The EMBO Journal Vol.18 No.8 pp.2092–2105, 1999

Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in *Dictyostelium*

Ruedi Meili, Charlene Ellsworth, Susan Lee, T.B.K.Reddy, Hui Ma and Richard A.Firtel[1]

Department of Biology, Center for Molecular Genetics, Room 225, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0634, USA

[1]Corresponding author
e-mail: rafirtel@ucsd.edu

Chemotaxis-competent cells respond to a variety of ligands by activating second messenger pathways leading to changes in the actin/myosin cytoskeleton and directed cell movement. We demonstrate that *Dictyostelium* Akt/PKB, a homologue of mammalian Akt/PKB, is very rapidly and transiently activated by the chemoattractant cAMP. This activation takes place through G protein-coupled chemoattractant receptors via a pathway that requires homologues of mammalian p110 phosphoinositide-3 kinase. *pkbA* null cells exhibit aggregation-stage defects that include aberrant chemotaxis, a failure to polarize properly in a chemoattractant gradient and aggregation at low densities. Mechanistically, we demonstrate that the PH domain of Akt/PKB fused to GFP transiently translocates to the plasma membrane in response to cAMP with kinetics similar to those of Akt/PKB kinase activation and is localized to the leading edge of chemotaxing cells *in vivo*. Our results indicate Akt/PKB is part of the regulatory network required for sensing and responding to the chemoattractant gradient that mediates chemotaxis and aggregation.

Keywords: Akt–PKB/chemotaxis/*Dictyostelium*/PI3 kinase

Introduction

The protein kinase Akt/PKB controls a variety of regulatory responses in mammalian cells, including inhibition of apoptosis, inhibition of GSK3 activity, and regulation of cellular metabolism and protein synthesis (Bellacosa *et al*., 1991; Coffer and Woodgett, 1991; Jones *et al*., 1991; Cross *et al*., 1995; Kohn *et al*., 1996; Lefebvre *et al*., 1996; Deprez *et al*., 1997; Dudek *et al*., 1997; Kauffmann-Zeh *et al*., 1997; Kennedy *et al*., 1997; Kulik *et al*., 1997) and is activated by both receptor tyrosine kinases and G protein-coupled receptors (Burgering and Coffer, 1995; Franke *et al*., 1995; Andjelkovic *et al*., 1997; Murga *et al*., 1998). In *Caenorhabditis elegans*, Akt/PKB is a negative regulator of dauer larval formation and is activated through the insulin receptor (Paradis and Ruvkun, 1998). In mammalian cells, Akt/PKB activation occurs through the binding of phosphatidylinositol-3,4-bis-phosphate [PtdIns-(3,4)-P_2] to the PH domain located at the N-terminus of the protein, and by phosphorylation by PDK1 and proposed PDK2 of a Ser/Thr residue in the activation loop and one near the C-terminus, respectively (Alessi *et al*., 1996, 1997a,b; Frech *et al*., 1997; Klippel *et al*., 1997; Stockoe *et al*., 1997). PDK1, in turn, is activated by the phosphoinositide-3 kinase (PI3K) product phosphatidylinositol-3,4,5-tris-phosphate [PtdIns-(3,4,5)-P_3] (Alessi *et al*., 1997a,b; Stephens *et al*., 1998).

In *Dictyostelium*, the transition from individual cells to a multicellular organism is controlled by the chemoattractant extracellular cAMP. Cyclic AMP activates second messenger pathways via the serpentine, cell surface receptor cAR1 coupled to the heterotrimeric G protein containing the Gα2 subunit (Firtel, 1995; Chen *et al*., 1996; Parent and Devreotes, 1996; Van Haastert and Kuwayama, 1997). Two pathways are central to regulating this response: (i) the activation of adenylyl cyclase ACA, which results in production of cAMP, the activation of PKA and the release of cAMP into the extracellular space, thereby relaying the chemoattractant signal; and (ii) the activation of guanylyl cyclase, producing cGMP, a second messenger essential for chemotaxis. Cyclic GMP, functioning through a proposed cGMP-dependent protein kinase, activates myosin II kinase, which controls myosin fiber assembly (Pagh *et al*., 1984; Abu-Elneel *et al*., 1996; Chen *et al*., 1996; Dembinsky *et al*., 1996; Kuwayama and Van Haastert, 1996; Van Haastert and Kuwayama, 1997). Cyclic GMP is also required for the reorganization of the actin cytoskeleton, via an as yet undefined mechanism. cAMP stimulation leads to an assembly of the actin cytoskeleton at the leading edge (Newell, 1995; Noegel and Luna, 1995; Chen *et al*., 1996). Activation of guanylyl and adenylyl cyclases is very transient, with the levels of cGMP and cAMP peaking at ~10 and ~90 s, respectively. Both pathways become refractory to subsequent stimulation until the cAMP is hydrolyzed by extracellular/cell surface phosphodiesterase, allowing the pathways to de-adapt.

The signaling pathways leading to the activation of the adenylyl cyclase ACA during aggregation are well understood. In addition to the Gβγ subunit, which appears to be the direct activator of ACA, proteins such as the cytosolic protein Pianissimo, the pleckstrin homology (PH) domain-containing protein CRAC (which translocates from the cytoplasm to the plasma membrane at the site of receptor activation) and the MAP kinase ERK2 play important roles in controlling the production of cAMP, but do not affect chemotaxis (Insall *et al*., 1994a; Segall *et al*., 1995; Wu *et al*., 1995; Chen *et al*., 1996, 1997; Firtel, 1996; Parent *et al*., 1998). Mutations in other genes required for ACA activation, including the gene encoding the putative Ras guanine nucleotide exchange factor (GEF) Aimless (*AleA*) and the Ras-interacting protein RIP3, also lead to chemotaxis defects (Insall *et al*.,

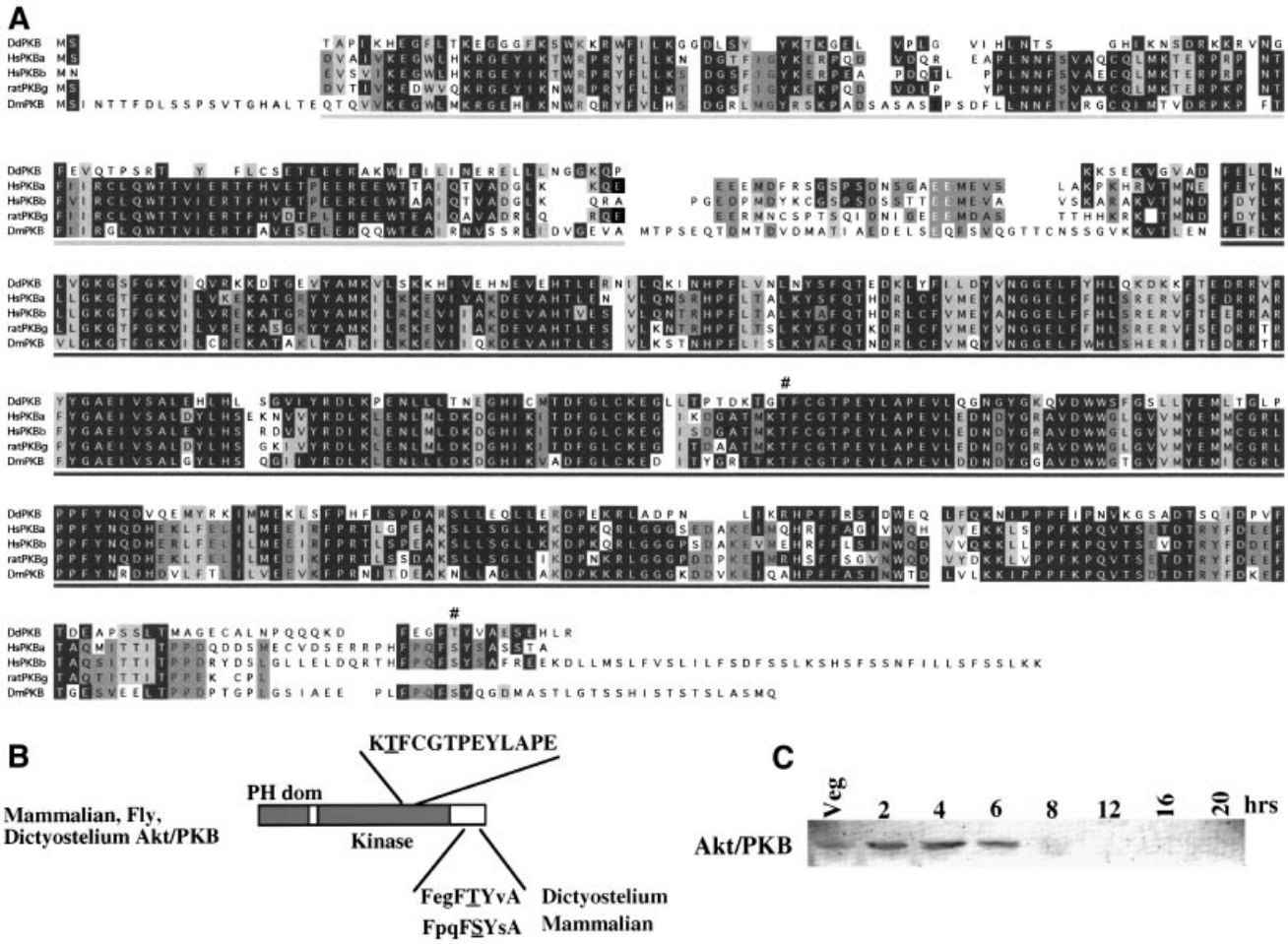


Fig. 1. Sequence, structure and expression of *Dictyostelium* Akt/PKB. (**A**) Sequence comparison of *Dictyostelium* Akt/PKB with human Akt/PKBα and Akt/PKBβ, rat PKBγ and *Drosophila melanogaster* PKB (Bellacosa *et al*., 1991; Coffer and Woodgett, 1991; Jones *et al*., 1991; Andjelkovic *et al*., 1995). Putative sites of phosphorylation in the activation loop and the C-terminal end are marked with a #; gray bar, PH domain; black bar, kinase domain. (DDBJ/EMBL/GenBank accession Nos: *Dictyostelium* Akt/PKB, U15210; *D.melanogaster* PKB, X83510; human Akt/PKBα, M63167; human Akt/PKBβ, M77198; rat Akt/PKBγ, D49836.) (**B**) Structure comparison of mammalian, *Drosophila* and *Dictyostelium* Akt/PKB. Proteins have an N-terminal PH domain followed by a conserved kinase domain, and a C-terminal regulatory domain. The consensus sequence around the two sites of phosphorylation are shown. (**C**) Western blot analysis of the developmental pattern of expression of *Dictyostelium* Akt/PKB. See Materials and methods for details.

1996; S.Lee, C.Parent, R.Insall and R.A.Firtel, manuscript in preparation). The null mutation in the gene encoding the Ras protein RasG shows defects in cell movement, being unable to properly regulate pseudopod extension (Tuxworth *et al*., 1997). Strains lacking the MAP kinase kinase DdMEK1 exhibit severe aggregation defects, forming extremely small multicellular aggregates (Ma *et al*., 1997). *ddmek1* null cells exhibit normal receptor activation of adenylyl cyclase, but the activation of guanylyl cyclase is impaired and the cells are defective in chemotaxis towards cAMP. Further analysis suggests that DdMEK1 does not lie between the receptor and guanylyl cyclase, but permits receptor activation of this pathway (Ma *et al*., 1997).

Previous studies identified a *Dictyostelium* homologue of mammalian Akt/PKB (*PkbA*; S.K.O.Mann and R.A.Firtel, unpublished observation; R.Dottin, DDBJ/EMBL/GenBank accession No. DDU15210). The encoded open reading frame (ORF) exhibits a high degree of amino acid sequence conservation and a domain organization that is indistinguishable from that of Akt/PKBs from metazoans (Bellacosa *et al*., 1991; Coffer and Woodgett, 1991; Jones *et al*., 1991), including an N-terminal PH domain, homologous kinase and C-terminal regulatory domains, and phosphorylation sites required for Akt/PKB activation. In this manuscript, we demonstrate that *Dictyostelium* Akt/PKB is activated in response to chemoattractant signaling. This activation is mediated by the cAMP receptor cAR1 and requires the coupled heterotrimeric G protein. In addition, the activation requires the function of PI3Ks that are related to mammalian p110, PI3K. We demonstrate that *pkbA* null cells exhibit aggregation-stage defects, including the inability to polarize properly in a chemoattractant gradient and a decrease in the rate of chemotaxis towards cAMP. Moreover, we find that an Akt/PKB PH domain–green fluorescent protein (GFP) fusion (PH–GFP) transiently translocates to the plasma membrane in response to receptor stimulation and localizes to the leading edge of chemotaxing cells. These data that suggest that transient membrane localization correlates with Akt/PKB activation by chemoattractants, and that this pathway is involved in controlling aspects of cell polarity and cell movement.

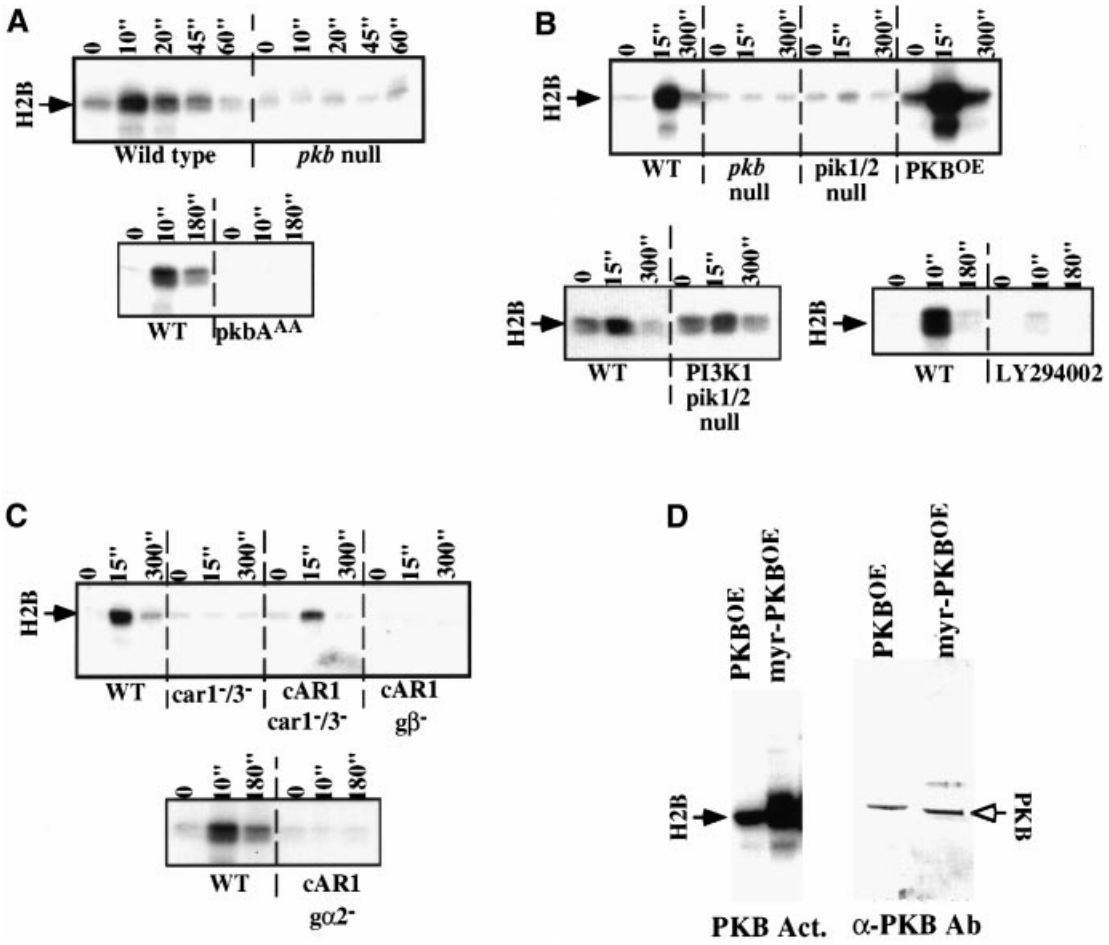


Fig. 2. Activation of Akt/PKB. (**A**) Akt/PKB activity of immunoprecipitates towards H2B as a substrate is shown. Aggregation-stage cells (see Materials and methods) are activated by cAMP. Aliquots were taken at the timepoints indicated. Cells were lysed, Akt/PKB was immunoprecipitated using a rabbit anti-*Dictyostelium* PKB peptide antibody, and kinase activity was measured as described in Materials and methods. Wild-type and *pkbA* null cells are shown. Activity peaks between 10 and 20 s. (**B**) PKB activity in wild-type cells, *pkbA* null cells, PI3K1/PI3K2 double knockouts (*pi3k1/2* null cells), PKB overexpressing cells and *pik1/2* double knockout cells constitutively expressing PI3K1. Wild-type cells were also treated with the PI3K inhibitor LY294002 (Calbiochem) at 12.5 μM for 60 s immediately prior to cAMP stimulation. Wild-type controls were done at the same time each mutant was analyzed as an internal control. (**C**) Akt/PKB activity is depicted in cAR1/cAR3 (*car1*$^{-}$*/3*$^{-}$) double knockout cells. *cAR1/cAR3* double knockout cells constitutively expressing cAR1 under the control of the *Act15* promoter, *g*β null cells and *gα2* null cells constitutively expressing cAR1, and wild-type cells (Insall *et al*., 1994b). (**D**) Left-hand panel shows H2B kinase activity of cells overexpressing wild-type Akt/PKB and Akt/PKB tagged with the N-terminus of c-Src which is sufficient for N-terminal myristylation and membrane localization. Right-hand panel depicts the Western blots of aliquots from the same experiment using the anti-PKB antibody. Wild-type Akt/PKB and the myristylated Akt/PKB are expressed at the same level. A closed arrowhead marks the position of the phosphorylated H2B. All experiments shown have been repeated independently. The data shown in all panels are representative. *car1*$^{-}$*/3*$^{-}$ double knockout cells expressing cAR1, and *g*β and *g*α null cells constitutively expressing cAR1 and their use in similar experiments have been described previously (Insall *et al.*, 1994b; Milne *et al.*, 1995; Schnitzler *et al.*, 1995; Wu *et al.*, 1995; Maeda *et al.*, 1996).

The conservation of regulatory pathways that control cell movement and cytoskeletal elements between *Dictyostelium* and mammalian cells (Chen *et al*., 1996; Zigmond *et al*., 1997) potentially allows one to use findings in one system to understand cell movement in another.

Results

Structure and expression pattern of Dictyostelium Akt/PKB

Akt/PKB (pkbA) was cloned using data from previous PCR screens (S.K.O.Mann and R.A.Firtel, unpublished observation) and sequence data in DDBJ/EMBL/GenBank (accession No. DDU15210). Figure 1A shows the derived amino acid sequence of *Dictyostelium* Akt/PKB compared with that of metazoan Akt/PKBs (Bellacosa *et al*., 1991; Coffer and Woodgett, 1991; Jones *et al*., 1991). There is strong amino acid sequence conservation in the N-terminal PH and kinase domains. The site of expected phosphorylation in the activation loop of the kinase is conserved. Comparison of the site that is phosphorylated near the C-terminus in mammalian Akt/PKBs identifies a possible phosphorylation consensus sequence (Figure 1B). In mammalian Akt/PKBs, this second phosphorylation site is a Ser, while the *Dictyostelium* Akt/PKB has a Thr at the comparable position.

Figure 1C depicts Western blot analysis of Akt/PKB protein levels (see Materials and methods). The protein is found in vegetative cells and levels increase during the first few hours of development. After 8 h, the time of mound formation, the levels have decreased significantly and the protein is barely detectably using our assay. The protein is not detected in the *Akt/PKB* null strain (data not shown).

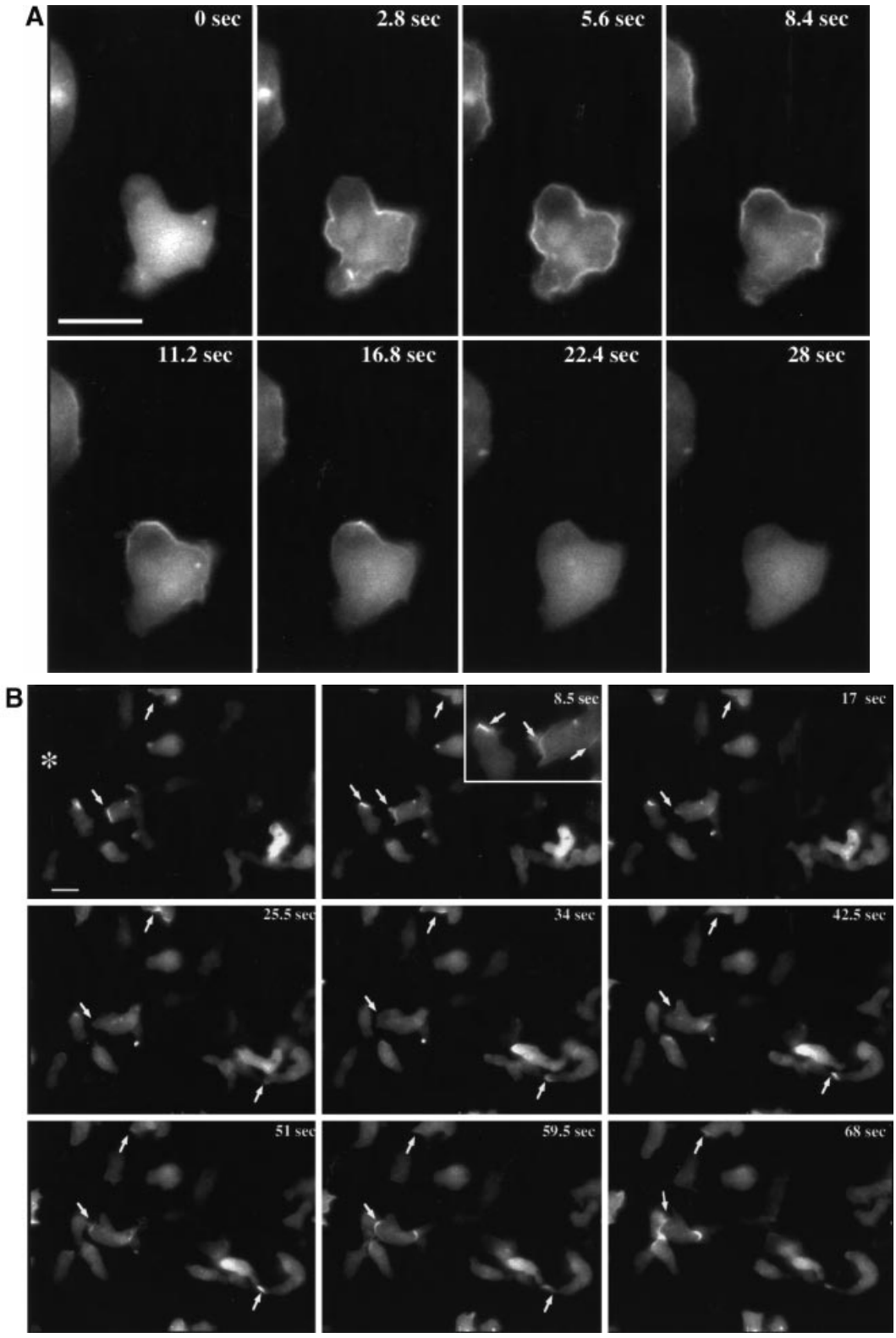


Fig. 3. Membrane localization of *Dictyostelium* Akt/PKB PH–GFP fusion protein. (**A**) Wild-type cells expressing the Akt/PKB PH domain fused to GFP expressed under the control of the Act15 promoter were pulsed for 4.5 h to make them aggregation-competent and then plated on a glass coverslip glued over a hole in a 30 mm Petri dish (see Materials and methods). By applying pressure to a glass capillary filled with 150 μM cAMP, a short pulse of cAMP was released at t_0 and the ensuing changes in GFP:PH distribution were recorded. The first image is taken immediately before the cAMP addition. One whole cell and part of another are in the field. Images were taken every 2.8 s. Every image is shown for the first 11.2 s and then every other image. (**B**) Aggregation-competent cells were plated on a coverslip and allowed to chemotax towards a micropipet containing 150 μM cAMP (approximate position of the tip indicated by the asterisk in the first image). Accumulation of PH–GFP can be seen at the leading edge of some cells. Three of these cells are marked with an arrowhead. The time for each image relative to when the recording was initiated. An enlargement of part of the second image (8 s) showing localization at the leading edge is shown. The CCD integration time for each image was ~8 s. See Materials and methods for details. All experiments have been repeated and the data shown are representative. Scale bar, 10 μm; the same scale is shown for all parts of the figure.

Activation of Akt/PKB by the chemoattractant cAMP

Cyclic AMP is the chemoattractant that regulates aggregation in *Dictyostelium* (see Introduction). To determine whether *Dictyostelium* Akt/PKB activity is stimulated in response to the chemoattractant, aggregation-stage cells were stimulated by cAMP, lysed, and Akt/PKB was immunoprecipitated with a rabbit anti-*Dictyostelium* Akt/PKB peptide antibody (see Materials and methods). Figure 2A shows that kinase activity towards histone 2B (H2B) is rapidly stimulated in response to cAMP, peaking at 10–20 s, after which the activity rapidly decreases. The kinetics of activation and subsequent adaptation are very similar to those of cAMP stimulation of guanylyl cyclase activity (Van Haastert and Van der Heijden, 1983). To determine whether the stimulated kinase activity is due to Akt/PKB, we created an *Akt/PKB* null (*pkbA* null) strain by homologous recombination (see Materials and methods). Immunoprecipitates from these cells only contain a low background activity that does not change in response to cAMP stimulation. Mammalian Akt/PKB kinase activity is activated through the phosphorylation of conserved Ser/Thr residues (see Introduction). To examine this indirectly, we mutated the conserved residues (Figure 1B) to Ala ($pkbA^{T278A,T435A}$) and overexpressed the mutant protein in *pkbA* null cells. As can be seen, cells overexpressing $pkbA^{T278A,T435A}$ show no receptor-mediated activation of Akt/PKB activity (Figure 2A). These data are consistent with the possibility that the *Dictyostelium* Akt/PKB is activated by phosphorylation in a similar way to mammalian Akt/PKB.

Cyclic AMP activation of Akt/PKB requires the PI3Ks, PI3K1 and PI3K2

In mammals, Akt/PKB is co-regulated by the phospholipid products of PI3K PtdIns-(3,4,5)-P_3 and PtIns-(3,4)-P_2. We previously identified three *Dictyostelium* PI3Ks (PI3K1–3) with extensive homology to members of the mammalian p110 family of PI3Ks (Zhou *et al*., 1995). PI3K1 and PI3K2 are most closely related to each other and appear to be genetically redundant. A single knockout of either gene has no overt phenotype, while the double knockout has multiple growth and developmental defects, including defects in the organization of the actin cytoskeleton (Zhou *et al*., 1995, 1998; Buczynski *et al*., 1997). PI3K3 is most homologous to mammalian p110γ (Vanhaesbroeck *et al*., 1997). A knockout of this gene has no visible consequences but is essential for growth in a genetic background lacking either PI3K1 or PI3K2.

Figure 2B demonstrates that *Dictyostelium* Akt/PKB is not activated in response to cAMP in PI3K1/2 double knockout cells (*pi3k1⁻/2⁻*). Activation is normal in PI3K1 or PI3K2 single knockouts or *PI3K3* null cells (data not shown). Expression of PI3K1 in *pi3k1⁻/2⁻* cells results in complementation of the growth and developmental defects of *pi3k1⁻/2⁻* cells (Zhou *et al*., 1995; T.B.K.Reddy, R.Meili and R.A.Firtel, unpublished observation). When assayed for cAMP-stimulated Akt/PKB activation, these cells

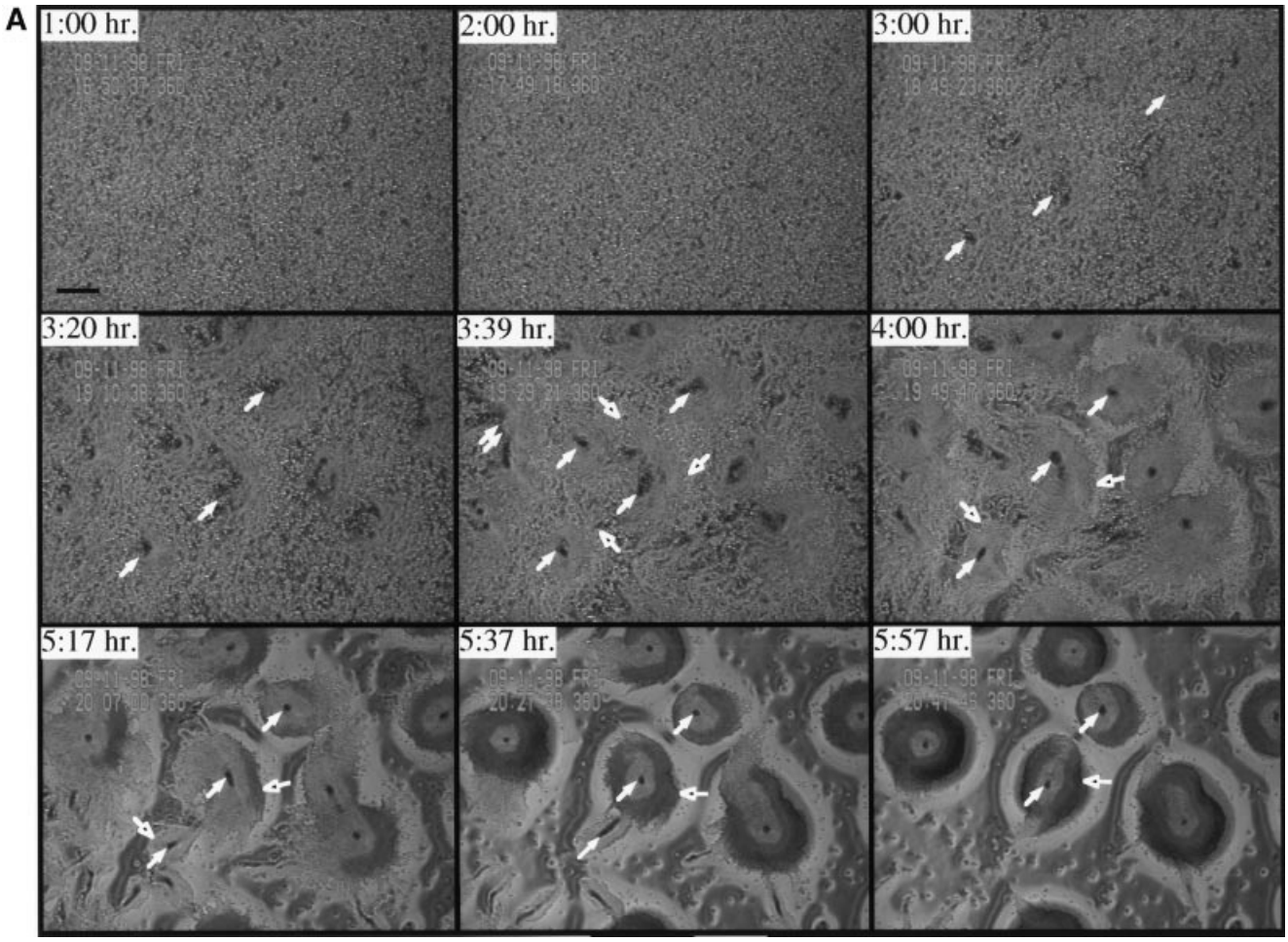


exhibit receptor stimulation of Akt/PKB activity (Figure 2B). LY294002 is an inhibitor of mammalian PI3Ks (Vlahos *et al*., 1994). Although use of the *pi3k1/2* double knockout strain provides a strong genetic test for the requirement of PI3K for Akt/PKB activation, we cannot rule out that this effect is due to a pleiotropic effect of disrupting PI3K1 and PI3K2. We therefore tested whether LY294002 can inhibit cAMP-stimulated Akt/PKB activation. As depicted in Figure 2B, addition of LY294002 1 min before cAMP decreased the level of Akt/PKB activation by >90%. Together, these data indicate that PI3K1 and PI3K2 are required for Akt/PKB activation in *Dictyostelium*.

Many, but not all cAMP-stimulated, receptor-dependent pathways in *Dictyostelium* require heterotrimeric G proteins containing the Gα2 protein subunit (Firtel, 1995; Chen *et al*., 1996; Parent and Devreotes, 1996). To investigate whether this is the case for Akt/PKB, we analyzed cAMP stimulation of Akt/PKB in *gα2* null and *g*β null cells that constitutively express cAR1 (to ensure that the receptor is not limiting). To minimize experimental differences between these cells and wild-type control cells,

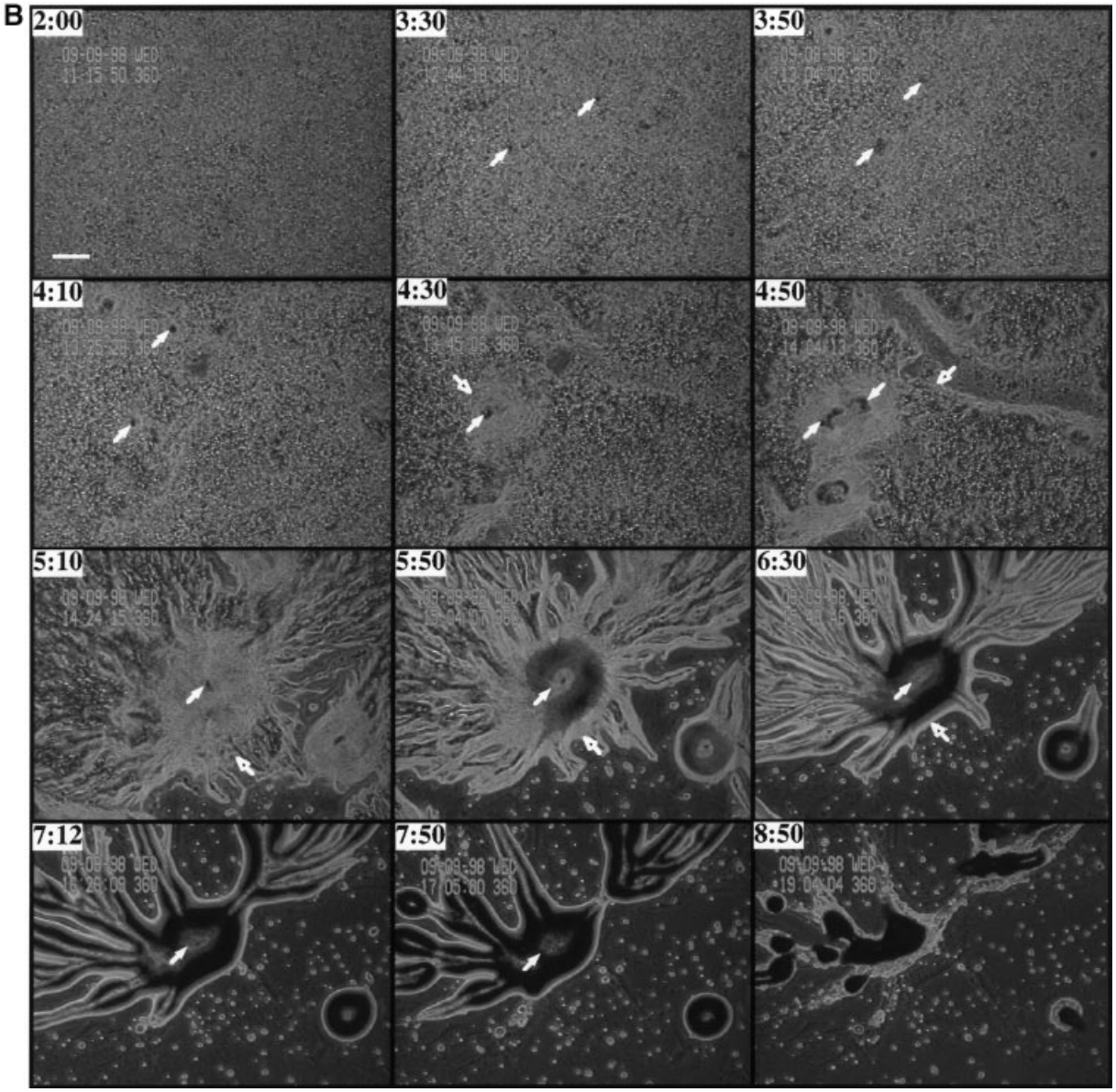


Fig. 4. Time-lapse phase-contrast video microscopy of wild-type and PKB cells during aggregation. (**A**) Aggregation of wild-type cells is depicted. Log-phase vegetative cells were washed and plated at ~95% confluency on agar as described previously (Ma *et al*., 1997). White arrowheads point to several aggregation centers. Arrowheads with black centers point to the perimeters of the aggregation domains. Aggregation centers can be seen by 3 h and the perimeters of the aggregation domains are observed by the 3 h 30 min timepoint. Loose aggregates are formed by 6 h.
(**B**) Corresponding experiments were performed using *pkbA* null cells. Aggregation centers are not observed until 3 h 30 min or later. These centers tend to be transient. Aggregation domains are visible by 4 h 30 min. The domains are larger than those in wild-type cells. Because of the location of the aggregation domains, the region of the plate being viewed was shifted at the 5 h timepoint to show a representative large aggregation center. This is representative of observations over the entire plate. Images from 5 h 10 min onward depict this extremely large aggregation center and a smaller one. Very long aggregation streams are observed for the large aggregate; the large aggregate breaks up into smaller aggregates which produce tips and undergo morphogenesis. Experimental details are described in Materials and methods. All experiments have been repeated and the data shown are representative. Scale bar, 100 μm; valid for all frames in the figure.

these cells were stimulated for 5 h with 30 nM cAMP (see Materials and methods). This stimulation activates receptor-dependent, G protein-independent pathways such as the activation of ERK2, calcium influx and receptor phosphorylation (Milne and Devreotes, 1993; Milne *et al*., 1995; Maeda *et al*., 1996; Kim *et al*., 1997). Neither *g*β nor *gα2* null cells constitutively expressing cAR1 show receptor stimulation of Akt/PKB activity (Figure 2C). *car1/car3* double knockout strains, which lack cAR1 and the other aggregation-stage receptor cAR3 (Insall *et al*., 1994b), do not exhibit cAMP stimulation of Akt/PKB activity (Figure 2C). These data indicate that cAMP stimulation of Akt/PKB activity functions through the cAMP serpentine receptor and heterotrimeric G proteins containing the Gα2 subunit. Our data suggest the induction of Akt/PKB activity requires the function of PI3K1 and PI3K2, and is activated through the cAMP receptors via a G protein-linked pathway.

Cyclic AMP-stimulated translocation of an Akt/PKB PH–GFP gene fusion

Akt/PKB translocates to the plasma membrane in response to receptor activation (Andjelkovic *et al*., 1997). In addition, the PH domain of mammalian Akt/PKB binds PtdIns-(3,4,5)-P_3 and PtdIns-(3,4)-P_2, leading to the activation of kinase activity (Burgering and Coffer, 1995; Frech *et al*., 1997; Klippel *et al*., 1997). As *Dictyostelium* Akt/PKB activation requires the function of PI3K1 or PI3K2, we examined whether the *Dictyostelium* Akt/PKB PH domain fused to GFP translocates to the plasma membrane with kinetics consistent with the activation of Akt/PKB kinase activity. As depicted in Figure 3A, the Akt/PKB PH–GFP fusion is uniformly distributed in the cytoplasm of resting, unstimulated cells. In response to cAMP stimulation, the fusion protein rapidly translocates to the plasma membrane. Partial membrane localization can be detected in the first image (2.8 s after stimulation) and is maximal at ~6–9 s. This translocation is very transient, being significantly reduced by 11 s, and not discernible by 22 s. These kinetics of PH–GFP translocation to the plasma are very similar to those of receptor-mediated activation of Akt/PKB kinase activity. Akt/PKB activity remains high for a short period of time after the PH–GFP fusion is no longer membrane-localized, suggesting that continued membrane localization may not be required for kinase activity.

We examined localization of Akt/PKB PH–GFP fusion in cells chemotaxing to cAMP (Figure 3B). Of the migrating cells (some cells are non-responsive and do not migrate at all; we expect this is due to photo damage), ~70% show a transient localization of the Akt/PKB PH–GFP fusion to the leading edge. A careful frame-by-frame viewing of the images reveals that this translocation coincides with the movement of these cells, as has been described for the PH domain-containing protein CRAC (Parent *et al*., 1998). Cell movement occurs by the protrusion of a leading edge followed by a lifting and retraction of the posterior of the cell (see Soll and Voss, 1998). The rapidity of the PH–GFP membrane translocation suggests that the Akt/PKB localization occurs during the initial phase of this two-step process, prior to the protrusion of a new pseudopod. The rapid and transient kinetics of this translocation, as depicted in Figure 3A, together with the integration times of ~8 s per frame, reduce the probability of synchronizing each image captured to visualize leading edge localization. We therefore do not expect our recordings to show the translocation for every cell as it moves. Moreover, as the cell lifts its leading edge during the initial forward movement, the leading edge is no longer in focus. Changing the plane of focus periodically shows cells with the PH–GFP fusion at the leading edge (R.Meili and R.A.Firtel, unpublished observation). In at least three of the cells (Figure 3B, marked by arrowheads), the timing of the recording and the response was fortuitously synchronized. The Akt/PKB PH–GFP fusion localizes to the leading edge, delocalizes and relocalizes as the cells move. When the cells form cell–cell contacts as they do *in vivo* during stream formation, a more intense fluorescence can be seen where the cells touch. This is also observed in experiments using CRAC–GFP (C.Parent and P.Devreotes, personal communication).

pkbA null cells exhibit aggregation defects

When *Dictyostelium* cells are plated for development on phosphate-buffered agar plates as an ~95% confluent monolayer, wild-type cells aggregate to form a multicellular aggregate in ~8 h (Ma *et al*., 1997). The properties of this aggregation can be visualized by time-lapse phase-contrast video microscopy, which permits the observation of wave patterns of cAMP moving through the field of cells that result in cell shape changes and chemotaxis (Ma *et al*., 1997). Aggregation centers become very well defined by 4 h, followed shortly thereafter by the chemotaxis of cells towards the aggregation center (Figure 4A). Examination of the videotapes of *pkbA* null cells reveals similar refractoral waves moving across the field of cells prior to the formation of aggregation centers (data not shown), indicating that *pkbA* null cells produce and respond to cAMP. As shown in Figure 4B, *pkbA* null cells exhibit refractoral patterns in the monolayer of cells as aggregation centers form in response to cAMP signaling. However, contrary to our observations for wild-type cells, the centers are not stable. Eventually, a few large aggregation centers emerge. Chemotaxis commences and very large aggregates form (Figure 4B). When we examine aggregation under conditions in which cells are plated at ~25% confluency, wild-type cells aggregate normally with only a slight delay and all of the cells participate in aggregate formation (data not shown). In contrast, *pkbA* null cells do not aggregate (data not shown).

Many of the components of the aggregation-stage signaling pathways are induced by nanomolar pulses of cAMP via an autoregulatory loop. Aggregation-stage cells are generated by pulsing cells in suspension with 30 nM cAMP for 5 h to maximally express receptors and other components of the pathways required for receptor activation of adenylyl and guanylyl cyclases (Devreotes *et al*., 1987; Mann and Firtel, 1987; Saxe *et al*., 1991). To determine whether *pkbA* null cells are unable to aggregate properly due to an inability to activate cAMP-induced aggregation-stage gene expression, *pkbA* null cells were pulsed with 30 nM cAMP. We examined expression of two aggregation-stage genes, those encoding cAR1 and the cell adhesion molecule csA, which are good indicators of the expression of this class of gene. Both genes are expressed similarly in *pkbA* null and wild-type cells

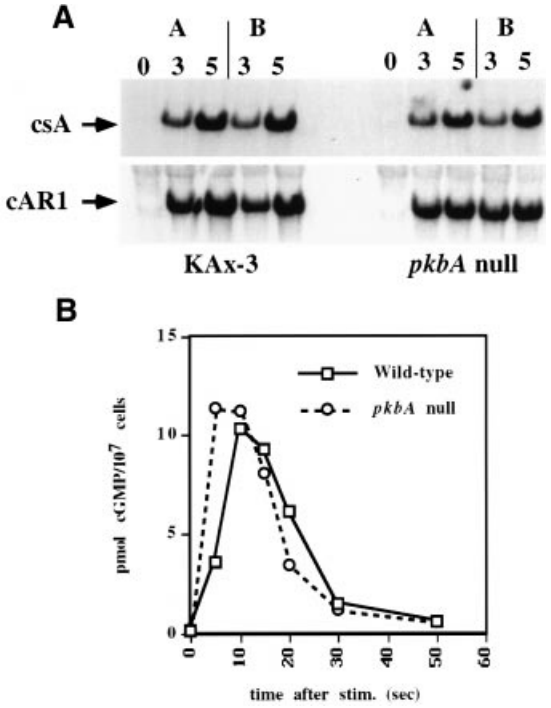


Fig. 5. Aggregation-stage gene expression and cAMP-induced activation of guanylyl cyclase in wild-type and *pkbA* null cells. (**A**) Expression of the pulse-induced aggregation-stage genes csA and cAR1. Cells were pulsed with either 25 (lanes A) or 80 nM (lanes B) cAMP every 6 s. RNA was isolated and analyzed by RNA blot hybridization. (**B**) Cyclic AMP stimulation of guanylyl cyclase was measured by quantitating cGMP production in response to cAMP stimulation. The experiment was repeated several times representative results are shown. See Materials and methods for details.

(Figure 5A). This finding strongly suggests that the components required for aggregation, except for Akt/PKB, are not limiting in *pkbA* null cells and that these cells respond to cAMP and activated aggregation-stage pathways. We cannot exclude that some genes required for chemosensory signaling are not fully expressed in *pkbA* null cells.

The kinetics of activation of *Dictyostelium* Akt/PKB in response to the chemoattractant cAMP are very similar to those of accumulation of cGMP (Van Haastert and Van der Heijden, 1983), a second messenger required for chemotaxis, and *pkbA* null cells show aggregation defects, suggesting that Akt/PKB function may be required for proper chemotaxis. To examine this question, we directly compared receptor-mediated cGMP production and the ability to chemotax in *pkbA* null and wild-type cells. As we show in Figure 5B, addition of cAMP to aggregation-stage cells results in a rapid stimulation of cGMP production with kinetics and levels of accumulation similar to those of control wild-type cells. Therefore, aggregation phenotypes exhibited by *pkbA* null cells are presumably not due to effects on guanylyl cyclase activation and cGMP production.

pkbA null cells exhibit defects in polarization and cell movement in chemoattractant gradients

To examine chemotaxis, we assayed the ability of aggregation-competent *pkbA* null cells (cells pulsed for 4.5 h with cAMP) to chemotax to cAMP emitted from a micropipet. Figure 6A (left panel) depicts wild-type cells chemotaxing towards a micropipet containing cAMP, a response that is conveniently analyzed by time-lapse video microscopy. Wild-type cells become very elongated (polarized) in the cAMP gradient and move with pseudopodia extending almost exclusively in the direction of the cAMP gradient (Figure 6B). In contrast, *pkbA* null cells are less polarized in the gradient and the majority of cells remain more rounded (Figure 6A, right panel, and C). Detailed analysis indicates that the *pkbA* null cells often extend pseudopodia in directions perpendicular to the direction of the cAMP gradient (Figure 6C), a response that is seldom seen with wild-type cells but is similar to observations in *rasG* null cells (Tuxworth *et al*., 1997). *pkbA* null cells move more slowly than wild-type cells, as illustrated in Figure 6A, where many fewer *pkbA* null cells accumulate by the micropipet tip at 20 min than wild-type cells (Figure 6A). To exclude the possibility that *pkbA* null cells are simply delayed in obtaining the ability to properly chemotax, we assayed *pkbA* cells that had been pulsed for as much as 7 h with 30 nM cAMP prior to assaying chemotaxis. We observed no difference in the response of *pkbA* null cells (data not shown).

Our results indicate that Akt/PKB function is required for the proper function of cells in a chemoattractant gradient and may play a role in suppressing lateral pseudopod extension. We examine this further by overexpressing either wild-type Akt/PKB or Akt/PKB carrying the N-terminal myristylation signal from mammalian Src, which localizes proteins to the plasma membrane (Buser *et al*., 1994; Sigal *et al*., 1994). As shown in Figure 2B, overexpression of wild-type Akt/PKB results in an increase in basal and cAMP-stimulated kinase activity in Akt/PKB immunoprecipitates. When the myristylated wild-type Akt/PKB is overexpressed to the same level as wild-type Akt/PKB as determined by Western blot analysis (Figure 2F), there is a significant increase in basal activity (Figure 2E). We envision this to be due to the constitutive localization of Akt/PKB to the plasma membrane leading to a constitutive activation of kinase activity, as has been demonstrated for mammalian Akt/PKB (Kohn *et al*., 1996). When we analyzed chemotaxis towards cAMP emitted from a micropipet, cells expressing the myristylated Akt/PKB polarized but moved extremely slowly, even compared with *pkbA* null cells (data not shown).

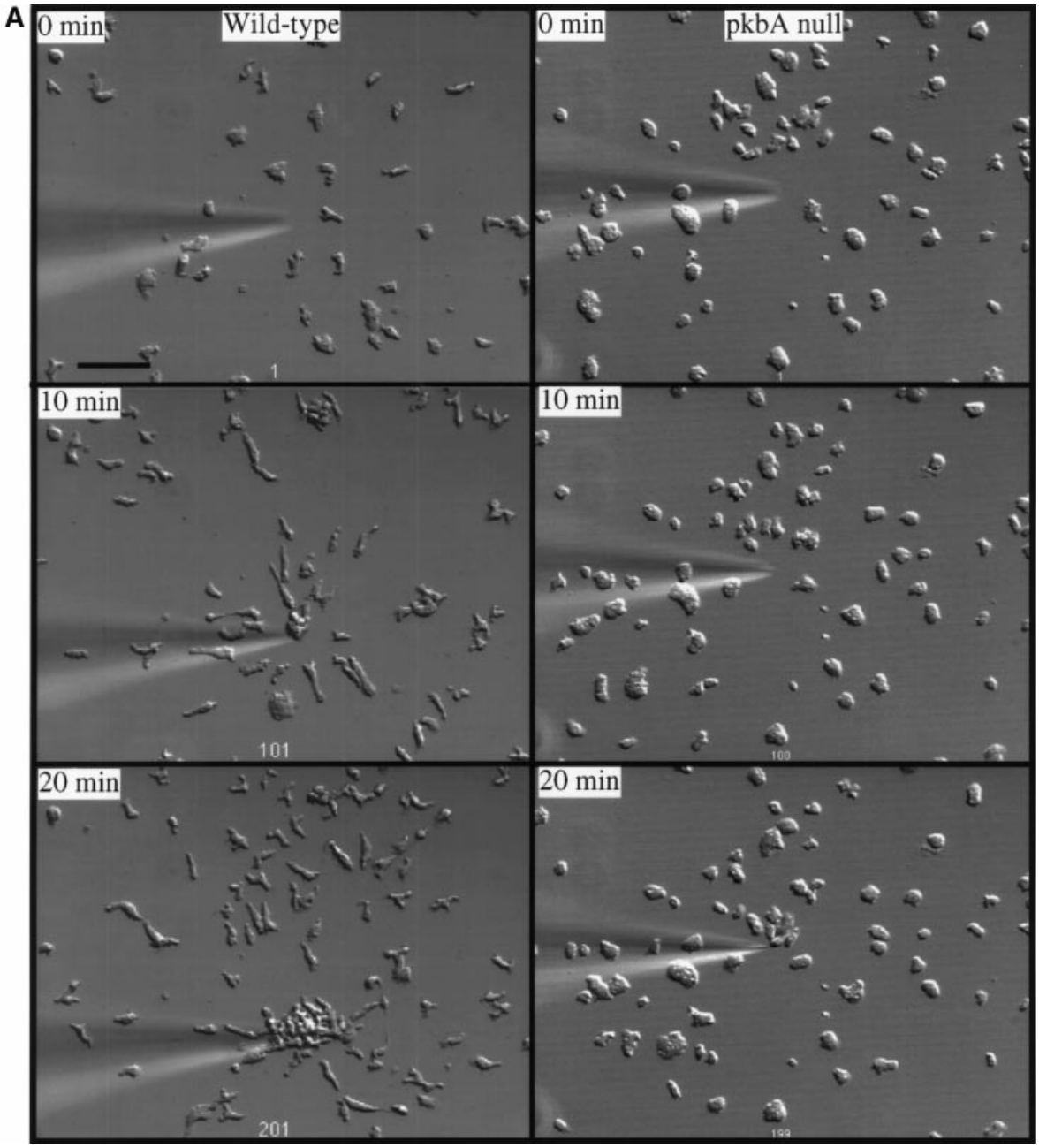
Discussion

Akt/PKB is transiently activated in response to the chemoattractant cAMP

We demonstrate that in response to the chemoattractant cAMP, *Dictyostelium* cells rapidly stimulate Akt/PKB activity. The activity peaks at 10–15 s and then quickly decreases, indicating that Akt/PKB activation, like many other aggregation-stage responses in *Dictyostelium*, rapidly adapts. We find that Akt/PKB activation requires cell-surface cAMP receptors and the heterotrimeric G protein that couples to other receptor-mediated aggregation-stage responses. Interestingly, activation of Akt/PKB in *Dictyostelium* requires the function of two closely-related PI3Ks that exhibit a high level of homology to the mammalian p110 family of PI3Ks, which are activated in response to a variety of extracellular signals (Vanhaesbroeck *et al*., 1997). The role of PI3K in Akt/PKB activation is supported by our results demonstrating that the PI3K inhibitor

LY294002, at concentrations similar to those that inhibit PI3K activity in mammalian cells, effectively blocks cAMP stimulation when it is given only 1 min prior to receptor stimulation. The genetic and inhibitor studies strongly suggest that the requirement of PI3K for Akt/PKB activation is direct. We suggest that receptor stimulation leads to the activation of PI3K via a G protein-coupled pathway, and the activation of Akt/PKB, possibly via a mechanism that is similar to Akt/PKB activation in mammalian cells. This occurs via a phosphorylation of conserved residues in the activation loop and C-terminal tail (see Introduction). Consistent with such a model, we show that *Dictyostelium* Akt/PKB, in which these two residues are mutated to Ala (pkbA^{T278A,T435A}), is not activated in response to cAMP.

We found that a GFP fusion with the Akt/PKB PH domain transiently translocates to the plasma membrane responding cells. We show that as cells form cell–cell contacts, as they would in the formation of aggregation stream *in vivo*, there is a sustained localization of PH–GFP at the position of the cell contact. We surmise that cell–cell contact may potentiate pathways leading to this localization. Although we do not directly demonstrate that the whole kinase undergoes a similar translocation in response to cAMP due to technical difficulties with the full-length Akt/PKB–GFP fusion, our data are consistent with a membrane translocation of *Dictyostelium* Akt/PKB in response to receptor stimulation and, in accordance with models describing the activation of mammalian Akt/PKB (see Introduction). Considering the requirement of PI3K1/2 for Akt/PKB activation, we hypothesize that PI3K1 and PI3K2 are transiently activated in response to cAMP and their activation is required for Akt/PKB activation. Recently, Parent *et al.* (1998) demonstrated



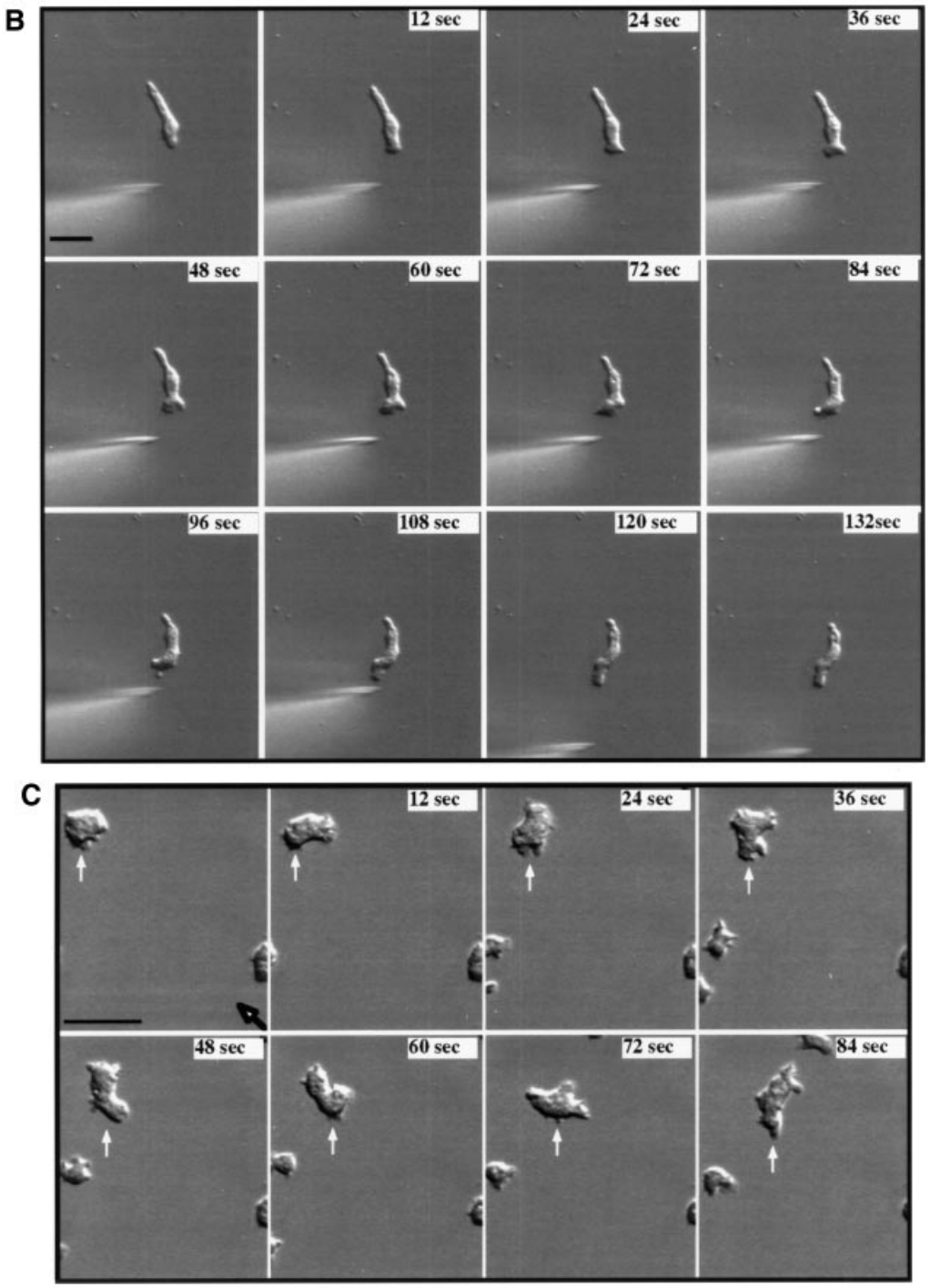


Fig. 6. Chemotaxis of wild-type and *pkbA* null cells. Wild-type and *pkbA* null cells were washed and pulsed for 4.5 h with 30 nM cAMP every 10 min (see Materials and methods), and plated on a Petri dish with a hole in the center over which a glass coverslip has been glued in place for the chemotaxis assay. The tip of a micropipet containing 150 μM cAMP is visible. (**A**) Chemotaxis of wild-type cells and *pkbA* null cells. The three left-hand panels of the figure show wild-type cells. Accumulation of cells at the micropipet tip is seen within 10 min and significant accumulation is observed after 20 min. The right-hand side of the figure shows the chemotaxis of *pkbA* null cells. *pkbA* null cells move very slowly and very few cells have accumulated at the tip after 20 min, even though the initial density of the *pkbA* null cells is slightly higher than for the wild-type cells (compare the '0 min' panels). (**B**) Chemotaxis of a single wild-type cell towards a micropipet containing cAMP recorded at 6 s intervals. The frames show the same area of the Petri dish (data courtesy of Chang Chung; Chung *et al.*, submitted). Note: after the 108 s time point, the needle was moved further away from the cells. For both wild-type cells and pkbA null cells (C), the properties of the cell movement was not dependent on how close the needle was to a specific cell as long as the cell was in the field of view shown in (A). (**C**) Movement of a *pkbA* null cell. A single cell is viewed at 12 s intervals. The position of the micropipet remains constant and points in the direction of the open arrowhead shown in the upper left panel. The cell being followed is marked by a white arrowhead. The cells do not polarize in the direction of movement. Pseudopodia are extended in multiple directions simultaneously and not in the direction of the chemoattractant gradient. All experiments have been repeated and the data shown are representative. Scale bars, 10 μm in (A) and (B), 20 μm in (C).

that a GFP fusion of the PH domain containing protein CRAC, which is required for receptor activation of adenylyl cyclase in *Dictyostelium*, shows a transient membrane translocation to the leading edge of cells with kinetics similar to those of the Akt/PKB PH–GFP fusion in response to cAMP stimulation. In polarized, chemotaxing cells, the Akt/PKB PH–GFP fusion exhibits a similar localization to the leading edge. As described in the Results section, our data suggest that in chemotaxing cells the Akt/PKB PH–GFP fusion transiently localizes to the leading edge, indicating this is a dynamic process that occurs during the process of chemotaxis. It is possible that the same second messenger regulates the membrane localization of both proteins, nucleating the formation of signaling complexes at the leading edge required for a series of signaling responses, including activation of adenylyl cyclase and pathways leading to cell movement. This would facilitate the interaction of the proteins involved in these pathways and localize the downstream responses to permit the directed cell movement required during aggregation. Such signaling complexes have been reported in *Dictyostelium* (Xiao and Devreotes, 1997), although it is not known how many components of these pathways are localized. It is expected that similar complexes would be formed in metazoans.

pkbA null cells exhibit aggregation defects

Our phenotypic analysis of *pkbA* null cells suggests that Akt/PKB has multiple functions during the aggregation stage of *Dictyostelium* development that include the regulation of chemotaxis. *pkbA* null cells are less polarized in cAMP gradients and the rate of movement of these cells is slower than for wild-type cells. Closer examination reveals that, unlike wild-type cells, many of the *pkbA* null cells produce lateral pseudopodia even though cAMP receptors are uniformly distributed along the surface of the cell and thus all parts of a cell in a cAMP gradient receive a signal (Xiao *et al*., 1997). *rasG* null cells have similar cell movement defects (Tuxworth *et al*., 1997). Cells must discriminate between small concentration differences between the front and back of a cell in a gradient to regulate directional responses such as chemotaxis. Previous results with CRAC–GFP similarly indicated that the activation of downstream pathways may be restricted to the leading edge of a migrating cell (Parent *et al*., 1998). It is therefore probable that an intracellular signal relay mechanism prevents pseudopodia formation in regions of the cells other than the front. Alternatively, some components of these pathways may be limiting and their accumulation in one part of the cells may limit the ability for the same pathway to be activated in another part of the same cell. While we cannot distinguish between these possibilities, we favor the former model, which is consistent with localization of CRAC–GFP and Akt/PKB PH–GFP at the leading edge. Our data are consistent with Akt/PKB being required for the suppression of lateral pseudopod formation perpendicular to the chemoattractant gradient. We demonstrate that overexpression of a membrane-targeted (myr) Akt/PKB results in a very high constitutive Akt/PKB kinase activity, and myr-Akt/PKB-expressing cells are unable to chemotax efficiently. Overexpression of a membrane-targeted Akt/PKB might result in a constitutive activation of Akt/PKB along the entire plasma membrane. We expect that such a non-spatially restricted active Akt/PKB may inhibit chemotaxis by preventing the cells from detecting the directionality of a subsequent cAMP signal. Although these cells become polarized, possibly due to a spatially-restricted activation of other cellular responses, the cells move more slowly than wild-type cells, suggesting that localized activation of Akt/PKB may be an important component of cell movement. In mammalian cells, PI3K is required for Ras-mediated reorganization of the actin cytoskeleton in non-motile cells (Ma *et al*., 1998). It is not known whether this function of PI3K requires downstream activation of Akt/PKB.

We found that *pkbA* null cells exhibit normal expression of two aggregation-stage genes that are representative of genes expressed during aggregation required for chemotaxis. Thus, we do not think the defects in cell movement that we have observed result from secondary effects on gene expression, although we cannot exclude the possibility that some genes required for chemosensory responses are not fully expressed. We demonstrated that cells pulsed for as long as 7 h have similar chemotaxis defects. Thus, if *pkbA* null cells are delayed in attaining chemotaxis competence, it cannot be overcome by pulsing the cells for a longer period of time. Moreover, cAMP activation of guanylyl cyclase in *pkbA* null cells, which is required for chemotaxis, is indistinguishable from that of wild-type cells, indicating that defects in this pathway are not responsible for cell movement defects of *pkbA* null cells and that the ability to activate this pathway is not delayed in *pkbA* null cells.

pkbA null cells have two other aggregation defects. First, *pkbA* null cells produce few stable aggregation centers and the formation of aggregates is delayed compared with wild-type cells. Aggregation requires each cell to respond to ~20 pulses of cAMP (one every 6–10 min) to produce a multicellular organism (Firtel, 1995; Ginsburg *et al*., 1995; Van Haastert, 1995; Parent and Devreotes, 1996). Analysis of video movies of *pkbA* null cells indicates that aggregation centers appear but are rapidly lost, with new centers appearing and disappearing. Eventually, a few dominant, large aggregation centers persist over an area in which wild-type cells normally have multiple stable aggregation domains. One possible explanation of this phenotype is that Akt/PKB affects the adaptation of the signaling pathways, although the available data do not prove this model. The second phenotype is an inability of cells to aggregate when plated at lower densities. We think this phenotype is associated with the reduced efficiency of chemotaxis. When cells are plated as a confluent monolayer, cell–cell contacts may facilitate chemotaxis, possibly through cell adhesion molecules that are induced during aggregation. It is thought that such processes allow the coalescing of *mek1* null cells to form aggregates (Ma *et al*., 1997).

We predict that Akt/PKB translocates to the plasma membrane in response to cAMP signaling, as this would be consistent with a proposed pathway for Akt/PKB activation and the present data. As many of the components required for chemotaxis are thought to be conserved between *Dictyostelium* and mammalian cells (Chen *et al*., 1996; Zigmond *et al*., 1997), it is possible that PI3K and Akt/PKB may play similar roles in the response of mammalian cells to chemokines.

Materials and methods

Materials

Sodium orthovanadate, β-glycerophosphate, aprotinin and leupeptin were obtained from Sigma. H2B was purchased from Boehringer Mannheim. [γ-^{32}P]ATP was from ICN. Protein A–Sepharose CL-4B was obtained from Pharmacia Biotech., Inc.

Antibodies

A rabbit polyclonal anti-*Dictyostelium* PKB antibody was raised against a glutaraldehyde conjugate of bovine serum albumin (BSA) with the peptide KGGVAESEHLER corresponding to the C-terminus of DdPKB.

Preparation of the affinity resin and affinity purification was essentially performed according to Harlow and Lane (1988). The peptide was coupled to immobilized diaminodipropylamine (Pierce) activated with glutaraldehyde (Sigma, Grade I) by rotating a slurry containing 5 mg peptide/ml beads for 2 h at room temperature.

Creation of pkbA null strain

A *pkbA* knockout construct was made by inserting the Blasticidin resistance (Sutoh, 1993) cassette into a *Bam*HI site created at base 520 of the PKB cDNA. Specifically, *pkbA* knockout plasmid was made by inserting a Blasticidin resistance (Sutoh, 1993) cassette into a *Bam*HI site inserted by PCR at a position 520 of the Akt/PKB ORF in the cDNA in the following construct: a 5′-fragment including a small intron was amplified from genomic DNA by PCR using the primers TAAATATGTCAACAGCACCAATTAAAC and TTTTTGGATCCTT-TGAAGAATATTACGCTCAC and subsequently digested with the enzymes *Hin*cII and *Bam*HI. A 3′ fragment was amplified using the primers TTTTGGATCCATCACCCATTTTTGGTTAATCTC and TTTTTTCTAGAGGGAACGAGCATCTGGAG and digested with the enzymes *Bam*HI and *Aat*II. These fragments were used to replace the *Hin*cII and *Aat*II fragment in the *pkbA* cDNA clone.

The vector was digested with *Hin*cII and *Aat*II and the DNA was transformed into *Dictyostelium* cells. After Bsr selection (Sutoh, 1993), cells were plated for the isolation of clones. Randomly selected clones were screened for a gene disruption by PCR, which was then confirmed by Southern blot analysis. Two independent clones were picked and examined. Both showed the same aggregation defects described in the Results section.

Plasmids

A full-length *pkbA* cDNA clone was obtained by screening a 12–16 h developmental λZAP library (Schnitzler *et al*., 1994) with a probe amplified from genomic DNA by PCR.

To introduce a membrane targeting epitope, a linker coding for the first 16 amino acids of chicken c-Src composed of the myristylation signal and the basic amino acid cluster sufficient for stable membrane association (Buser *et al*., 1994; Sigal *et al*., 1994) was added to the N-terminus using the unique *Hin*cII site.

For the PH–GFP fusion construct, GFP was amplified by PCR using a template obtained from Roger Tsien (UCSD) using the two primers GTTTTTACTAGTAAAAAATGAGTAAAGGTGAAGAACTTTTC and TTTTTGATATCTTGTATAGTTCATCCATGC. This product was digested with *Spe*I and *Eco*RV. DNA encoding the first 113 amino acids of Akt/PKB comprising the PH domain was amplified from the *pkbA* cDNA using primers TAAATATGTCAACAGCACCAATTAAAC and CCCCCCCTCGAGAAAAATTAAGATTCCGATTTCTTTGGTT-GTTTACC. This product was digested with *Hin*cII and *Xho*I. In a triple ligation, the two PCR products were subcloned into pBluescript, sequenced, and inserted into the expression vector DIP-j expression (Gaskins *et al*., 1996).

The putative non-activatable mutant, pkbA^{T278A,T435A}, in which the putative phosphorylation sites of Akt/PKB were mutated to Ala, was generated using the 'Transformer site-directed mutagenesis kit' (Clonetech) with the oligonucleotides GTACCACAGAAAGCACCA-GTTTTG and GATTCAGCGACATAAGCAAATCCTTCAAAATC. Successful mutagenesis was confirmed by sequencing.

All *pkbA* constructs were subcloned into DIP-j for expression in *Dictyostelium* (Gaskins *et al*., 1996).

cAMP stimulation of Dictyostelium cells

To produce aggregation-competent cells, log-phase vegetative cells were washed three times with Na/K phosphate buffer and resuspended at a density of 2–3×10^6 cells/ml in Na/K phosphate buffer and pulsed for 5 h with 30 nM cAMP every 10 min (Devreotes *et al*., 1987; Mann and Firtel, 1987; Saxe *et al*., 1991). The cells were collected by centrifugation and resuspended at a density of 2–3×10^7 cells/ml. Cells were allowed to resensitize to being able to be stimulated by cAMP by bubbling air through the cell suspension for 10 min (Devreotes *et al*., 1987; Ma *et al*., 1997). A sample of 200 μl was taken before the cells were stimulated with cAMP (100 nM final concentration). Samples were taken at the times indicated.

Lysis and immunoprecipitation

The 200 μl samples were lysed by mixing with an equal volume of 2× lysis buffer [50 mM Tris pH 7.6 at room temperature, 200 mM NaCl, 20 mM NaF, 2 mM vanadate, 50 mM β-glycerophosphate, 6 mM sodium pyrophosphate, 2 mM EDTA, 2 mM EGTA, 4 μg/ml leupeptin, 4 μg/ml aprotinin, 2% Nonidet P-40 (NP-40), 20% glycerol, 2 mM dithiothreitol (DTT)]. The lysate was pre-cleared by centrifugation after addition of 20 μl pansorbin (Calbiochem). One microliter of antibody was added to 200 μl supernatant and incubated on ice for 1 h. The formed immune complexes were collected with 50 μl of a 1:1 slurry of protein A beads in lysis buffer by incubation under agitation for 1 h at 4°C. The beads were washed twice with lysis buffer and twice with kinase buffer (25 mM MOPS pH 7.4 at room temperature, 25 mM β-glycerophosphate, 20 mM magnesium chloride, 1 mM DTT).

PKB/Akt activity assay

PKB/Akt activity was measured in a immunocomplex kinase assay following immunoprecipitation with the anti-PKB antibody. The beads were incubated with 75 μl kinase buffer containing 5 μCi [γ-^{32}P]ATP, 5 μM cold ATP, and 5 μg H2B as substrate. The reaction was stopped by the addition of 25 μl 4× sample buffer and boiling for 5 min. The samples were separated by SDS–PAGE (12.5%), blotted onto a PVDF membrane (Millipore), and exposed to film. All assays were repeated independently.

cGMP assay

To measure cGMP production in response to cAMP stimulation, cells were prepared and stimulated as described in the section entitled 'cAMP stimulation of *Dictyostelium* cells' and assayed as described previously (Van Haastert and Van der Heijden, 1983; Ma *et al*., 1997). One-hundred microliter samples were taken at appropriate intervals and processed using the 'Cyclic GMP [^{3}H] assay system' (Amersham) following the manufacturer's instructions. The assay was independently repeated. A representative experiment is shown.

Developmental time course of Akt/PKB levels: Western blot analysis

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. For each time point, 1.5×10^7 cells were harvested. The samples for Western blotting were processed as described under 'Lysis and immunoprecipitation'. PKB was released from the protein A beads by boiling in 100 μl 1× SDS sample buffer. Materials were sized by SDS–PAGE.

After separating the samples (10 μl/lane) by SDS–PAGE and blotting onto PVDF membranes (Millipore), the membranes were probed using standard Western blotting protocols. For detection of the primary anti-Akt/PKB antibody, we used an alkaline phosphatase protein A conjugate (Calbiochem). We developed the membranes using a colorigenic substrate combination (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; Boehringer Mannheim).

Chemotaxis assay

Log-phase vegetative cells were washed three times with Na/K phosphate buffer and resuspended at a density of 2–3×10^6 cells/ml in Na/K phosphate buffer and pulsed for 5 h with 30 nM cAMP every 10 min (Devreotes *et al*., 1987; Mann and Firtel, 1987; Saxe *et al*., 1991). Pulsed cells were plated in Na/K phosphate buffer at a density of 6×10^4 cells/cm^2 onto a plate with a hole covered by a 0.17 mm glass coverslip. A Eppendorf Patchman micromanipulator with a glass capillary needle (Eppendorf Femtotip) filled with 150 μM cAMP solution was brought into the field of view of an inverted microscope. The response of the cells was followed by time-lapse video recording.

Image acquisition

Dictyostelium cells were developed on Na/K phosphate agar plates at a density of 4×10^6 cells/cm^2 and recorded with a CCD72S video camera (DAGE MTI, Michigan City, MI) using a Nikon Optiphot-2 microscope and a 4× phase-contrast lens. Individual frames were captured into a

image processing program (NIH Image) with the help of a SCION frame-grabber board.

Visible light images of chemotaxing cells were taken with a Nikon Eclipse TE 300 inverted microscope equipped for DIC-imaging with a Plan Fluor ELWD 2×/0.45 or Plan Fluor ELWD 4×/0.60 lens. Individual frames were captured from a CCD72S video camera into a image processing program (NIH Image) with the help of a SCION frame-grabber board.

Fluorescence pictures were obtained with the same equipment but using a RC300 video camera (DAGE MTI, Michigan City, MI) capable of on-chip integration. The pictures were acquired with IPLab Spectrum, Scanalytics, by integrating for 2–8 s.

Acknowledgements

We would like to thank members of the Firtel laboratory, particularly Chang Chung and Jason Brown, for helpful discussions. We would also like to thank Jennifer Roth and Jason Brown for helpful suggestions on the manuscript. R.M. was funded, in part, by grants from the Swiss National Science Foundation and the Novartis Stiftung. This work was funded by research grants from the USPHS to R.A.F.

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Received December 14, 1998; revised and accepted February 24, 1999