Integrin-mediated Protein Kinase A Activation
at the Leading Edge of Migrating Cells

Running Head: Integrins Specify Localized PKA Activation

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Förster resonance energy transfer (FRET)
cAMP-dependent protein kinase A (PKA) is important in processes requiring localized cell protrusion, such as cell migration and axonal path finding. Here we used a membrane-targeted PKA biosensor to reveal activation of PKA at the leading edge of migrating cells. Previous studies show that PKA activity promotes protrusion and efficient cell migration. In live migrating cells, membrane-associated PKA activity was highest at the leading edge, and required ligation of integrins such as $\alpha_4\beta_1$ or $\alpha_5\beta_1$ and an intact actin cytoskeleton. $\alpha_4$ integrins are Type I PKA-specific A-Kinase Anchoring Proteins (AKAPs) and we now find that Type I PKA is important for localization of $\alpha_4\beta_1$ integrin-mediated PKA activation at the leading edge. Accumulation of 3’ phosphorylated phospho-inositides (PtdIns(3,4,5)P$_3$) products of PI3-kinase is an early event in establishing the directionality of migration; however, polarized PKA activation did not require PI3-kinase activity. Conversely, inhibition of PKA blocked accumulation of a PtdIns(3,4,5)P$_3$-binding protein, the AKT-PH domain, at the leading edge; hence PKA is involved in maintaining cell polarity during migration. In sum, we have visualized compartment-specific PKA activation in migrating cells and used it to reveal that adhesion-mediated localized activation of PKA is an early step in directional cell migration.
**Introduction**

Directional cell migration is important for processes such as wound healing, leukocyte trafficking, axonal path finding and cancer cell metastasis. The initial step in most mammalian cell migration is the establishment of cell polarity (Etienne-Manneville and Hall, 2001; Ridley et al., 2003). Once polarity is established, leading edge protrusions, often seen as broad lamellipodia, are pushed forward by actin polymerization and are stabilized by integrin adhesion to the extracellular matrix (ECM) (Ridley et al., 2003). The cell pulls itself along these connections which provide the traction necessary for migration while simultaneously dissociating adhesive structures at the cell rear. During migration, localized signaling events lead to the topographically restricted formation and dissolution of attachment structures. Signaling molecules promoting cell protrusion and adhesion accumulate at the front of the cell; these include N-WASP, profilin, the Arp 2/3 complex, 3’ phosphorylated phospho-inositides (PtdIns (3,4,5) P$_3$), and the Rho family GTPases; Rho, Rac and Cdc42 (Ridley et al., 2003; Horwitz and Webb, 2003; Pertz et al., 2006). These molecules regulate actin polymerization at the leading edge which drives membrane protrusion. Thus, understanding the basis for the localization of cellular signaling activities to specific regions of the cell is central to understanding cell migration.

Integrins, transmembrane receptors that mediate the adhesion of migrating cells to the ECM, consist of one $\alpha$ and one $\beta$ chain whose large extracellular domains form a headpiece that binds to components of the ECM (Ridley et al., 2003). In addition to mediating adhesion, integrins initiate and coordinate the signaling events necessary for cell polarization and motility (Etienne-Manneville and Hall, 2001). $\alpha$4 integrins ($\alpha$4$\beta$1 and $\alpha$4$\beta$7) are expressed on highly migratory cells such as leukocytes and neural crest cells (Rose et al., 2002). This class of integrins enhances directional cell migration by the binding of an adaptor, paxillin, to the $\alpha$4 cytoplasmic domain (Liu et al., 1999). Phosphorylation of the $\alpha$4 cytoplasmic domain by c-AMP-dependent protein kinase A (PKA) inhibits paxillin binding and is restricted to the leading edge of broad protrusive lamellipodia and absent from the sides and rear of migrating cells (Goldfinger et al., 2003). This localized phosphorylation of $\alpha$4 at the leading edge is necessary for efficient $\alpha$4$\beta$1-mediated migration (Goldfinger et al., 2003) because inducing global $\alpha$4 phosphorylation or global blockade of $\alpha$4 phosphorylation both inhibit migration. Furthermore,
blocking PKA inhibits protrusion and cell migration (Goldfinger et al., 2003; Howe et al., 2005; Lim et al., 2007). Thus, PKA-mediated polarized phosphorylation of α4 integrin is necessary for directional cell migration.

The localization of PKA determines its effects on a variety of cellular processes. PKA phosphorylates numerous protein substrates such as ion channels, GTPases, and transcription factors. The substrate specificity of PKA in vivo is controlled by its subcellular compartmentalization and is mediated by interactions of its regulatory subunits with A Kinase Anchoring Proteins (AKAPs) (for review see (Wong and Scott, 2004)). Activation of the cAMP/PKA pathway can induce loss of stress fibers (Dong et al., 1998), activation of Rac and Cdc42 (Feoktistov et al., 2000; O'Connor and Mercurio, 2001), filopodia and lamellipodia formation (O'Connor and Mercurio, 2001; Nishiya et al., 2005) microfilament assembly (Whittard and Akiyama, 2001b) and inhibition of Rho activity (Lang et al., 1996; Ellerbroek et al., 2003); events which occur at the leading edge of migrating cells. PKA-associated AKAPs, such as gravin, WAVE1 and ezrin localize to the leading edge. PKA influences integrin-dependent migration in a variety of cells [for review see (Howe, 2004)] and PKA and its phosphorylated substrates are enriched in cell protrusions (Howe et al., 2005; Lim et al., 2007). The presence of AKAPs at the leading edge, the role of PKA in migration, and the localization of α4 phosphorylation to the leading edge suggest that the localized activation of PKA is important in cell migration.

We have developed Förster Resonance Energy Transfer (FRET)-based PKA kinase activity reporters (AKAR1, AKAR2 and AKAR3) that permit the real time measurement of bulk PKA activity in the cytoplasm of a living cell (Zhang et al., 2001; Zhang et al., 2005; Allen et al., 2006). Here we examine the localization of PKA activity at the plasma membrane of living cells using a membrane-targeted reporter, pmAKAR3 (Allen and Zhang, 2006). We show that PKA is selectively and transiently activated at the leading edge of polarized cells during migration. This localized PKA activity requires the engagement of integrins such as α4β1 or α5β1 and polymerized actin. Localized PKA activity does not require the activity of phosphatidylinositol 3-kinase (PI3-kinase) but is required for the normal polarized accumulation of PtdIns(3,4,5)P3 at the leading edge. Thus, integrins localize PKA activation to the leading edge and this localized PKA activation is an early event in directional cell migration.
Results

PKA is activated preferentially at the front of cells during $\alpha_4\beta_1$ integrin-mediated migration.

Spatial restriction of integrin $\alpha_4$ phosphorylation by PKA to the leading edge of migrating cells plays an important role in $\alpha_4\beta_1$-dependent cell migration (Goldfinger et al., 2003; Lim et al., 2007). We used a PKA activity biosensor (AKAR) to determine whether localized PKA activity could account for the spatial patterning of $\alpha_4$ phosphorylation. Initial attempts using AKAR2.2 (Sachs et al., 2007), a soluble cytoplasmic biosensor, prevented visualization of a change in PKA activity at the plasma membrane due to its intense fluorescence in the cytoplasm. Subsequently, we utilized a compartment-specific reporter targeting the indicator to the plasma membrane by adding a membrane localization sequence from K-Ras, giving rise to plasma membrane localized pmAKAR2.2 (Sachs et al., 2007). Further improvements resulted in pmAKAR3 (Allen and Zhang, 2006), a third generation plasma membrane localized PKA activity reporter that was used for the studies described here.

To learn if localized activation of PKA at the leading edge can account for localized $\alpha_4$ phosphorylation, we transfected CHO cells expressing integrin $\alpha_4$ (CHO-$\alpha_4$) with pmAKAR3 and plated the cells on CS1, a fragment of fibronectin that serves as a specific ligand for $\alpha_4\beta_1$ (Jongewaard et al., 1996), and time-lapse FRET micrographs of the cells were taken. A cell that was randomly polarized exhibited increased FRET emission ratio at the membrane protrusions concurrent with the formation of lamellipodial protrusion (arrows, Figure 1A and Supplemental Movie 1). Addition of 25 $\mu$M forskolin, an agonist for adenylate cyclase, resulted in increased FRET ratio throughout the cell with a saturated maximal response after 40s (Figure 1A, 1C and Supplemental Movie 1). As a control for the specificity of the biosensor probe, CHO-$\alpha_4$ cells expressing pmAKAR3(TA), containing a threonine to alanine point mutation in the PKA substrate region and thus rendering it resistant to PKA phosphorylation (Allen and Zhang, 2006), exhibited low overall FRET, even within membrane protrusions (arrows, Figure 1B) and stimulation with forskolin did not affect the FRET ratio (Figure 1B and 1C), establishing that the forskolin-induced increase in FRET requires phosphorylation of the biosensor’s substrate domain. Whereas high FRET ratio values, indicative of increased PKA activity, were consistently observed in the lamellipodial protrusions of polarized cells (Figure 1A), non-
polarized cells did not exhibit elevated FRET ratios at the cell periphery relative to the central areas of the cell (Figure 1D). Thus, membrane-localized PKA activity, as indicated by the plasma membrane anchored FRET-based reporter, pmAKAR3, is increased in protrusions of polarized cells.

We scratch-wounded CHO-α4 cell monolayers plated on CS1 to achieve robust directional cell migration (Goldfinger et al., 2003; Lim et al., 2007). Along the margin of the wound, the cells rapidly polarized and extended a broad advancing lamellipodium toward the wound as they began to migrate (Supplemental Movie 2). Similar to the randomly protruding cells shown in Figure 1A, the leading edge of migrating CHO-α4 cells exhibited increased FRET that was sustained during protrusion, indicative of an enrichment of PKA activity (Figure 2A, Supplemental Movie 3). A line profile analysis of FRET emission ratios within the individual migrating cell indicated a gradient of PKA activity that was highest at the leading edge tapering gradually to basal levels at the middle and back end of the cell (Figure 2B). A quantitative analysis of FRET ratios in 3 zones, corresponding to the front, middle and rear of polarized cells, revealed that the differences in PKA activity between the front and back were statistically significant (Supplemental Figure S1). We consistently observed leading edge enrichment of PKA activity in all cells that exhibited persistent protrusive behavior indicative of active migration (n>50 cells). Furthermore, leading edge FRET was sustained for the duration of protrusive activity that was observed to persist for up to 6 minutes (not shown). Addition of 30 μM H89, an inhibitor of PKA, to the actively migrating cells resulted in dramatic loss of FRET activity at the leading edge membranes within 60 seconds, indicating that the FRET signal depended on continuing PKA activity and confirming that the signal from the pmAKAR3 probe is rapidly reversible. As expected from previous studies (Goldfinger et al., 2003; Howe et al., 2005; Lim et al., 2007), inhibition of PKA activity also resulted in reduced protrusive activity (Figure 2A and Supplemental Movie 3) and cessation of cell migration. A line profile analysis for the same region 60 seconds after H89 addition exhibited a loss of the front-to-back PKA activity gradient (Figure 2B).

Since pmAKAR3 is a rapidly reversible reporter of PKA activity, the present data suggest that increased PKA activity is continuously present at the leading edge. Furthermore, treatment of cells with H89 prior to wounding prevented the increased FRET ratios at the wound edge (data not shown). Addition of H89 to actively migrating cells led to reduction of protrusive
activity and loss of the gradient of FRET ratios, indicating that the spatial polarization of FRET was reflective of PKA activity. Furthermore, the increased FRET at the leading edge was not due to a path length effect or registration artifact because it was not observed in cells expressing pmAKAR3(TA), the probe that lacks the PKA phosphorylation site (Supplemental Figure S2).

Cells located within the monolayer several rows away from the wound edge (Figure 2C and Supplemental Figure S3) were not polarized and did not exhibit the high levels of localized PKA activity observed for cells at the wound edge. Thus, only the polarized cells migrating out from the wound edge manifested increased PKA activity in the leading edge. Furthermore, blocking PKA markedly suppressed cell protrusion and migration, confirming the importance of this kinase in cell migration.

**Integrins mediate the activation of PKA at the leading edge of migrating cells.**

Previous studies show that β1 integrin-mediated adhesion can activate PKA (Meyer et al., 2000; Kim et al., 2000; Plopper et al., 2000; Whittard and Akiyama, 2001a; Howe et al., 2005; Lim et al., 2007). Since migrating cells are continuously forming new integrin-mediated contacts at the leading edge (Ridley et al., 2003), we examined the role of integrin ligation in polarized PKA activation. CHO-α4 cells plated on the α4β1-specific ligand, CS1, were scratch wounded and allowed to migrate in the presence of either HP2/1 or PB1, function-blocking antibodies for integrins α4β1 and α5β1, respectively. When α4 integrins were blocked by treatment with HP2/1, cells at the wound edge failed to migrate and exhibited little to no enrichment of PKA activity at the leading edge (Figure 3A, top panels and Supplemental Figure S4). In contrast, blockade of α5β1 with PB1 neither blocked migration nor reduced FRET in leading edge protrusions (Figure 3A, bottom panels). Thus, α4 integrins mediate the activation of PKA at the leading edge of migrating cells.

The foregoing experiments showed that α4β1-mediated cell migration was associated with activation of PKA at the leading edge of migrating cells. To assess whether this phenomenon was α4-specific, we studied the migratory behavior of CHO cells plated on fibronectin. Since these cells do not express α4β1, they adhere and migrate on fibronectin using primarily integrin α5β1 (Goldfinger et al., 2003). When a monolayer of CHO cells plated on fibronectin was wounded, an increase in the FRET ratio occurred along the leading edge (not shown). This phenomenon was sustained when CHO cells were allowed to migrate in the
presence of HP2/1 antibody (Figure 3B, top panels) indicating that treatment with HP2/1 did not result in non-specific inhibition of PKA activity or migration. In contrast, blockade of integrin α5β1 with PB1 inhibited the migration of CHO cells on fibronectin and blocked the increased PKA activity (Figure 3B, bottom panels and Supplemental Figure S5) at the leading edge. Thus, multiple integrins can mediate the activation of PKA at the leading edge of migrating cells.

**Type I PKA specific modulation of PKA activity at the leading edge.**

The cytoplasmic domain of integrin α4 functions as an A-kinase anchoring protein (AKAP) to tether Type I PKA at the plasma membrane (Lim et al., 2007). To investigate the role of Type I PKA in PKA activation at the leading edge, mouse embryonic fibroblasts (MEF) expressing α4 integrins and pmAKAR3 were adhered on CS1 and assayed by FRET ratio imaging. Wild type MEFs extend multiple protrusions that exhibited high levels of PKA activity (Figure 4A and 4B). In sharp contrast, loss of the Type Iα PKA regulatory subunit (PKA-RIα) in prkar1a--/ MEFs led to increased PKA activity observed throughout the cell (Figure 4A and 4B). This was not unexpected, as loss of PKA-RIα leads to increased basal PKA activity as a result of excess free PKA catalytic subunits (Amieux et al., 2002; Lim et al., 2007). This de-regulation of PKA activity could complicate interpretations of the role of PKA-RIα in spatial control of PKA activity. Consequently, we used mis-localization of Type I PKA as an alternative strategy to examine its role in polarized PKA activation. We employed a mitochondrial-targeted TypeI-specific AKAP peptide, mitoAKB-RI, to sequester Type I but not Type II PKA on mitochondria (Burns-Hamuro et al., 2003; Lim et al., 2007). Expression of mitoAKB-RI in CHO-α4 cells (Lim et al., 2007), resulted in marked reduction of PKA activity at the leading edge of migrating cells (Figure 4C). In contrast, expression of mitoAKB-RII, that specifically sequesters Type II PKA to the mitochondria, had no discernible effect on enrichment of leading edge PKA activity in CHO-α4 cells migrating on CS1 substrate (Figure 4D). This shows that in cells undergoing integrin α4β1-dependent migration, Type I PKA is largely responsible for the localized PKA activity at the leading edge membrane.

**Role of F-actin and cellular protrusion in PKA activation at the leading edge.**

The foregoing studies showed that global blockade of integrin engagement prevented the activation of PKA at the leading edge of migrating cells. Because high affinity integrins are
preferentially localized to the leading edge (Kiosses et al., 2001), this is a site at which many integrins are newly engaging ligands. To directly assess the role of local engagement of integrins in the localized PKA activation, we utilized a micro-patterned matrix substratum of alternating 5 μm wide strips of CS1 and uncoated surface (Figure 5A). The pattern was formed by reversibly bonding to a microfluidic chip made of PDMS (polydimethylsiloxane) with an array of parallel 5 μm wide microchannels 5 μm partitions between them and perfusing through the microchannels a solution of CS-1. When CHO-α4 cells were seeded on this micro-patterned substrate, increased FRET was observed primarily where the cells made contact with the adhesive substrate (Figure 5A and Supplemental Figure S6) and was absent from regions that made contact with CS1 ligand-free zones. This increased FRET activity was blocked by addition of H89 (Figure 1D and 5A). Thus, both antibody inhibition experiments (Figure 3) and cell adhesion to micro-pattened substrates indicate that integrin engagement by ligands is required for increased membrane-associated PKA in cell protrusions.

The micropatterned substrates also revealed a close association between regions of the cells that were protruding and increased PKA activity (Fig. 5A). The association of protrusions with increased PKA activity raised the possibility that actin polymerization, the principal biochemical mechanism that drives protrusion, might play a role in maintaining this adhesion-dependent PKA activity. To test this idea, we treated CHO-α4 cells adherent to CS1 with Latrunculin-A. Following addition of Latrunculin-A, the greatest decline in PKA activity was observed at the leading edges and protrusive structures (Figure 5B). Consistent with loss of new actin polymerization, protrusive activity ceased in the presence of Latrunculin-A. This decline in PKA activity in protrusions occurred in spite of the fact that the Latrunculin-A-treated cell maintained a polarized morphology. Indeed, PKA activity in protrusions decreased to the low level seen at the lateral and central compartments of the cell by 17 min after Latrunculin-A addition (Figure 5B). Thus, increased PKA activity at the membrane protrusions is sustained by actin polymerization.

**PKA activation at the leading edge does not require PI3-kinase activity.**

The foregoing results indicate that the increase in PKA at the leading edge is specific for protruding areas, implying that PKA activation might be the result of forward protrusion during migration. The localized accumulation of PtdIns(3,4,5)P3 by activated PI3-kinase at the leading
edge has been implicated as an early step in the polarization and forward protrusion of cells migrating in chemoattractant gradients (Wang et al., 2002; Funamoto et al., 2002; Huang et al., 2003). This raises the possibility that active PI3-kinase at the leading edge is necessary for the polarized activation of PKA. Surprisingly, while inhibition of PI3-kinase with LY294002 altered lamellipodial dynamics and reduced the migration of α4β1-expressing CHO cells into a wound, it did not block PKA activation at the leading edge (Figure 6A and Supplemental Figure S7). In the majority of cells treated with Ly294002, PKA FRET appeared elevated at both the front, lateral sides, and rear, demonstrating the ability of PKA to become activated in the absence of PI3K activity with some disruption of normal polarity. As a control for the efficacy of Ly294002 treatment, we examined the subcellular localization of GFP-PH-Akt, which functions as a biosensor for PI3-kinase activity since the PH domain of Akt is recruited by PtdIns(3,4,5)P₃ (Meili et al., 1999; Servant et al., 2000). Following wounding, approximately 60% of cells exhibited increased fluorescence of GFP-PH-Akt at the leading edge in untreated cells whereas all cells treated with Ly294002 exhibited no accumulation of GFP-PH-Akt to the leading edge (Figure 6B). Furthermore, we also observed no accumulation of GFP-PH-Akt to the leading edge in cells treated with H89 (Figure 6B). To further confirm the observation that PKA activation was independent of PI3-kinase activity, we examined the phosphorylation of PKA substrates at the leading edge by staining fixed cells with phospho-PKA substrate specific antibodies, as an indicator of PKA activity. Leading edge staining for phospho-PKA substrates was observed in Ly294002 treated CHO-α4 cells migrating on CS1 (Figure 6C). In contrast, treatment with H89 completely abolished leading edge staining for PKA phosphorylated substrates. When we stained cells to detect phosphorylated α4 integrins, a similar result was obtained in that Ly294002 did not inhibit leading edge accumulation of phosphorylated α4, while H89 did so (Figure 6D). Identical results were obtained for CHO-α4 cells treated with wortmannin to inhibit PI3-kinase (data not shown).

We next examined the requirement of PKA activity for the accumulation of PtdIns(3,4,5)P₃ by measuring phosphorylation and activation of Akt (PKB) (Franke et al., 1995; Burgering and Coffer, 1995), a kinase regulated by PtdIns(3,4,5)P₃. Adhesion of cells induced phosphorylation of Akt in a time-dependent manner (Figure 6E). The adhesion mediated stimulation of phosphorylated Akt was not blocked by H89 treatment (Figure 6E) although the same concentration of H89 prevented PKA phosphorylation of substrate proteins.
(Figure 6F). Taken together, the data suggests that PKA activation at the leading edge is independent of PI3-kinase activity and in contrast to the accumulation of GFP-PH-Akt at the leading edge, activation of Akt, a reflection of global levels of PtdIns(3,4,5)P$_3$, does not require PKA activity. Thus, blocking PKA activation prevents the accumulation of GFP-PH-Akt in protrusions, but does not block activation of a PtdIns(3,4,5)P$_3$-dependent enzyme.

**Polarized cells exhibit a front-to-back gradient of cAMP.**

The foregoing studies established that PKA activation at the leading edge of migrating cells is dependent on integrins and the integrity of the actin cytoskeleton, but not on PI3-kinase activity; however, these results did not explain why the PKA activity is markedly increased at the leading edge. Because PKA is activated by cAMP, we hypothesized that increased cAMP might be generated at the front of migrating cells. To address this possibility, we transfected CHO-α4 cells with a cytoplasmic FRET-based biosensor for cAMP termed ICUE2 (DiPilato *et al.*, 2004; Violin *et al.*, 2008). These cells were attached to CS1 substrate and imaged as they underwent migration into a wound. In a majority of migrating cells, a linear front-to-back cAMP gradient was observed (Figure 7). Thus, we conclude that the localization of increased cAMP activity can partially account for the enrichment of PKA activity in protrusions during integrin-mediated cell migration.

**Discussion**

**Leading edge PKA activity in cell migration.**

Cell migration requires the precise localization of cellular signaling events. Here, we utilize a membrane-targeted reporter of PKA activity and show that transient PKA activation is localized to the leading edge of migrating cells. Furthermore, integrin ligation is necessary, but not sufficient, for the localized activation of PKA. This effect was not specific for an alpha integrin subunit type, as it was mediated by either α4β1 or α5β1 integrins. However, leading edge PKA activity is mediated primarily by Type I PKA for cells undergoing α4β1-dependent migration. High levels of localized PKA activation at leading edge protrusions require polymerized actin as evidenced by loss of leading edge FRET upon Latrunculin-A treatment. Leading edge PKA activity was not abolished in cells treated with PI3-kinase inhibitors; on the
contrary, PKA FRET increased in the lateral and back compartments of the cell. Our data also establish a mechanism for localized PKA activity at the cell leading edge since migrating cells exhibit a front-to-back decreasing gradient of cAMP. These results establish that integrin-dependent activation of PKA at the leading edge of cells is independent of PI3-kinase activity and plays a role in maintenance of cell polarity and directional cell migration.

The specificity of PKA signaling is achieved by its compartment-specific tethering mediated by AKAPs. The advent of the FRET-based PKA activity biosensor (Zhang et al., 2001), coupled with compartment specific targeting (Allen and Zhang, 2006), afforded us the opportunity to observe PKA signaling in specific compartments in living cells. The specific targeting of pmAKAR3 to the plasma membrane both enhanced the resolution of AKAR FRET by eliminating cytoplasmic background, and allowed for analysis of PKA activity in the vicinity of membrane-associated substrates. Previous work showed preferentially phosphorylation of PKA substrates at the front of migrating cells; our studies now show that this is due to increased PKA activity and is an integrin-dependent process. For example, PKA can phosphorylate proteins that are at or near the leading edge including VASP (Butt et al., 1994; Howe et al., 2002; Howe et al., 2005), LASP-1 (Chew et al., 2000), Rho GTPase (Dong et al., 1998), and c-Src (Abrahamsen et al., 2003). Our finding that PKA is activated at the leading edge suggests a role for this localized activation in the preferential phosphorylation of these substrates here. By utilizing FRET and real time imaging in moving cells, we see that PKA activity is sustained during the forward migration of the cell. Furthermore, changes in global PKA activity can modulate signaling enzymes such as Rac (O'Connor and Mercurio, 2001) or Cdc42 (Feoktistov et al., 2000). Cdc42-mediated filopodia and Rac-mediated lamellipodia are the usual morphological consequences of forward protrusion. PKA contributes to Rac activation in growing pseudopodia (Howe et al., 2005) and we had previously found that restricted α4 integrin phosphorylation contributes to the selective activation of Rac at the front of migrating cells (Nishiya et al., 2005). The present results show that PKA activity is localized to the leading edge, accounting for restricted α4 phosphorylation and Rac activation. Furthermore, these results are consistent with the idea that PKA substrates, which can contribute to forward protrusion by regulating Rac and CDC42, are selectively phosphorylated at the leading edge (Howe et al., 2005; Lim et al., 2007).
We find that integrin ligation is necessary for the localized activation of PKA at the leading edge; however, this effect is not specific for a particular alpha subunit. PKA activation was evident at the leading edge in cells migrating via either α4β1 or α5β1 integrins. Activation of PKA upon α5β1 mediated adhesion is necessary for appropriate regulation of calcium channels in smooth muscle cells (Lopez et al., 1987). Furthermore, adhesion mediated by β1 integrins leads to an increase in intracellular cAMP (Plopper et al., 2000; Whittard and Akiyama, 2001a), transient PKA-mediated phosphorylation of VASP (Howe et al., 2002) and an associated increase in polymerized F-actin (Whittard and Akiyama, 2001b). Interestingly, cell detachment (Howe and Juliano, 2000) and mechanical strain on β1 integrins (Meyer et al., 2000) can activate PKA. Cells are continuously forming new integrin-mediated attachments at the cell anterior and exerting force on those attachments (Ridley et al., 2003). Meyer et al. (Meyer et al., 2000) found that Gs proteins mediated this form of integrin-mediated mechanotransduction. Combined with our data, these studies suggest that forces exerted on integrin attachments at the leading edge activate PKA by stimulating cAMP synthesis through Gs-mediated activation of adenylate cyclase. The abundance of AKAPs at the leading edge, and our finding that localized PKA activation is an important early determinant of cell polarity underscores the need for further analysis of the relative roles of AKAPs and local cAMP synthesis in the localized activation of PKA.

α4 integrins are strongly pro-migratory and have an unusual relationship with PKA signaling. α4 integrins bind directly to Type I PKA (Lim et al., 2007) and are phosphorylated by PKA at the leading edge to promote α4β1-dependent migration. This direct relationship suggests that Type I PKA might be of particular significance for PKA activation when cells migrate via α4 integrins. In support of this idea, we found that sequestration to the mitochondria of Type I, but not Type II PKA, resulted in reduced leading edge PKA activity, complementing and explaining previous studies (Lim et al., 2007) that identified the selective importance of Type I PKA in α4 phosphorylation and α4β1-dependent migration (Lim et al., 2007). The fact that Type II PKA has been implicated in directional migration in fibroblasts and smooth muscle cells (Howe et al., 2005), and in growth cone motility (Han et al., 2007), indicates other AKAPs are able to couple PKA activation to migration. Indeed, in contrast to α4β1-dependent migration, we find that α5β1-dependent migration and PKA activation at the leading edge is inhibited by sequestration of Type I PKA, and to a lesser extent by Type II PKA (data not shown). Similarly,
Howe and co-workers (Howe et al., 2005) found that HT31, a peptide that is a preferential inhibitor of Type II PKA-AKAP interactions, blocked preferential phosphorylation of PKA substrates in pseudopodia. Taken together, these data suggest that localized PKA activation is important in migration; however, different integrins use differing mechanisms of tethering PKA to effect this localized activation.

We found that cell migration was associated with a gradient of increased cAMP towards the leading edge. The work of Ingber and colleagues (Meyer et al., 2000) elegantly demonstrated that G-protein dependent cAMP synthesis requires both ligation of integrins and mechanical strain transmitted through the cytoskeletal network. We observed little spatial enrichment of FRET in non-migrating, non-polarized cells indicating that integrin ligation alone is not sufficient for activating PKA. Elevated PKA FRET was consistently seen in sites of active protrusions and required polymerized actin, suggesting that force generated by motile cells exerted upon integrin adhesions constitutes one mechanism to sustain cAMP synthesis. Thus, we propose that cAMP gradients resulting from integrin ligation in association with protrusive forces contribute to the localized PKA activation at the leading edge.

Localized PKA activity is involved in leading edge localization of PtdIns(3,4,5)P$_3$.

The PI3-kinase product, PtdIns(3,4,5)P$_3$, accumulates preferentially at the leading edge of some polarized cells (Wang et al., 2002) and is required for polarization and migration of certain cells in response to chemotactic gradients (Wang et al., 2002; Funamoto et al., 2002; Huang et al., 2003; Schneider and Haugh, 2005). PtdIns(3,4,5)P$_3$ can positively regulate fundamental components of the migration machinery, such as Rac activation [for review see (Welch et al., 2003)]. In our experiments, PI3-kinase inhibition altered lamellipodial behavior and partially inhibited migration; yet, it failed to block the polarized activation of PKA or phosphorylation of its substrates. On the contrary, PI3-kinase inhibition increased PKA activity at the lateral sides and back of the cell. These results suggest that the integrin-dependent activation of PKA at the leading edge is independent of PI3-kinase, however PI3-kinase activity is important for maintaining polarized PKA signaling. It is possible that PI3-kinase is necessary for maintaining proper cAMP gradients in cells since, cardiomyocytes lacking PI3K$_\gamma$ have increased cAMP levels (Kerfant et al., 2005) resulting from loss of phosphodiesterase activity (Kerfant et al.,...
Spatial restriction of PKA and PI3-kinase activity may act together to maintain polarity and promote directional cell migration.

Surprisingly, PKA activity was necessary for leading edge accumulation of GFP-PH-Akt at the cell front. PKA inhibition did not inhibit Akt phosphorylation, which is dependent on PI3-kinase-mediated synthesis of PtdIns(3,4,5)P_3. While PKA is not required for global accumulation of PtdIns (3,4,5)P_3, it appears to control the bulk quantity of the PtdIns(3,4,5)P_3 at the leading edge as reported by GFP-PH-Akt. In Dictyostelium and human neutrophils, the localization of PtdIns(3,4,5)P_3 during migration is specified by the localization and activation of enzymes that synthesize the lipid, e.g. PI3-kinases, and those that degrade it, e.g. PTEN (Wang et al., 2002; Funamoto et al., 2002; Li et al., 2005). Our finding that PKA is upstream of the accumulation of GFP-PH-Akt in migrating cells, point the way to studies to analyze the role of PKA in the regional regulation of PI3-kinases and PTEN. Our finding that integrin-mediated localized PKA activation can control polarized protrusion suggests that the pathway elucidated here will be widely used in directional cell migration.
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Materials and Methods

Plasmids
The following constructs have been described previously: pmAKAR3 (Allen and Zhang, 2006), ICUE2 (Violin et al., 2008) and GFP-PH-Akt (Watton and Downward, 1999). To generate pmAKAR3(TA), the yfp gene was PCR amplified with primers to introduce a 3' sequence encoding the K-Ras plasma membrane targeting motif KKKKKSKTKCVIM as well as a 5' SacI site and 3' EcoRI site. This YFP with the targeting motif replaces the original YFP in the construct AKAR3(TA). pmAKAR3(TA) was then subcloned into a mammalian expression vector derived from pcDNA3. To generate mitoAKB-xx-mcherry constructs, mcherry was PCR amplified to introduce a 5’ SalI site and a 3’ STOP codon and NotI site. The resulting SalI/NotI fragment was subcloned into pCI-mitoAKB-xx-Flag constructs (Burns-Hamuro et al., 2003), replacing the Flag epitope with mcherry.

Cell Culture and Monolayer Wounding
Chinese hamster ovary cells (CHO) and CHO cells stably expressing the integrin α4 (CHO-α4) subunit (Goldfinger et al., 2003) were maintained in DMEM complete (supplemented with 10% FBS, 1% non-essential amino acids [Sigma], 50 units of penicillin/ml and 50 μg of streptomycin sulfate/ml) at 37°C with 6% CO2. Cells were transfected with 2 μg DNA/60mm dish using Lipofectamine™ Plus (Invitrogen) 24h before imaging according to the manufacturer’s instructions. Wound assays were performed as described previously (Goldfinger et al., 2003) with minor modifications. Imaging dishes (35mm) with glass cover slips bottoms were coated overnight at 4°C with either 5 μg/ml of the CS1 fragment of fibronectin fused to GST which lacks the RGD sequence and specifically engages the α4 integrin as previously described (Jongewaard et al., 1996) or 5 μg/ml human fibronectin at 4°C and blocked with 1% heat-denatured BSA. Cells were plated and allowed to form a monolayer in imaging media (Phenol red free-DMEM/10% FBS/20mM HEPES pH7.4) for 2h before imaging. A single scratch was made through the monolayer using a 200 μl pipet tip. Cells were washed 2X in imaging media to remove cell debris and allowed to migrate for 15 mins prior to live imaging or for 30 mins prior to fixation and immunofluorescence labeling. Function-blocking antibodies directed against integrins α4, HP2/1, or integrin α5, PB1, were applied at 3.0 μg/ml at the time of scratch
wounding. The following pharmacological activators or inhibitors were obtained from Biomol (Plymouth Meeting, PA) and used to treat cells at the following concentrations: forskolin at 25 μM, H89 at 30 μM, wortmannin at 10 μM, Ly294002 at 30 μM, and Latrunculin-A at 1 μM.

5 FRET Imaging
Cells were imaged on a Nikon Eclipse TE-2000 microscope with a 60x/1.4NA oil-immersion objective and a cooled charge-coupled device (CCD) camera (Coolsnap HQ, Photometrics, Tucson, AZ) controlled by Invivo (Media Cybernetics, Bethesda, MD). Simultaneous imaging of both the CFP and YFP emission utilized a Dual View (Optical Insights) optical splitter equipped with a 505DCXR (Chroma Technology) beam splitter. The following excitation filters, altered by a Ludl (Ludl Electronic Products, Hawthorne, NY) wheel changer, were from Semrock Corporation (Rochester, NY): 427/10 for CFP, 504/12 for YFP and 589/15 for mcherry. For imaging of cells expressing pmAKAR3 or ICUE2, the filter set up consisted of the emission filters 464/23 for CFP and 547/31 for YFP paired with FF440/520 multi-edge dichroic beam splitters. For imaging of cells co-expressing pmAKAR3 and mcherry, the set up consists of the emission filters, 464/23 for CFP, 542/27 for YFP and 639/42 for mcherry paired with FF444/521/608 multi-edge dichroic beam splitters. The entire imaging assembly (stage, objective and sample) were encased in a Plexiglas environmental chamber maintained at 37°C. For prolonged imaging experiments, cells were sub-housed in a humidified chamber supplied with 6% CO₂. Exposure time was 300 ms and images were acquired at 2x2 binned resolution. Only cells exhibiting comparable expression of pmAKAR3 were selected for imaging and further analysis. Post acquisition analyses were carried out on ImageJ (NIH) with the Ratio Plus plugin as follows: CFP and YFP emission images were background corrected by subtracting an averaged value calculated from a cell-free region. The FRET ratio values was calculated by dividing YFP by CFP fluorescence (after background subtraction) and the pseudocolored FRET images generated by viewing the ratiometric data using the Ratio LUT (Look Up Table) plugin running in ImageJ. To maintain the same pseudocolored visual scheme, ratio images for ICUE2 were generated by dividing CFP by YFP fluorescence, since binding of cAMP to this reporter results in loss of FRET.

Immunofluorescence Imaging
Cells were rinsed briefly in TBS (50 mM Tris, 150 mM NaCl pH7.4) and fixed for 10 mins in 3.7% formaldehyde/TBS* (TBS* is 50mM Tris, 100mM NaCl, 20mM Na₄P₂O₇, 2mM NaF, pH7.4). Cells were permeabilized with 0.1% Triton X-100/TBS* for 5 mins, blocked with 3%BSA/2% normal goat serum/TBS* for 1h, then incubated with either 10 \( \mu \text{g/ml} \) \( \alpha \)-PS-\( \alpha \)₄ (mouse monoclonal antibody clone 633.1, (Goldfinger et al., 2003), or 1:150 diluted \( \alpha \)-phospho-(S/T) PKA substrate rabbit polyclonal antibody (Cell Signaling, Danvers, MA) in block overnight at 4°C. Bound antibodies were detected by the corresponding FITC-conjugated goat secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and mounted in Prolong Gold (Invitrogen). Widefield immunofluorescence images were acquired on the Nikon TE-2000 microscope with a 60x/1.4NA oil-immersion objective. Acquired images were processed using ImageJ (NIH) and Photoshop (Adobe, version 6.0).

**Integrin substrate micro-patterning**

The microfluidic chip used to produce the 5 \( \mu \text{m} \) wide CS1 strips on a glass substrate was cast out of PDMA (Sylgard 184 by Dow Corning) with a lithographically fabricated master mold. The mold was produced by spin-coating a silicon wafer with a 5 \( \mu \text{m} \) layer of a UV-curable epoxy (SU8-2005 by Microchem, MA), exposing it to UV-light through a photomask (laser-plotted at a resolution of 50,800 dpi), and removing uncured epoxy. The mold was treated with TMCS to make its surface non-sticky, then a ~5 mm layer of PDMS pre-polymer was poured onto the wafer, and cured for 1.5 h at 80°C. The PDMS cast was separated from the mold, cut into individual chips and inlet and outlet holes were punched using a gauge 20 luer stub. A complete PDMS chip (not shown) had an array of 300 parallel channels, each 5 \( \mu \text{m} \) wide, 5 \( \mu \text{m} \) deep, and 10 mm long, connected to a common inlet and a common outlet.

To create the pattern of CS1 strips, a PDMS chip was reversibly bonded to a microscope cover glass by overnight baking at 80°C, and a 10 \( \mu \text{g/ml} \) solution of CS1-Alexa-Fluor647 (GST-CS1 directly conjugated to Alexa-Fluor647 using a protein labeling kit (Invitrogen, Carlsbad, CA)) in PBS was perfused through the microchannel array for ~10 min by applying a differential pressure of 2 psi between the inlet and outlet of the chip (flow rate of ~0.6 \( \mu \text{L/min} \)). The microchannels were further perfused with 1% BSA in PBS for 30 min to purge them from the CS1-Alexa-Fluor647 solution and to block the surface of the glass substrate. The PDMS chip
was then removed and the cover glass further blocked with 1%BSA in PBS for ½ h before seeding of cells. Cells were adhered for 1 h before imaging.

**Immunoblot analysis**

Western blots for Akt phosphorylation were performed using CHO cells grown to 40% confluence and serum starved (0.5% serum) overnight. Cells were removed with trypsin and rinsed in 2% BSA containing media and held in suspension for 30 min before plating in wells coated with 5 μg/ml of FN for the indicated lengths of time in 2% BSA containing media. Blots were first probed with pS473Akt, then stripped and reprobed for total Akt. Both antibodies were purchased from Cell Signaling Technology (9271 and 9272 respectively) (Boston, MA). LY294002 and H89 were used at concentrations of 50 μM and 30 μM respectively and cells were pretreated with these inhibitors for 1h before trypsinization and kept in their continued presence for the duration of the experiment.

Western blots were also performed on CHO cells plated overnight in 10% serum after treatment 50 μM Fsk. Briefly, cells were pretreated with H89 (30 μM) or control media in the presence of 10% serum for 1h before the exposure to Fsk for 15 min. Cells were lysed in either control or H89 containing lysis buffer and immediately frozen at -20°C. Phospho-PKA substrates were identified using antibody 9621 from Cell Signaling Technology (Boston, MA).
Figure Legends

**Figure 1. Spatial distribution of PKA activity in polarized cells visualized by pmAKAR3.**
CHO-α4 cell expressing (A) pmAKAR3 or (B) pmAKAR3(TA) were plated at low densities on the α4β1-specific ligand, CS1, and allowed to adhere for 30 min before image acquisition. Pseudocolored FRET images are shown in the top panels with the corresponding YFP/CFP emission ratio value color coded scale. The corresponding YFP emission images are shown in the bottom panels for reference. Yellow arrowheads indicate regions of protrusive behavior visible by time-lapse imaging. Cells were stimulated with 25 μM forskolin at T=0. (C) FRET ratio values of whole cell area (as represented in A and B) were computed and plotted against the indicated time points. Depicted is the mean and standard deviation of data from 3 replicate measurements (n=3 cells) (D) CHO-α4 cell expressing pmAKAR3 were plated on CS1 at a low density for 15 min prior to imaging. The representative cell as shown was selected based on a lack of a polarized morphology.

**Figure 2. PKA activity gradient in cells migrating into a scratch-induced wound.** (A) CS1-adhered monolayers of CHO-α4 cells expressing pmAKAR3 were wounded by scratching, and imaged 15 min later by time-lapse microscopy. Images were acquired before and after the addition of 30 μM H89 at T=0. (B) Plots of the line profile scans for the cell shown in (A) with the front-to-back cell orientation depicted on the x-axis. As shown are the averaged FRET ratio values for 5 successive image frames (5 s intervals) centered at -30s (i) and 120s (ii) as shown in (A). Error bars are the standard deviations. (C) Representative FRET image of a cell embedded within the monolayer located 2-3 rows behind the wound edge.

**Figure 3. Activation of PKA at the leading edge is integrin dependent.** (A) CHO-α4 cell monolayers adhered on CS1 and expressing pmAKAR3 were wounded, and migration allowed to proceed in the presence of 2 μg/ml HP2/1 (anti-α4) or PB1 (anti-α5) monoclonal antibodies as indicated. (B) CHO cell monolayers adhered on fibronectin and expressing pmAKAR3 were wounded, and migration allowed to proceed in the presence of 2 μg/ml HP2/1 or PB1 monoclonal antibodies as indicated.
Figure 4. Integrin α4β1-specific activation of PKA at the leading edge is Type I PKA dependent. (A) Wildtype or prkar1a-/- mouse embryonic fibroblasts stably expressing α4β1 integrins were transfected with pmAKAR3, adhered on CS1 and imaged as isolated cells. Yellow arrowheads indicate regions of cell protrusion. (B) Profile plots shown are the averaged ratiometric values for six parallel line scans centered around the white line tracing as seen in (A) with the front-to-back cell orientation indicated on the x-axis. (C) CHO-α4 cells doubly transfected with mcherry-mitoAKB-RI (C) or mcherry-mitoAKB-RII (D) and pmAKAR3 were adhered as monolayers on CS1 and migration was induced by wounding. The mitoAKB constructs express Type I or Type II specific PKA binding peptides that sequester the relevant PKA isoform to the mitochondria.

Figure 5. Enrichment of PKA activity occurs at cellular protrusions and requires actin polymerization. (A) A microfluidic chamber was used to coat a glass cover slip with Alexa647-labelled CS1 substrate in 5 μm wide strips (blue) interspersed with uncoated glass surface (black in panel 1). Shown is a representative CHO-α4 cell expressing pmAKAR3 adhered on the micropatterned substrate and extending protrusions along the CS1 coated surface. The pseudocolored FRET images as shown were overlayed upon the image of the coating to facilitate its alignment relative to the cell. (B) An isolated adhered pmAKAR3-transfected CHO-α4 cell was treated with 1 μM Latrunculin-A at T=0 and imaged for FRET. Yellow arrowheads indicate regions of cell protrusion visible in time lapse image sequence. Profile plots shown are the averaged ratiometric values for six parallel line scans centered around the white line tracing.

Figure 6. PKA activation at the leading edge is independent of PI3-kinase activity and involved in GFP-PH-Akt accumulation during cell migration.

A) CHO-α4 cell monolayers on CS1 expressing pmAKAR3 were wounded, then treated with 30 μM Ly294002 for 15 mins and imaged in the continued presence of the inhibitor. (B) Wounded monolayers of GFP-PH-Akt-expressing CHO-α4 cells on CS1 were fixed and imaged to visualize accumulation of PtdIns(3,4,5)P3. (C-D) CHO-α4 cell monolayers adhered on CS1 were wounded, allowed to migrate for 15 mins, and then treated with 30 μM Ly294002, 30 μM H89, or left untreated for another 15 mins before fixation and staining for (C) phosphorylated PKA substrates (p(S/T)substrate PKA) and (D) phosphorylated α4 (phospho-α4) using the appropriate
antibodies. Bottom panels show the magnified and merged images of the regions bounded by yellow rectangles. (E) CHO-α4 cells were adhered to fibronectin coated wells for the indicated times, lysed, fractionated by SDS-PAGE and immunoblotted to detect Akt phosphorylation at serine 473 (pS473-Akt). The blot was reprobed with antibody recognizing total Akt protein. (LY = Ly294002) (F) To ensure the efficacy of H89 inhibition of PKA, cells were treated with 50μM Fsk in the presence or absence of H89 and immunoblotted with phospho-PKA substrate antibody.

**Figure 7. Polarized cells exhibit a front to back cAMP gradient.** (A) CHO-α4 cells expressing ICUE2, a FRET-based cAMP binding biosensor, were grown as monolayers on CS1, were wounded, and imaged as described above. The pseudocolor coding scheme as shown is for CFP divided by YFP emission ratio to reflect the fact that this reporter exhibits loss of FRET upon cAMP binding. (B) Plots of the line profile scans for the cell shown in (A) with the front-to-back cell orientation depicted on the x-axis. Profile plot is the averaged ratiometric values and data distribution for six line scans as shown in the inset. (C) The ratiometric image was subdivided into 4 zones as shown in the inset and ratio values randomly sampled within these zones (n=5 per zone). Error bars are standard deviations and p values, using the Student’s paired t-Test, are shown for the indicated comparisons.
Supplemental Figure S1. Ratiometric images for the cell shown in Figure 2A was subdivided into 3 zones as shown (left panels) and ratio values randomly sampled within these zones (n=5 per zone). The averaged values are plotted for measurements made before and after treatment with H89 (right panel). Error bars are standard deviations and p values, using the Student’s paired t-Test, are shown for the indicated comparisons.

Supplemental Figure S2. CS1-adhered monolayers of CHO-α4 cells expressing pmAKAR3(TA) were wounded by scratching, and imaged 15 min later.

Supplemental Figure S3. Ratiometric image for the cell shown in Figure 2C was subdivided into 3 zones as shown (left panels) and ratio values randomly sampled within these zones (n=5 per zone). Error bars are standard deviations and p values, using the Student’s paired t-Test, are shown for the indicated comparisons.

Supplemental Figure S4. Ratiometric image for the cells shown in Figure 3A were subdivided into 3 zones as shown (left panels) and ratio values randomly sampled within these zones (n=5 per zone). Error bars are standard deviations and p values, using the Student’s paired t-Test, are shown for the indicated comparisons.

Supplemental Figure S5. Ratiometric image for the cells shown in Figure 3B were subdivided into 3 zones as shown (left panels) and ratio values randomly sampled within these zones (n=5 per zone). Error bars are standard deviations and p values, using the Student’s paired t-Test, are shown for the indicated comparisons.

Supplemental Figure S6. Ratiometric image for the cells shown in Figure 5A were subdivided into 5 zones as shown (left panels) and ratio values randomly sampled within these zones (n=5 per zone). Error bars are standard deviations and p values, using the Student’s paired t-Test, are shown for the indicated comparisons.

Supplemental Figure S7. Ratiometric image for the cell shown in Figure 6A was subdivided into 4 zones as shown (left panels) and ratio values randomly sampled within these zones (n=5 per
zone). Error bars are standard deviations and p values, using the Student’s paired t-Test, are shown for the indicated comparisons.

Movie 1. CHO-α4 cell expressing pmAKAR3 were plated at low densities on the α4β1-specific ligand, CS1, and allowed to adhere for 30 min before image acquisition. Pseudocolored FRET images are shown in the left panel with the corresponding YFP/CFP emission ratio value color coded scale. The corresponding YFP emission images are shown in the right panel for reference. Cells were imaged at 10 s intervals between frames and stimulated with 25 μM forskolin as indicated between 90s - 100 s.

Movie 2. CS1-adhered monolayers of CHO-α4 cells were wounded by scratching, and imaged at 10 min intervals between frames for 5 hours.

Movie 3. CS1-adhered monolayers of CHO-α4 cells expressing pmAKAR3 were wounded by scratching, and imaged 15 min later by time-lapse microscopy. Images were acquired before and after the addition of 30 μM H89 at T=100 s.
Reference List


Lim et. al.


