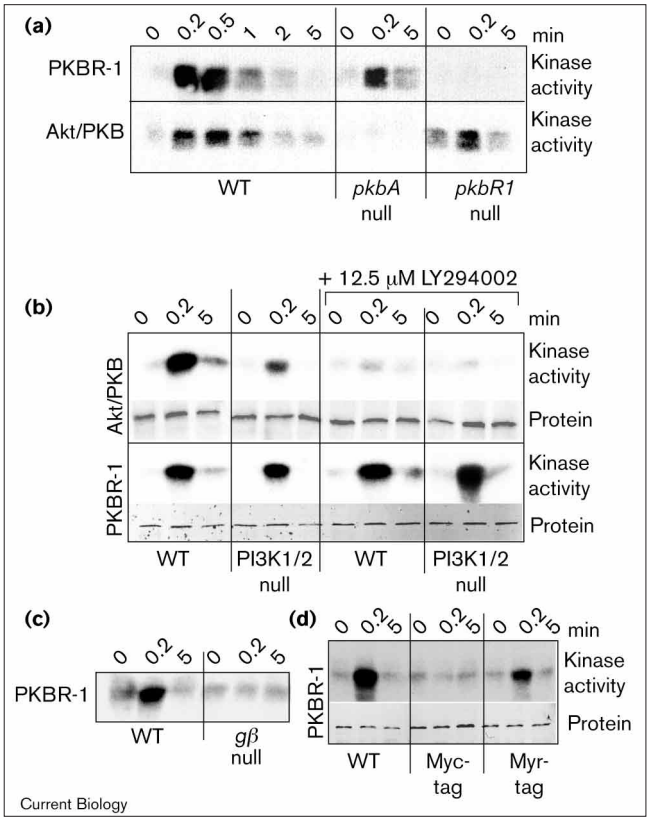
PKBR-1 RNA blots show two developmentally regulated transcripts (Figure 3a). The smaller mRNA species is found at low levels during vegetative growth and is induced by starvation at the onset of development, with transcript levels peaking at 4–8 hours during the aggregation stage. By 8 hours of development, the larger transcript is induced and peaks at the mound stage (12 hours), suggesting that PKBR-1 transcription is regulated by two different pathways. Both mRNAs are derived from the same gene, as they are both absent from *pkbR1* null cells (data not shown).

In contrast to the mRNA, there is a single protein species. The overall protein levels increase upon the initiation of development and are maximal during aggregation, consistent with the expression of the first transcript. Protein levels then decrease ~2–3-fold and remain constant at this level throughout the slug stage (Figure 3b). Examination of the PKBR-1 expression pattern by immunohistochemistry reveals a switch from ubiquitous expression in all cells during growth and aggregation in early development to expression restricted to cells found at the top of the mound during tip formation (data not shown). This expression pattern is similar to that of *ecmA*, which is a marker for the prestalkA cells that eventually form the anterior 10% of the slug [30]. The appearance of the larger mRNA species and the change to spatially restricted protein expression occur at about the same time during development, suggesting a possible causal relationship between these two events.

pkbR1 null cells arrest at the mound stage

To investigate the role of PKBR-1, we generated a knockout strain by homologous recombination. No PKBR-1 gene product was detectable on both mRNA and protein levels (data not shown). *pkbR1* null cells aggregate with kinetics similar to those of the parental wild-type strain, forming a tight aggregate around 10 hours of development (Figure 4a). The subsequent development of *pkbR1* null mounds deviates markedly from that of wild-type mounds, however; most *pkbR1* null mounds arrest without forming a tipped aggregate, whereas wild-type strains form fruiting bodies by 25 hours (Figure 4a). Although most of the mounds show no further morphogenesis, some mounds round up and become almost spherical (Figure 4a, panel '*pkbR1* null 50 h'). Depending on the conditions, some of the mounds form an aberrant sorus atop an irregular stalk-like structure without proceeding through the normal intermediate stages. Dissection of the fruiting body-like structures that form reveals mature spores and stalk cells; however, these are less organized than in normal fruiting bodies and no stalk tube is observed (data not shown).

Figure 2



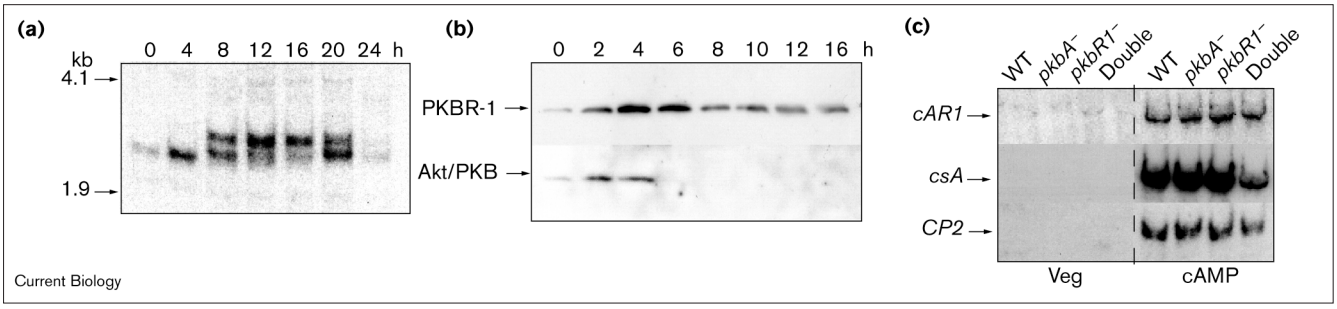
Activation of PKBR-1 by the chemoattractant. **(a)** Wild-type (WT), *pkbA* null and *pkbR1* null aggregation-stage cells were stimulated with cAMP. Samples were taken at the times indicated and PKBR-1 and Akt/PKB kinase activity were visualized by autoradiography of an immunocomplex *in vitro* kinase assay with histone 2B as substrate separated on SDS–PAGE. **(b)** Kinase activation in response to cAMP stimulation in wild-type (WT) and PI3K1/PI3K2 null cells (PI3K1/2) with or without a 60 sec pretreatment with 12.5 μM of the PI3-kinase inhibitor LY294002. (The Akt/PKB and PKBR-1 assays were done on the same samples so the relative effect of PI3-kinase can be compared.) **(c)** PKBR-1 kinase activity in *gβ* null cells constitutively expressing the cAMP receptor cAR1. See Materials and methods for details. **(d)** Activation of PKBR-1 (WT), Myr–PKBR-1 and Myc–PKBR-1 expressed in *pkbR1* null cells from the *Act15* promoter. The upper panel shows the kinase activity. The lower panel is a western blot and shows protein levels.

A *pkbA/pkbR1* double knockout strain does not grow axenically or aggregate

Because of the structural similarity of PKBR-1 to Akt/PKB, we investigated the impact of the absence of both kinases on *Dictyostelium* growth and development. As described previously, *Akt/PKB* (*pkbA*) null cells do not polarize properly in a chemoattractant gradient, move slowly, and do not aggregate at lower cell densities, densities at which the parental strain aggregates normally [1]. However, *pkbA* null cells exhibit no obvious growth defects and multicellular aggregates form normal fruiting bodies.

We generated a PKBR-1 Akt/PKB double knockout strain (*pkbR1*/*pkbA* null cells, see Materials and methods).

Figure 3

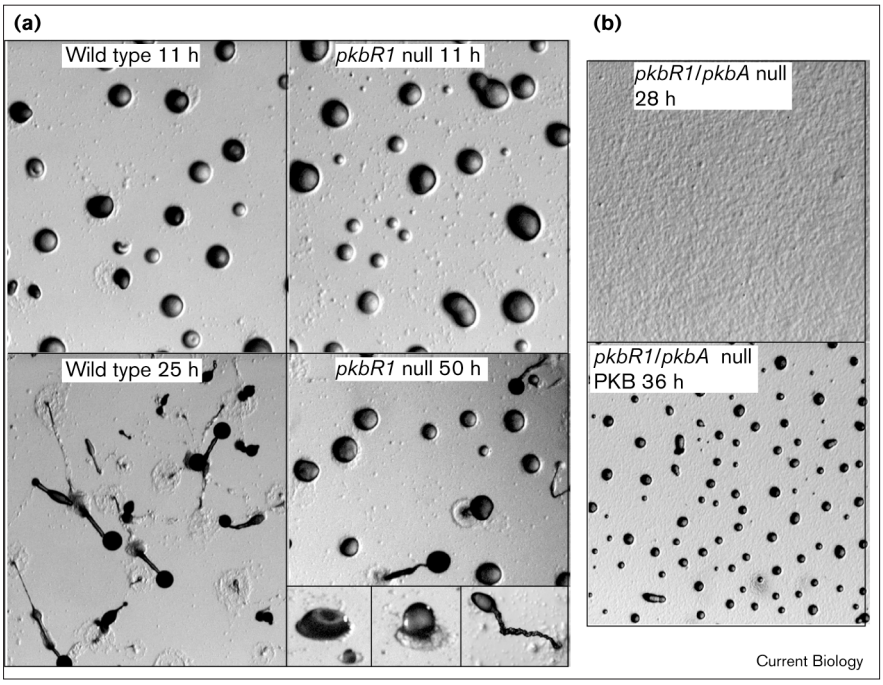


Temporal expression pattern of *Dictyostelium pkbR1* and cell-type-specific genes during development. **(a)** Northern blot analysis of *pkbR1* RNA during development. **(b)** Western blot analysis of PKBR-1 protein using an antibody raised against the carboxyl terminus. **(c)** RNA blot analysis examining responsiveness to cAMP of the wild-type (WT), *pkbA* null (*pkbA*$^-$), *pkbR1* null (*pkbR1*$^-$) and double knockout (Double) cells. Top two rows: RNA was isolated from log-phase vegetative cells (Veg) or from cells pulsed for 5 h with 30 nM cAMP (cAMP) and probed for the pulse-induced genes *cAR1* and *csA*. Bottom row: for the post-aggregative gene *CP2*, RNA from cells pulsed for 5 h and then given high cAMP (300 μM) was used.

It became apparent that disruption of both genes severely impairs cell growth under axenic conditions, even when the cells grow attached to plastic Petri dishes. To isolate clones and grow *pkbR1/pkbA* null cells, it was necessary to supplement the axenic medium with bacteria (*Klebsiella aerogenes*). When we examined the developmental potential of *pkbR1/pkbA* null cells, no aggregation occurred and the cells remained in a monolayer indefinitely (Figure 4b, and data not shown). Re-expression of Akt/PKB under the control of an *Act15* promoter partially rescues the developmental defect, allowing aggregation and formation of smaller than wild-type mounds, most of which arrest at this stage (Figure 4b).

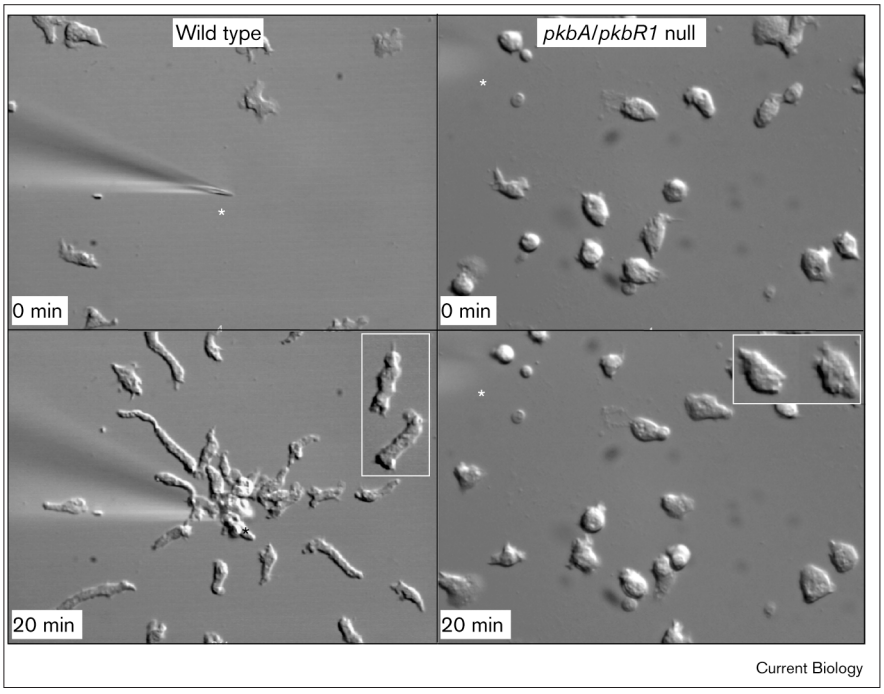
To determine whether the double null cells are either unable to detect the developmental signals or cannot respond chemotactically, we assayed the ability of the double null cells to respond to cAMP signaling by examining cAMP-mediated gene expression and the ability of 'aggregation-competent' double null cells pulsed for 5 hours with cAMP (to maximize expression of the signaling components required for responses to cAMP [1,29],

Figure 4



Developmental phenotype of the *pkbR1* single knockout and *pkbR1/pkbA* double knockout. **(a)** Developmental morphology of wild-type (left panels) and *pkbR1* null cells (right panels) at the mound stage (11 h) and at the terminal stage of development (25 h for wild-type cells; 50 h for *pkbR1* null cells). The inset illustrates the range of terminal structures formed by the knockout strain. **(b)** Aggregation-deficient phenotype of *pkbR1/pkbA* double knockout cells after 28 h (upper panel). Partial complementation was achieved by re-expression of Akt/PKB from the *Act15* promoter (lower panel).

Figure 5



Chemotaxis of wild-type and *pkbR1* null cells. Cells were pulsed for 5 h with 30 nM cAMP every 6 min to induce cAMP receptors and other components of the cAMP chemotaxis pathway and plated on a glass coverslip covering a hole in a small plastic Petri dish. Chemotaxis of the cells to a micropipette containing 150 μM cAMP was assayed as previously described [1]. The cells were visualized on a Nikon microscope with differential interference contrast imaging as described in the Materials and methods and analyzed using the DIAS program [38]. The double knockout cells exhibit a speed of 1.4 ± 0.3 μM/min, wild-type cells showed a mean speed of 8.9 ±1.0 μM/min, and the *pkbA* null cells exhibited a mean speed of 3.3 ± 0.3 μM/min.

see below) to undergo chemotaxis towards cAMP emitted from a micropipette. Figure 3c shows the expression in wild-type, *pkbA* null, *pkbR1* null, and the double knockout cells of the aggregation-stage genes encoding the cAMP receptor cAR1 and the cell adhesion protein contact sites A (CsA) in response to 30 nM pulses of cAMP, a signal that mimics cAMP signaling during aggregation [29]. All four strains showed a similar induction of cAR1 expression. The expression of CsA, which is dependent on cAR1 expression and pathways regulated through cAR1, is similar in wild-type cells and the single knockout strains, but is reduced in the double null cells. We also examined the expression of *CP2*, a gene induced by high levels of cAMP at the mound stage via a cAR1-dependent pathway. The expression level in the double knockout cells is slightly reduced compared to that in the other three strains. These results indicate that the double knockout cells can respond to cAMP signals, but at a level that is slightly reduced compared with either null strain or wild-type cells.

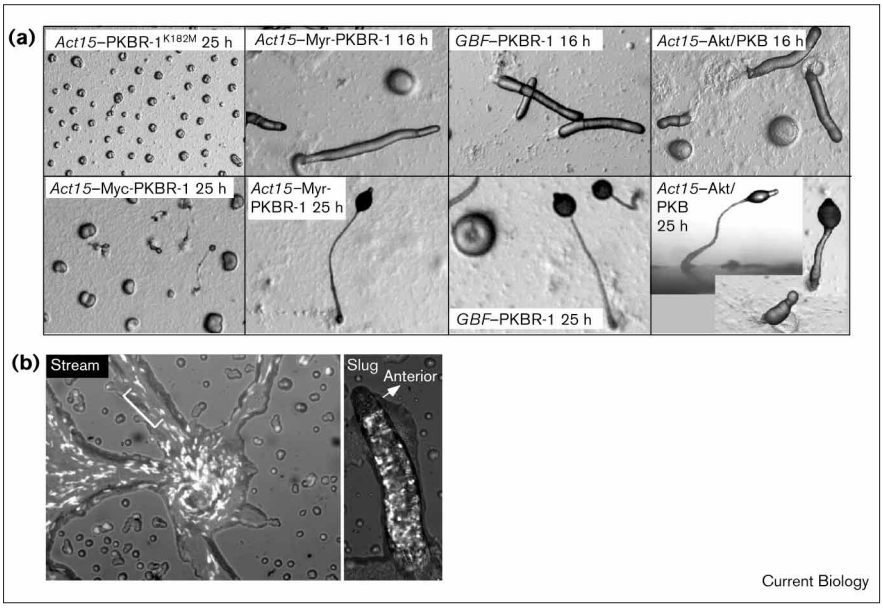
As shown in Figure 5, the double knockout cells undergo chemotaxis, but at a significantly reduced rate compared with either the wild-type or *pkbR1* null cells (which show normal chemotaxis, data not shown). The rate is also reduced compared to *pkbA* null cells, which already exhibit a significantly reduced rate of chemotaxis and cell polarization compared to wild type ([1], and see legend to Figure 5). In addition, *pkbR1*/*pkbA* null cells undergoing chemotaxis do not polarize (Figure 5, inset; compare with inset of wild-type cells). This defect is not unexpected, considering the polarization defect of *pkbA* null cells [1]. The growth and aggregation defects and more severe chemotaxis deficiencies of *pkbR1*/*pkbA* null cells compared with *pkbA* null cells suggest that PKBR-1 and Akt/PKB may co-regulate multiple pathways and there may be some functional redundancy between the genes. We note that the double knockout cells exhibit strong growth defects and thus the reduced response of these cells to cAMP signaling could be due to pleiotropic effects of the growth defects or actual defects in the response pathways.

Expression of either PKBR-1 or Akt/PKB can complement the mound-arrest *pkbR1* null phenotype

The hallmarks of *pkbR1* null strain development are mound arrest, the absence of a slug stage for those aggregates that develop further, and formation of a few, aberrant, fruiting bodies. *pkbA* null cells, on the other hand, exhibit aggregation-stage defects, with the aggregates that form producing normal fruiting bodies [1]. Akt/PKB protein is preferentially expressed during growth and aggregation [1] and becomes undetectable at the mound stage, the time at which *pkbR1* null strain developmental defects are first observed. One explanation for this is that PKBR-1 function is mostly redundant in the presence of Akt/PKB protein but becomes essential after the disappearance of Akt/PKB protein.

To test this hypothesis, we expressed Akt/PKB or PKBR-1 in *pkbR1* null cells from the constitutive *Act15*

Figure 6



Development of PKBR-1 mutant strains. **(a)** The developmental phenotypes of *pkbR1* null strains expressing PKBR-1, Akt/PKB, a kinase-inactive mutant of PKBR-1 (PKBR-1^{K182M}) and a non-myristoylatable mutant (Myc–PKBR-1) at either 15 h (slug stage) or 25 h (terminal development). Expression from the *Act15* and *GFP* promoters are shown for Akt/PKB and PKBR-1. **(b)** *pkbR1* null cells expressing GFP under the control of the *Act15* promoter were mixed with wild-type (KAx-3) cells in a ratio of 1:10 and plated for development. Structures are visualized by admixing some visible light to show nonfluorescent cells more clearly.

promoter and the *GBF* promoter, which is preferentially induced after aggregation [31] (J. Brown and R.A.F., unpublished observations). Clones were selected and their development analyzed. Strains expressing PKBR-1 from the *GBF* promoter (*GBF*–PKBR-1) develop like the wild-type *Dictyostelium* parental strain, proceeding through a slug stage to the formation of well-proportioned fruiting bodies (Figure 6a). Strains expressing Akt/PKB from the *Act15* (*Act15*–Akt/PKB) or *GBF* promoter exhibit almost normal development, with a slug stage and formation of fruiting bodies, although the slugs and the fruiting bodies do not appear completely normal (Figure 6a, data for *GBF* promoter not shown). A putative kinase-dead mutant of PKBR-1 (Act15/PKBR-1^{K182M}), which has lysine in the ATP-binding site mutated to methionine, is unable to restore development (Figure 6a). These cells aggregate abnormally and form very small mounds which arrest at this stage, suggesting that the kinase-dead PKBR-1 functions as a dominant-negative during aggregation and may affect Akt/PKB as well as PKBR-1 function. Consistent with this, expression of this construct in wild-type cells resulted in a similar phenotype (data not shown). Likewise, expression of a mutant lacking the amino-terminal myristoylation site (*Act15*–Myc–PKBR-1) does not restore normal development, although expression of a mutant in which the native myristoylation site is replaced with the one from c-Src (*Act15*–Myr–PKBR-1) does (Figure 6a), indicating that PKBR-1 membrane localization is required for *in vivo* function.

To determine the ability of the amino-terminally tagged Myc– and Myr–proteins to be activated, we repeated the kinase activation experiments with *pkbR1* null strains constitutively expressing Myr–PKBR-1 and Myc–PKBR-1 at approximately the same levels as endogenous PKBR-1 is expressed in wild-type cells (Figure 2d). As shown, Myr–PKBR-1 but not Myc–PKBR-1 is activated, suggesting that activation requires the protein to be membrane localized. The level of activation of Myr–PKBR-1 is approximately a third to a half that of endogenous PKBR-1 expressed at approximately the same levels in wild-type cells. This reduced activation may result from structural changes in the protein that may stem from the added amino-terminal domain.

PKBR-1 knockout cells exhibit patterning defects in chimeras with wild-type cells

The mound-arrest phenotype of the *pkbR1* null strain could indicate that these cells have either a cell movement defect, a defect in cell-type differentiation, or a combination of both. To examine if cell-type-specific gene expression is induced in *pkbR1* null mounds, we probed developmental RNA blots with the prestalk-specific and prespore-specific genes *ecmA* and *SP60/cotC* [19,32]. Both genes are induced in *pkbR1* null cells with kinetics similar to those of induction and expression in wild-type cells, indicating that *pkbR1* null cells are able to induce cell-type differentiation (data not shown), although the level of expression is reduced. This is probably a result of the *pkbR1* null cells arresting at the mound stage, which is before the maximal level of *ecmA* and *SP60/cotC* expression is reached in wild-type strains. We cannot, however, exclude a direct role for PKBR-1 in maximal expression of these genes.

To follow movement and position of *pkbR1* null cells during morphogenesis, we tagged these cells by expressing

GFP under the control of the *Act15* promoter and allowed them to form chimeric aggregates with wild-type cells. In aggregation streams, *pkbR1* null cells participate normally in aggregate formation (Figure 6b), suggesting that there is no cell movement defect at this stage. Moreover, the *pkbR1* null cells have a normal elongated shape, suggesting that they are not defective in cell polarization at this stage of development. The formation of the anterior–posterior axis and spatial patterning of cell types in *Dictyostelium* result from a differential sorting of prestalk cells to the apical tip to form a discrete anterior prestalk domain [19,30,32]. But while the distribution of the *pkbR1* null cells remains uniform in early mounds (data not shown), the null cells are not found in the tip or in the resulting anterior prestalk domain in the slug (Figure 6b, data not shown). *pkbR1* null cells form spores and are part of the basal disc but are not found in the stalk (data not shown).

Discussion

Regulation and localization of PKBR-1

PKBR-1 is highly homologous to Akt/PKB, including the presence of the two conserved phosphorylation sites required for kinase activation. Alanine substitutions at these sites abolish cAMP-mediated activation of PKBR-1. A major difference between PKBR-1 and Akt/PKB is the absence of a PH domain and the presence of a myristoylation site in PKBR-1. This myristoylation site is required for constitutive localization of PKBR-1 on the plasma membrane, cAMP-mediated activation of PKBR-1, and the ability of PKBR-1 to complement the *pkbR1* null phenotype, and can be replaced by the c-Src amino-terminal myristoylation site. The requirement for membrane localization for PKBR-1 activation and function is consistent with the requirement for the PH domain and membrane localization for Akt/PKB activation.

Our results suggest that some aspects of the activation pathway are conserved between PKBR-1 and Akt/PKB, including activation in response to cAMP via a heterotrimeric G-protein-dependent pathway with similar kinetics, and the requirement of the conserved consensus sites for phosphorylation. Unlike Akt/PKB, however, PKBR-1 activation does not require PI3-kinase. This is not so surprising, as PKBR-1 has a myristoylation site which functions as a constitutive membrane-localization signal. Interestingly, the addition of an amino-terminal myristoylation signal from c-Src to either *bona fide* mammalian or *Dictyostelium* Akt/PKB, but not PKBR-1, results in a constitutively active kinase [1,10]. It has been proposed that Akt/PKB is held in an inactive state through a negative regulatory function of the PH domain and that binding of the PH domain to the PI3-kinase product $PI(3,4,5)P_3$ in the plasma membrane results in the localization of the protein to the plasma membrane. This possibly causes a conformational change in the protein [33] and allows access to PDK1 (or the equivalent kinase in *Dictyostelium*). *Dictyostelium* Akt/PKB either lacking the PH domain or containing a point mutation in the PH domain which abrogates its ability to translocate to the plasma membrane in response to cAMP signaling, shows significantly reduced cAMP-mediated activation (R.M. and R.A.F., unpublished observations), which is consistent with a requirement for membrane localization for activation. An amino-terminal myristoylation site would place Akt/PKB constitutively at the membrane, where basal levels of phospholipids might release the negative inhibition, allowing PDK1 to have access to Akt/PKB. As PKBR-1 is not activated by constitutive membrane localization, it is possible that the amino-terminal domain of PKBR-1 may be an inhibitory domain similar to the PH domain, but one not regulated by phospholipids. We suggest that in *Dictyostelium*, and possibly in metazoans, there are two upstream regulatory pathways for Akt/PKB: a PI3-kinase-dependent pathway that controls plasma membrane localization, and a distinct PI3-kinase-independent pathway that regulates kinase phosphorylation and activation.

Function of PKBR-1 and redundancy with Akt/PKB

PKBR-1 protein is present throughout growth and development, with levels increasing upon starvation and being maximal during aggregation. Protein levels then decrease ~2–3 fold, at the time that expression of the gene from a second promoter is induced by cAMP signaling. This corresponds temporally with a change in the expression pattern of the protein. Initially, all cells have a similar level of PKBR-1 protein, as determined by *in situ* staining; during tip formation, however, cells expressing PKBR-1 are spatially restricted to a subpopulation of cells at the top of the mound. This restriction is similar to that observed with some prestalk-specific proteins [32]. Thus, the reduction in the level of PKBR-1 as the mound forms coincides with the loss of the protein in the cells that will not be forming the tip. In *Dictyostelium*, spatial patterning is established, in part, through the differential sorting of prestalk cells to the apex of the mound, forming the tip [19,32]. Because of the changes in protein levels and the distribution of PKBR-1-expressing cells in the mound, we suggest that the protein expressed during early development is turned over in all cells, or possibly only in cells that will not sort to form the tip. New PKBR-1 protein would then be expressed in this tip cell population from the late promoter. This shift from the presence of PKRB-1 in all cells to the localization of the protein only in prestalk cells, which form the tip and represent ~20% of the organism, presumably accounts for the reduced levels of this protein in the whole organism at the mound stage. As the cells that preferentially have PKBR-1 in the mound are those that have started to form the tip, and which extend to form a standing finger, we suggest that potential cell motility or polarity defects of *pkbR1* null cells are responsible for the mound-arrest phenotype.

The first observed developmental defect of *pkbR1* null cells is the mound-arrest phenotype. In chimeric organisms containing predominantly wild-type cells, *pkbR1* null cells are uniformly distributed during aggregation and in the early mound. As cell sorting starts to form the apical, anterior prestalk domain, *pkbR1* null cells do not participate, however. These results, combined with the observation that these cells can induce prestalk and prespore genes, are consistent with a defect in the ability of these cells to sort and form this developing tissue, rather than to a defect in the ability of the cells to induce prestalk cells. Several other mutant strains with known cell movement defects have a similar phenotype, including strains in which the genes encoding myosin II, myosin regulatory light chain and the LIM domain protein LIM2 have been disrupted [34–37]. As *pkbA* null cells have polarity defects during aggregation, it is possible that the morphogenesis defect of the *pkbR1* null strain is due to defects in cell movement at this stage of development. *pkbR1* null cells do not exhibit polarity defects in aggregation streams. A role for PKBR-1 in controlling cell movement and polarity is, however, supported by the strong chemotaxis and polarity defects exhibited by the PKBR-1 and Akt/PKB double knockout strain, which are presumably masked in the *pkbR1* single knockout by the presence of Akt/PKB. The severe growth and aggregation defects exhibited by the double null strain are consistent with PKBR-1 functioning at these stages of the life cycle and with PKBR-1 and Akt/PKB being at least partially redundant. Our results suggest that at least one of the kinases must be present for the cells to aggregate.

We also demonstrate that Akt/PKB can complement most of the morphogenetic phenotypes of *pkbR1* null cells. This is consistent with PKBR-1 and Akt/PKB having at least some overlapping functions and common downstream substrates. We also demonstrate that expression of a potential dominant-negative, kinase-dead form of PKBR-1 in wild-type cells results in aggregation defects, possibly due to the inhibition of Akt/PKB as well as PKBR-1 function. This is also consistent with PKBR-1 and Akt/PKB having common substrates. The expression patterns of PKBR-1 and Akt/PKB and the phenotypes of single and double knockout strains suggest that whereas Akt/PKB and PKBR-1 have overlapping functions during early development, PKBR-1 provides a unique function in the multicellular stages, when Akt/PKB protein is no longer present. If Akt/PKB and PKBR-1 have overlapping functions, why do *akt/pkb* null cells exhibit aggregation-stage polarization and chemotaxis defects? One possible reason is that PKBR-1 may not effectively interact with all the Akt/PKB substrates, as PKBR-1 is membrane-tethered. Alternatively, this difference may result from the uniform localization of PKBR-1 around the plasma membrane. We have shown that the PH domain of Akt/PKB is preferentially localized at the leading edge of cells undergoing chemotaxis and suggested that this leading edge localization and activation of Akt/PKB may have an important function in regulating cell polarization in a chemoattractant gradient [1]. Myristoylated PKBR-1 may not be able to provide this localized function. Moreover, when myristoylated Akt/PKB is expressed during aggregation, the cells show a loss of cell polarization similar to that observed for *pkbA* null cells [1]. One explanation is that a myristoylated protein is not locally activated at the leading edge, which may be important for the function of Akt/PKB. Our results do not explain why PKBR-1, a novel form of Akt/PKB with an amino-terminal myristoylation site but lacking a PH domain, would have evolved to function during the multicellular stages of development. We expect that the regulatory circuitry that controls Akt/PKB and PKBR-1 activation is optimized in the multicellular stages by using a different amino-terminal regulatory domain, which may involve differences in the activation pathway.

Materials and methods

Antibodies

A rabbit polyclonal anti-*Dictyostelium* PKBR-1 antibody was raised against the carboxy-terminal peptide CGADSILKD coupled via the cysteine to bovine serum albumin (BSA). An affinity resin was prepared as follows: a *Hin*cII/*Xho*I cDNA fragment coding for the carboxyl terminus of PKBR-1 was expressed as a glutathione-*S*-transferase (GST) fusion protein in *Escherichia coli,* purified by glutathione affinity chromatography according to the manufacturer's (Pharmacia) instructions, and coupled to Affigel10 (BioRad) according to the manufacturer's protocol. Affinity purification was essentially performed as described for the rabbit polyclonal anti-*Dictyostelium* Akt/PKB antibody [1]. Rabbit polyclonal anti-HA-tag antibody was purchased from Santa Cruz and mouse monoclonal anti-c-Myc antibody (9E10) was obtained from Invitrogen.

Molecular biochemistry and cell biology

A full-length *pkbR1* cDNA clone was obtained by screening a 12–16 h developmental λZAP library [31] with a probe amplified from genomic DNA by PCR. Epitope-tagged versions of PKBR-1 were made by PCR by amplifying the appropriate regions of the *pkbR1* cDNA with primers containing the necessary sequences. The nucleotide sequence of all amplified DNA and the final constructs were confirmed by DNA sequence. A *pkbr1* knockout construct was made by inserting the blasticidin-resistance cassette into a *Bam*HI site created at base 484 of the *pkbR1* cDNA by a PCR-based approach as described previously [1]. Randomly selected clones were screened for gene disruption by Southern blot analysis. Two independent clones were chosen for further analysis. To make a *pkbA/pkbr1* double knockout strain, *pkbr1* was first knocked out in *Dictyostelium* JH10 cells using the Thy1 expression cassette using the approaches described previously [1]. The double knockout cells grow only in the presence of bacteria. They were either propagated on bacterial lawns or in HL5 axenic medium supplemented with 5% of a *K. aerogenes* overnight culture. Western blot analysis was done as previously described [1]. PKBR-1 kinase assay was performed using an essentially identical protocol to that described previously for *Dictyostelium* Akt/PKB. Aggregation-competent cells were prepared by pulsing washed log-phase vegetative cells for 5 h with 30 nM cAMP every 10 min as described [1,29]. After bubbling the cells to reduce basal kinase activities [1,29], they were stimulated with cAMP (100 nM final concentration). Cell lysis, sample preparation and the kinase assay were as previously described for Akt/PKB [1], except that a PKBR-1-specific antibody was used to immunoprecipitate PKBR-1 from the lysates.

Plating cells for development and in situ *staining*

Exponentially growing cells were harvested and washed twice with 12 mM Na/K phosphate buffer pH 6.1 and plated on Na/K phosphate agar plates at a density of 4×10^6 cells/cm^2. Mixed cell populations of 1 part *pkbr1* cells tagged with *Act15*–GFP and 9 parts wild-type cells

were plated in the same way. For *in situ* staining, cells were developed on filters and fixed in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) containing 0.5% glutaraldehyde and 0.1% Nonidet-P40 for 10 min. The filters were blocked for 1 h with 2% gelatin in TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl), washed briefly with TTBS (TBS + 0.05% Tween 20) and incubated with the primary antibody in 1% gelatin TTBS for 1 h. All further incubation steps were done using the reagents from the Vectastain Elite ABC kit (Vector Laboratories) in 1% gelatin TTBS according to the manufacturer's instructions. The substrate for color development was Fast Red from Sigma. Chemotaxis assays and image acquisition were done as previously described [1].

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