JCB Article

Spatial restriction of α4 integrin phosphorylation regulates lamellipodial stability and α4β1-dependent cell migration

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Integrins coordinate spatial signaling events essential for cell polarity and directed migration. Such signals from α4 integrins regulate cell migration in development and in leukocyte trafficking. Here, we report that efficient α4-mediated migration requires spatial control of α4 phosphorylation by protein kinase A, and hence localized inhibition of binding of the signaling adaptor, paxillin, to the integrin. In migrating cells, phosphorylated α4 accumulated along the leading edge. Blocking α4 phosphorylation by mutagenesis or by inhibition of protein kinase A drastically reduced α4-dependent migration and lamellipodial stability. α4 phosphorylation blocks paxillin binding in vitro; we now find that paxillin and phospho-α4 were in distinct clusters at the leading edge of migrating cells, whereas unphosphorylated α4 and paxillin colocalized along the lateral edges of those cells. Furthermore, enforced paxillin association with α4 inhibits migration and reduced lamellipodial stability. These results show that topographically specific integrin phosphorylation can control cell migration and polarization by spatial segregation of adaptor protein binding.

Introduction

Cell migration is essential for all stages of development, for wound healing, and immune responses. For a cell to migrate, a precisely coordinated series of biochemical and physical events must be regulated in time and space. A migrating cell polarizes and extends forward processes (lamellipodia and filopodia), which must then attach to the substratum. Movement occurs when the cell–ECM connections at the front of the cell exert tension on the cell body through transmembrane linkages to the cytoskeleton, concurrent with a release of cell–ECM attachments at the rear of the cell (Lauffenburger and Horwitz, 1996). These localized morphological events are coordinated by spatially restricted biochemical signals. For example, proteins involved in regulating actin assembly and lamellipodial protrusions, such as WASP, profilin, the Arp 2/3 complex, the small GTPase Rac and its effector PAK, localize to the leading edge in nascent protrusions in migrating cells (for review see Webb et al., 2002).

Integrins, receptors which mediate cell–ECM attachments, also initiate and coordinate biochemical signaling pathways required for cell migration. Integrin signals maintain the polarity of migrating cells, although the precise biochemical mechanisms that account for this function are unclear (Lauffenburger and Horwitz, 1996). Phosphorylation of integrin cytoplasmic tails can modulate binding of accessory proteins (Tapley et al., 1989; Baker et al., 1997; Cowan et al., 2000; Han et al., 2001) and thus, influence the signaling activities of these receptors (Zhang et al., 2001); however, the topographic distribution of integrin phosphorylation has not been assessed during cell migration.

The α4 subfamily of integrins (α4β1 and α4β7) is of particular interest in regards cell migration. These integrins are expressed on leukocytes, neural crest cells, and developing skeletal muscle, and are essential for embryogenesis, hematopoiesis, and immune responses (Hemler, 1990; Yang et al., 1995; Arroyo et al., 1996). Furthermore, these integrins are promising therapeutic targets in a wide variety of chronic inflammatory diseases (von Andrian and Engelhardt, 2003; Rose et al., 2002). The α4 integrin subunit dramatically enhances cell migration in comparison with other integrin α subunits (Chan et al., 1992; Kassner et al., 1995). Thus, we

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Key words: α4 integrin; paxillin; polarization; lamellipodia; PKA

Abbreviation used in this paper: PKA, protein kinase A.

reasoned that an understanding of how α4 integrins promote cell migration could provide insight into the integrin-dependent signaling events that control migration.

The capacity of α4 integrins to enhance cell migration is a function of the α4 cytoplasmic tail (Chan et al., 1992). The α4 tail binds tightly to paxillin, a cytoplasmic adaptor protein, and paxillin binding is required for the ability of α4 to enhance migration (Liu and Ginsberg, 2000; Liu et al., 1999). Furthermore, phosphorylation of Ser^{988} in the α4 tail inhibits association of paxillin with the α4 tail in vitro and in vivo (Han et al., 2001). Because efficient cell migration requires control of integrin-dependent signaling functions, we hypothesized that phosphorylation of the α4 tail may be a regulator of α4-dependent cell migration. To address this hypothesis, we generated mAbs specific for α4 phosphorylated at Ser^{988}, and localized phospho-α4 and paxillin in migrating cells. In addition, we used a combination of pharmacological and mutational analyses to evaluate the role of phosphorylation-dependent regulation of paxillin–α4 association in cell polarization and migration. Here, we report that efficient α4-mediated cell migration requires precise spatial control of α4 phosphorylation by protein kinase A (PKA), and hence, of paxillin binding to the α4 integrin tail. The spatial regulation of paxillin–α4 interaction contributes to suppression of lamellipodia at the sides and rear, but not at the leading edge of migrating cells, and thus, to more efficient cell migration. Thus, we have defined a topographically specific integrin phosphorylation, identified the relevant kinase, and established the biochemical basis by which the phosphorylation event controls cell migration. Furthermore, we provide direct evidence that paxillin recruitment to α4 integrins can de-stabilize lamellipodia.

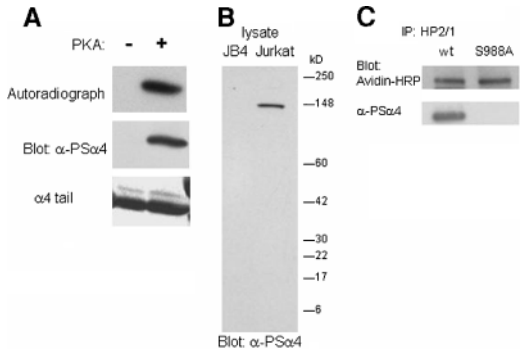

Figure 1. **Characterization of a phospho-specific α-PSα4 mAb.** (A) The α4 integrin–cytoplasmic tail recombinant proteins were incubated with protein kinase A (PKA) in the presence of [γ^{32}P]ATP. The autoradiograph in the top panel shows that the α4 tail is efficiently phosphorylated by PKA. Only PKA-phosphorylated α4 tail protein shows reactivity with the α-PSα4 mAb. Coomassie stained gels are shown to indicate equal loading of α4 tail proteins. (B) Western blot of cell extracts prepared from Jurkat cells and JB4 cells, an α4-deficient Jurkat variant cell line, indicating specific reactivity of the α-PSα4 antibody with endogenous α4 integrin. (C) α4 integrins were immunoprecipitated from stably transfected CHO cells that had been surface labeled with biotin before lysis for detection of α4 protein using avidin-HRP. α-PSα4 antibody reacts with immunoprecipitated wt α4, but does not recognize α4 containing a Ser^{988} to Ala mutation.

Results

Phosphorylated α4 integrin is preferentially localized to the leading edge of migrating cells

To evaluate the role of α4 phosphorylation in cell migration, we first sought to localize phosphorylated α4 integrin in migrating cells. To do this we generated mAbs specific for an α4 cytoplasmic domain phosphorylated at Ser^{988}. This phospho-specific antibody, designated α-PSα4, reacted with the phosphorylated but not unphosphorylated α4 integrin tail, demonstrating that α-PSα4 reacts specifically with α4 when it is phosphorylated (Fig. 1 A). The antibody was also α4-specific, because it reacted with the 150-kD α4 integrin polypeptide in Jurkat cells, and failed to react with lysates of JB4 cells, an α4-deficient Jurkat variant cell line (Fig. 1 B). Specificity was also confirmed by Western blotting α4 immunoprecipitates

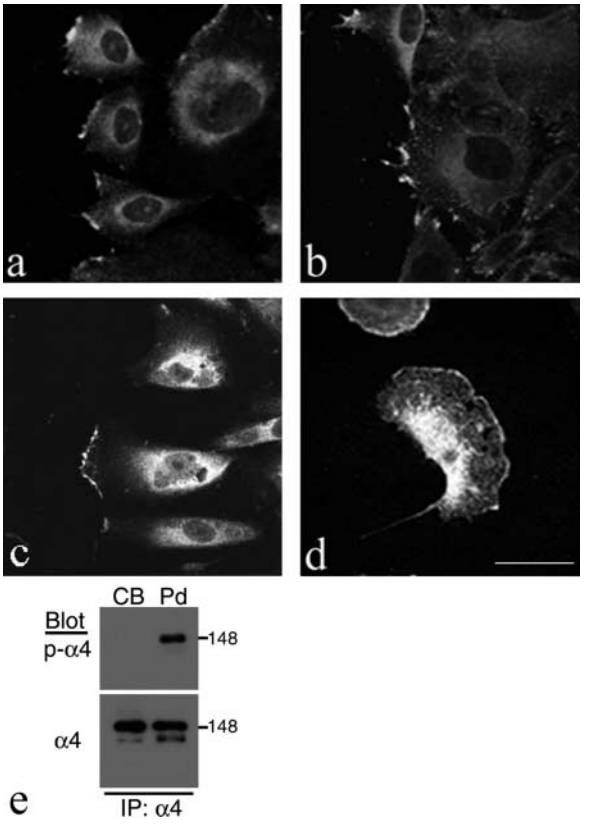

Figure 2. **α4 integrin is preferentially phosphorylated at the leading edge of migrating cells.** (a–c) CHO cells expressing human α4 integrin were plated onto dishes coated with 2 μg/ml CS-1 (α4β1-binding) fragment of fibronectin and scratch wounded. The localization of the phosphorylated α4 was assessed by staining with α-PSα4. Serine phosphorylated α4 is localized to clusters at the leading edges of polarized cells, and in the perinuclear region. (d) Primary human peripheral blood monocytes migrating on CS-1 show leading edge localization of phosphorylated endogenous α4. Bar, 25 μm. (e) A7r5 rat smooth muscle cells were plated on fibronectin on 3-μm porous filters by the method of Cho and Klemke (2002). Pseudopodia (Pd) and cell bodies (CB) were isolated, lysed and α4 was immunoprecipitated with HP2/1 mAb for α4. Immunoprecipitates were blotted with α-PSα4, then stripped and reprobed with a rabbit polyclonal antibody (Rb038) raised against α4. Phosphorylated α4 integrin is highly enriched in leading pseudopodia in polarized cells.

with α-PSα4. α-PSα4 reacted with a 150-kD polypeptide in α4 immunoprecipitates from cells expressing wild-type α4, but not α4 in which Ser[988] has been mutated to a nonphosphorylatable Ala (Fig. 1 C). Thus, this mAb is both α4 sequence specific and phosphorylation specific.

We used α-PSα4 to assess the distribution of phosphorylated α4 in migrating cells. We examined CHO cells expressing recombinant α4, and used scratch wound assays to induce polarized migration. Scratch wounds were made in confluent cultures plated on coverslips coated with the CS-1 fragment of fibronectin (CS-1), an α4-specific ligand. The phosphorylated α4 was present predominantly along the leading edge of polarized cells migrating into the wound space (Fig. 2, a–c), but was consistently absent at the lateral and trailing edges (Fig. 2, a–c). Phosphorylated α4 was also localized to the leading edge of polarized primary human monocytes migrating on CS-1 toward a chemoattractant gradient of stromal-derived factor-1 (Fig. 2 d), indicating that the polarization of α4 phosphorylation occurs with the native protein at natural abundance. A perinuclear pool of phospho-α4 was also noted in both cell types. In confluent cell cultures, phospho-α4 staining was limited to the perinuclear regions, with no detectable antibody reactivity at cell borders (unpublished data). To provide further confirmation that α4 phosphorylation is up-regulated in the leading edge of polarized cells, we isolated pseudopodia and cell bodies from α4-expressing smooth muscle cells. Although α4 integrin was present in cell bodies and pseudopodia, phosphorylated α4 was specifically enriched in the pseudopodia (Fig. 2 e). Thus, membrane-associated phosphorylated α4 is enriched at the leading edge of migrating cells.

Blockade of α4 phosphorylation inhibits lamellipodial extension during cell migration

The distinct localization of phospho-α4 at the leading edge of migrating cells suggested that α4 phosphorylation might be involved in the ability of cells to migrate on α4 integrin ligands. To test this idea, we examined the effect of mutating the serine phosphorylation site to alanine (α4(S988A)), a mutation that eliminates phosphorylation of the α4 tail (Han et al., 2001). Confluent CHO cells expressing α4(S988A) or wild-type α4 were plated on CS-1 and scratch wounded. The closure of the wound was quantified by phase microscopy as a measure of directed cell migration. The cells expressing α4(S988A) failed to extend lamellipodia into the wound and showed markedly reduced migration and wound closure relative to cells expressing wild-type α4 (Fig. 3). Both cell types express similar levels of α4 integrins at the cell surface, adhered to a similar extent to CS-1, and bound similar amounts of soluble VCAM-1, an activation-specific ligand (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200304031/DC1; and not depicted). Thus, the migratory defect in the S988A mutant was not due to a loss of adhesion or a reduction in α4 affinity.

To further confirm the specificity of staining with the α-PSα4 antibody, both cell types were fixed and stained with antibodies to α4 (HP2/1) and phospho-α4. Whereas both cell types showed strong staining for α4, only cells expressing wild-type α4, but not α4(S988A), showed reactivity with the α-PSα4 antibody (Fig. 3 B). Notably, the perinuclear staining was also absent in the cells expressing α4(S988A). Thus, a mutation that precludes phosphorylation of α4 blocks lamellipodial extension and cell migration.

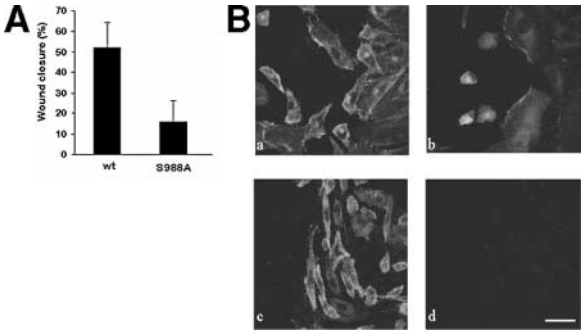

Figure 3. **The phosphorylation-resistant α4(S988A) mutation inhibits α4-dependent cell migration.** (A) CHO cells stably expressing wt α4 or α4(S988A) were plated onto dishes coated with 2 μg/ml CS-1, scratch wounded at confluence, and allowed to migrate into the wound space for 16 h. The ratio of the final wound area to the area immediately after scratching is indicated as percent closure. The cells bearing α4(S988A) manifest drastically reduced α4-dependent migration. (B) CHO cells expressing α4wt (a and b) or α4(S988A) cells (c and d) were fixed 16 h after scratch wounding and stained with antibodies to α4 (a and c) or with α-PSα4 (b and d). α4 is localized around the cell periphery, but is phosphorylated only at the leading edge. α-PSα4 does not react with CHO cells bearing α4(S988A). Bar, 25 μm.

The α4(S988A) mutation blocked lamellipodial extension and migration in a scratch wound assay. To obtain additional insight into the mechanism of this effect, we examined the effect of this mutation on the random migration and edge dynamics of cells using real time video microscopy. Cells expressing this mutant ruffled and extended protrusions in various directions from the cell body; however, the protrusions rapidly collapsed and the cells failed to stably polarize and migrate (migration rate = 4.3 μm/h ± 0.58; Fig. 4; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200304031/DC1). In sharp contrast, cells expressing wild-type α4 developed stable leading lamellipodia and exhibited clear directional migration (migration rate = 14.7 μm/h ± 1.06; Fig. 4; and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200304031/DC1). Lamellipodial extensions in wild-type α4 cells persisted for an average of 3.4 min ± 0.28, with 65 ± 3.3% of lamellipodia lasting >3 min. In contrast, cells expressing α4(S988A) extended protrusions that persisted for an average of 1.1 min ± 0.36, with 91 ± 0.8% of protrusions collapsing after 1 min (Fig. 4). Thus, the α4(S988A) mutation interfered with the ability of the cells to develop stable polarized lamellipodia.

PKA is a kinase that phosphorylates α4 Ser[988] (Han et al., 2001). Therefore, we used pharmacological inhibition of PKA activity as an alternative approach to evaluate the role of α4 phosphorylation in α4-dependent cell migration. We examined the effect of Rp-cAMP, an inhibitor of PKA activity, on α4 phosphorylation (Gjertsen et al., 1995). Cells were fixed and stained with α-PSα4 to detect the distribution of phosphorylated α4. Inhibition of PKA activity abrogated phospho-α4 staining at the leading edge membrane

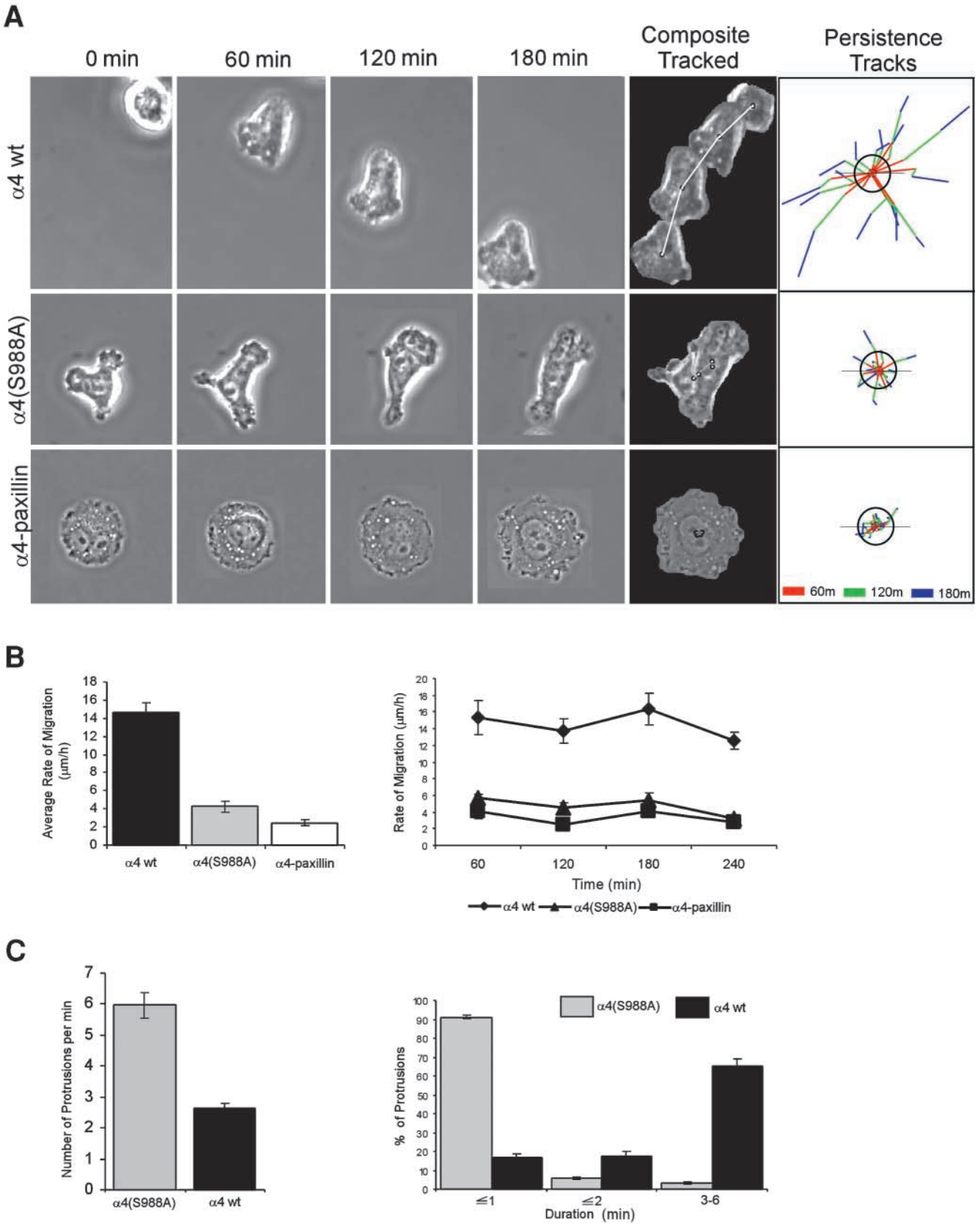

Figure 4. **α4 phosphorylation is required for cell polarization, lamellipodial stabilization, and directed migration.** (A) CHO cells bearing α4 wt, α4(S988A), or α4 fused to paxillin, plated on dishes coated with 2 μg/ml CS-1, were observed in random migration assays by phase-contrast microscopy and photographed every 10 min for 4 h. Representative cells are shown at times 0, 60, 120, and 180 min. Persistence tracks indicate displacements of cell centroids over 240 min. CHO cells bearing α4 polarize, extend lamellipodia and migrate in the direction of the lamellipodium. Those bearing α4(S988A) or an α4-paxillin chimera do not polarize and do not migrate. (B) Migration rates are shown

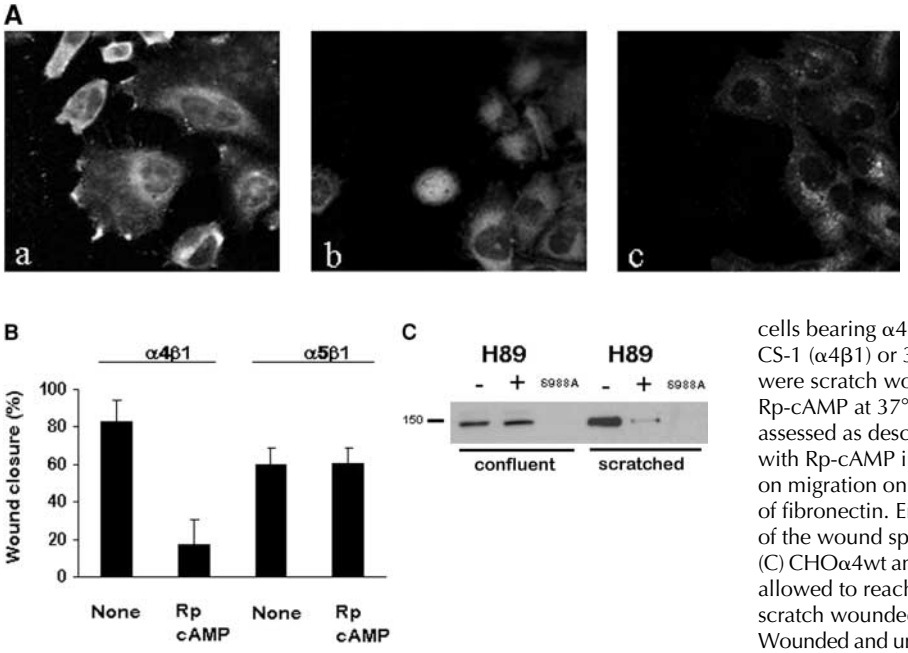

Figure 5. **Inhibition of PKA activity blocks α4 phosphorylation at the leading edge and α4-dependent migration.** (A) CHO cells bearing α4 were plated on dishes coated with 2 μg/ml CS-1 and confluent monolayers were scratch wounded, and incubated with 100 μM Rp-cAMP (b), 30 μM H-89 (c), or without inhibitors (a) for 30 min, and then fixed and stained with α-PSα4. Rp-cAMP or H-89 treatment eliminates phosphorylation of α4 at the leading edge. (B) CHO cells bearing α4 were plated on dishes coated with 2 μg/ml CS-1 (α4β1) or 3Fn(9-11) (α5β1) and confluent monolayers were scratch wounded, and incubated with or without Rp-cAMP at 37°C for 16 h. Migration into the wound was assessed as described in Materials and methods. Treatment with Rp-cAMP inhibits migration on CS-1, but has no effect on migration on the α5 integrin–binding 3Fn(9–11) fragment of fibronectin. Error bars are the SD from the average width of the wound space measured in three independent trials. (C) CHOα4wt and CHOα4(S988A) cells were plated on CS-1, allowed to reach confluence, and either left unscratched, or scratch wounded with multiple scratches in a grid pattern. Wounded and unwounded cultures were incubated for 30 min in the presence or absence of 30 μM H89, and then extracted in lysis buffer. Lysates were adjusted to identical protein concentrations and analyzed by SDS-PAGE followed by Western blotting with α-PSα4.

(Fig. 5 A). Interestingly, inhibition of PKA did not abolish a perinuclear pool of phospho-α4 staining. However, this staining was phospho-α4 specific, as it was not seen in cells transfected with α4(S988A) (Fig. 3 B). We observed the same loss of leading edge phospho-α4 staining in cells incubated with another specific inhibitor of PKA, H-89 (Fig. 5 A). To confirm that PKA activity is required for promoting increased levels of phosphorylated α4 in cells, lysates from scratch-wounded and confluent cultures were subjected to Western blotting with the α-PSα4 antibody. Phosphorylated α4 levels in scratch-wounded cultures increased relative to confluent cultures (Fig. 5 C). Furthermore, incubation of scratch-wounded cells with H-89 markedly reduced the total level of phosphorylated α4 in scratch wounds. However, inhibition of PKA had no effect on the levels of phosphorylated α4 in unwounded, confluent cultures (Fig. 5 C), indicating that PKA activity is required for the increase of α4 phosphorylation that follows wounding.

The ablation of phospho-α4 staining at the leading edge of migrating cells by inhibition of PKA activity blocked the α4-dependent migration of CHOα4 cells (Fig. 5 B). In contrast, such treatment had no effect on the already reduced migration of cells expressing α4(S988A) (unpublished data). Inhibition of cell migration by Rp-cAMP is α4 specific, as treatment of CHOα4 cells did not reduce the migration of these cells on an α5β1 ligand, the central cell binding domain of Fn (Fig. 5 B). Thus, inhibition of α4 phosphorylation blocks α4β1 integrin–dependent cell migration.

Phospho-α4 and paxillin are localized in distinct regions in migrating cells

The foregoing experiments showed that phosphorylated α4 is enriched at the leading edge of migrating cells, and this α4 phosphorylation is required for optimal polarization, lamellipodial stabilization, and migration of cells on an α4 ligand. Because α4 phosphorylation inhibits the binding of paxillin to the α4 cytoplasmic domain, we hypothesized that the role of α4 phosphorylation in cell migration is to reverse the paxillin–α4 association. Therefore, we stained migrating cells for paxillin and either total α4 or phospho-α4, and monitored basal localization in 0.1-μm thick basal confocal sections. Paxillin was observed in focal complexes in migrating and nonmigrating cells (Fig. 6; and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200304031/DC1). In migrating cells, paxillin consistently localized in clusters throughout the basal cell surface, including at the leading edge, and also in streaks along the lateral edges. Total α4 colocalized with paxillin staining along lateral edges (Fig. 6 and Fig. S2). However, no phospho-α4 staining could be detected along lateral edges of migrating cells, sites where paxillin was present (Fig. 6 and Fig. S2). At the leading edge, paxillin localized to focal complexes adjacent to, but not co-

based on cell centroid assignments for at least 40 cells/trial, $n = 3$. Migration rates in the right panel indicate average cell displacement between successive time points. CHO cells bearing α4wt exhibit persistent, high migration rates over 4 h, whereas cells bearing α4(S988A) or an α4-paxillin chimera maintain significantly lower migration rates over the experimental time frame. (C) The number and persistence of protrusions was tracked by phase-contrast microscopy. CHOα4wt and CHOα4(S988A) cells plated on CS-1 were photographed every minute for 10 min, and protrusions were scored visually in successive images ($n = 10$ cells for each cell type). (B and C) Error bars represent SEM for all cells counted.

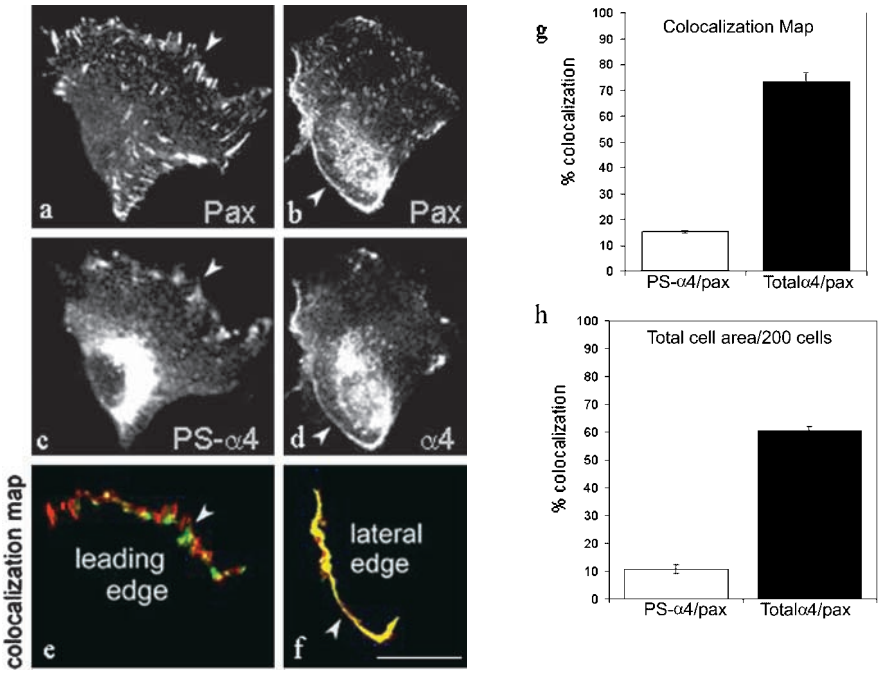

Figure 6. **Paxillin colocalizes with nonphosphorylated α4 at the lateral and trailing edges but not with phospho-α4 at the leading edge.** CHO cells expressing α4wt were plated on CS-1, scratch wounded and stained with antibodies to paxillin (a) and total α4 (b), or paxillin (c) and phospho-α4 (d). Images shown are confocal micrographs of 0.1-μm basal sections. Cells that are separated from the monolayer are shown in a through d to demonstrate colocalization of paxillin (red) with total α4 (green) at the lateral and trailing edges. Colocalization maps are shown in e and f. Yellow pseudocolor indicates overlap of red and green fluorescence. Percent colocalization in the indicated regions is shown in g. (h) Percent colocalization between paxillin and phospho-α4 or total α4 staining across the whole cell area, in 200 cells analyzed per case (*t* test = 1.15×10^{-8} significance). (g and h) Error bars represent SEM of colocalization of red and green per pixel. Bar, 25 μm.

incident with, clusters of phospho-α4. These focal complexes also did not contain β1 integrins, as evidenced by double staining with antibodies to β1 integrin and paxillin (unpublished data). Conversely, no phospho-α4 was detected in paxillin-rich clusters at the leading edge (Fig. 6 and Fig. S2). These staining patterns were observed both in cells that had migrated out from the monolayer (Fig. 6), as well as in polarized cells at the leading edge of the monolayer (Fig. S2). Colocalization of paxillin with phospho-α4 staining or with total α4 staining in migrating cells was also assessed morphometrically, for 200 cells in each case. 61 ± 1.6% of total α4 staining colocalized with paxillin in migrating cells, versus 11 ± 1.4% colocalization for phospho-α4 and paxillin (Fig. 6). Thus, phospho-α4 and paxillin do not colocalize along the leading edge of migrating cells, whereas nonphosphorylated α4 and paxillin do colocalize along the lateral and trailing edges.

To confirm that the observed colocalization of paxillin and α4 is dependent on the absence of α4 phosphorylation, we costained CHO cells expressing α4(S988A) or α4(S988D)—a phospho-mimicking mutant which inhibits paxillin binding to the α4 tail (Han et al., 2001)—with antibodies to α4 and paxillin. CHO cells bearing α4(S988A) remained rounded and did not spread (Figs. 3 and 7), and in these cells paxillin was observed in clusters throughout the basal cell surface, and concentrated around the cell perimeter. α4 staining colocalized with paxillin around the edges of these cells, but was not seen in the paxillin-rich central clusters (Fig. 7). In contrast, CHO cells bearing the pseudo-phosphorylation mutant, α4(S988D), showed pronounced spreading, and in these cells α4 was localized in clusters across the basal cell surface, and did not show appreciable colocalization with the paxillin-rich clusters (Fig. 7). Thus, nonphosphorylated α4 colocalizes with paxillin, whereas phosphorylated or pseudo-phosphorylated α4 fails to do so.

Enforced association of α4 and paxillin inhibits α4-dependent migration

The preceding experiments confirmed that α4 phosphorylation prevents its association with paxillin, supporting the

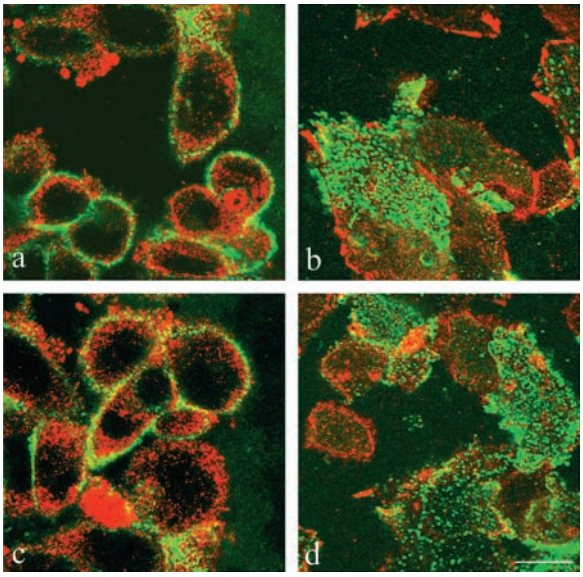

Figure 7. **Colocalization of unphosphorylated α4 with paxillin.** CHO cells stably expressing nonphosphorylatable α4(S988A) (a and c) or pseudo-phosphorylated α4(S988D) (b and d) were plated on CS-1 and scratch wounded. The cells were fixed and stained with antibodies to paxillin (red) and α4 (green). In a and c, the cells remain rounded. Paxillin and α4 are coclustered around the cell perimeters, and paxillin is also found in α4-deficient clusters at the basal surface. In (b and d), CHO cells expressing α4(S988D) are spread, and paxillin is localized in clusters throughout the basal surface and at the perimeter. However, α4(S988D) staining shows widespread basal localization but virtually no colocalization with paxillin. Bar, 25 μm.

hypothesis that α4 phosphorylation is required for cell migration because it inhibits the paxillin–α4 association. To directly test this hypothesis, we enforced paxillin association with the α4 tail by covalently fusing paxillin to the α4 COOH terminus. The α4-paxillin fusion protein becomes phosphorylated at Ser^{988} (Fig. 8 B), indicating that fusing paxillin to the COOH terminus of the α4 tail does not disrupt α4 phosphorylation. Furthermore, paxillin staining colocalized with α4 staining around cell perimeters when these cells were plated on CS-1, confirming that the chimera did enforce the α4-paxillin association. Endogenous paxillin was also present at the basal cell surface in clusters which did not contain α4 (Fig. 8 C). Two independent clones of cells expressing α4 integrin fused at its intracellular COOH terminus to paxillin migrated poorly on CS-1, consistent with the hypothesis that constitutive direct binding of paxillin to α4 integrin inhibits α4-dependent migration (Fig. 8 A). Joining a 25-kD affinity tag to the COOH terminus of α4 had no effect on α4-dependent cell migration (unpublished data), indicating that the effect of the paxillin fusion was specific. In random migration assays, cells expressing the α4-paxillin chimera formed ruffles and extended protrusions (Video S3, available at http://www.jcb.org/cgi/content/full/jcb.200304031/DC1). However, similar to CHOα4(S988A) cells, the protrusions quickly collapsed, the cells failed to stably polarize, and they did not migrate (migration rate = 2.5 μm/h ± 0.30; Fig. 4 and Video 3).

Discussion

α4 integrins strongly promote cell migration through their interaction with paxillin. We now find that efficient α4-mediated cell migration requires precise spatio-temporal regulation of paxillin binding to the α4 tail specified by topographically localized PKA-mediated α4 phosphorylation. First, staining of migrating cells with phospho-specific anti-α4 antibodies showed that phosphorylated α4 integrin accumulates along the leading edge of migrating cells. Second, blockade of α4 phosphorylation by substitution of Ser^{988} with a nonphosphorylatable Ala led to drastically reduced α4-dependent cell migration by inhibiting lamellipodial extension at the leading edge. Third, inhibition of PKA blocked both α4 phosphorylation at the leading edge and α4-dependent cell migration. Fourth, α4 phosphorylation blocks paxillin binding in vitro; we now find that paxillin is excluded from areas of clustered phospho-α4 at the leading edge of migrating cells, whereas paxillin colocalizes with nonphosphorylated α4 along the lateral edges of those cells. Finally, α4 phosphorylation is required for efficient cell migration because it blocks paxillin binding to the α4 tail. Enforced paxillin association with α4 inhibits migration in a

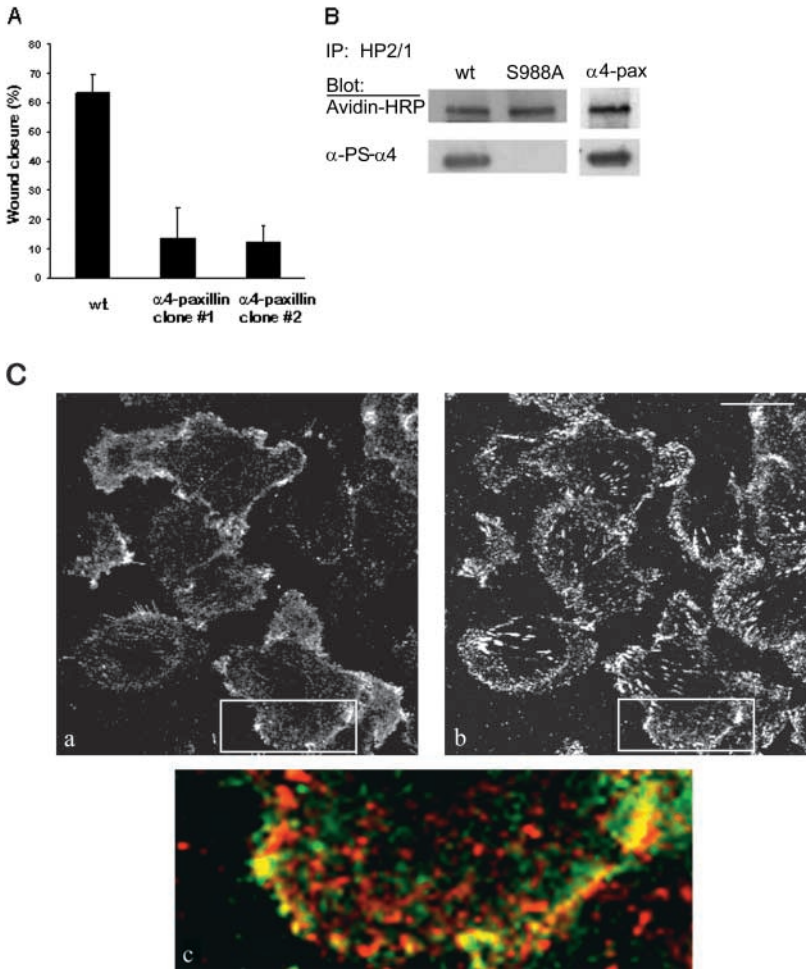

Figure 8. **Enforced association of paxillin with α4 integrin inhibits migration.** (A) CHO cells expressing α4 or an α4-paxillin chimera, were plated onto dishes coated with 2 μg/ml CS-1, scratch wounded at confluence, and allowed to migrate into the wound space for 16 h. The ratio of the final wound area to the area immediately after scratching is indicated as percent closure. Direct paxillin fusion to α4 integrin drastically reduces α4-dependent migration. Error bars are the SD from the average width of the wound space measured in three independent trials. (B) α4 integrins were immunoprecipitated from stably transfected CHO cells that had been surface labeled with membrane-impermeable biotin before lysis. Surface α4 was detected by staining immunoblots with avidin-HRP, and phospho-α4 was detected by staining with α-PSα4. The antibody reacts with immunoprecipitated α4 and the α4-paxillin chimera but not α4(S988A). (C) α4-paxillin chimera–transfected CHO cells were plated on CS-1, allowed to spread, and fixed and stained for α4 (a, green) and paxillin (b, red); the indicated region is shown as overlay in (c). Paxillin colocalizes with α4 at the cell periphery, but is also present in clusters across the basal surface that do not contain α4. Bar, 25 μm.

similar fashion to the α4(S988A) nonphosphorylatable mutation; it markedly reduces the stability of lamellipodia. These results show that efficient α4-mediated cell migration requires precise spatial control of α4 phosphorylation, and hence, of paxillin binding to α4 integrin. The spatial regulation of paxillin–α4 interaction contributes to suppression of lamellipodia at the sides and rear, but not at the leading edge of migrating cells, and thus, to more efficient cell migration.

The phosphorylated form of α4 integrin is preferentially localized along the leading edge of migrating cells and this localization is required for optimal cell migration. This conclusion is based on the strong staining of phosphorylated α4 at the leading edge of cells. Importantly, total α4 staining showed no such preferential localization and phosphorylated α4 was enriched in isolated pseudopodia from migrating smooth muscle cells. Thus, a path length artifact does not account for the increased phospho-α4 staining at the leading edge. Furthermore, pharmacologic or mutagenic blockade of α4 phosphorylation inhibited cell migration. Because cells expressing α4 containing a nonphosphorylatable α4(S988A) mutation were unable to extend stable lamellipodia, it is likely that α4 phosphorylation is important for stable lamellipodial protrusion. Conversely, the absence of α4 phosphorylation along the lateral edges of polarized, migrating cells was also required for optimal cell migration because pharmacologically enforced phosphorylation or a phosphorylation-mimicking α4 mutant also blocks migration in Jurkat T cells (unpublished data) and in CHO cells (unpublished data).

How is α4 phosphorylation localized to the leading edge? PKA phosphorylates α4 in vitro at a consensus PKA phosphorylation site (Han et al., 2001) and inhibition of PKA blocked α4 phosphorylation at the leading edge. Thus, selective localization of PKA could lead to preferential localization of phospho-α4 to the leading edge. Indeed, Howe has reported biochemical evidence for the enrichment of active PKA in the leading pseudopodia of migrating cells (unpublished data). This is a site at which integrins are engaging ligands, and engagement of β1 integrins can activate PKA (O'Connor and Mercurio, 2001). Furthermore, the greatest protrusive forces are exerted at the leading edge, and such forces can lead to PKA activation (He and Grinnell, 1994; Ihlemann et al., 1999). Alternatively, we noted an intracellular perinuclear pool of phospho-α4, presumably localized in vesicles. Insertion of membrane vesicles occurs at the front of migrating cells (Nabi, 1999); such vesicles could deliver phospho-α4 to this site. However, inhibition of PKA did not lead to de-phosphorylation of the internal pool of α4, but blocked the appearance of phospho-α4 at the leading edge. Furthermore, PKA inhibition specifically reduced the levels of phosphorylated α4 in scratch-wounded cells, but not in unwounded, confluent cultures. These results suggest that in response to scratch wounding, PKA phosphorylates α4 at the front of migrating cells. The maintenance of a phosphorylated internal pool of α4 in the face of inhibition of PKA may be because the internal α4 is phosphorylated by kinases other than PKA or is inaccessible to de-phosphorylation by phosphatases. In any case, the data presented here reveal that topographically localized PKA-mediated α4 phosphorylation is required for efficient α4-mediated cell migration.

The spatial patterning of α4 phosphorylation contributes to cell migration by regulating paxillin binding. We previously showed that PKA-mediated phosphorylation of α4 at Ser^{988} inhibits binding of paxillin to α4 integrins in vitro (Han et al., 2001). Here, we report that, at the leading edge, phosphorylated α4 is not colocalized with paxillin, indicating that α4 phosphorylation disrupts paxillin binding to α4 in vivo. Pinco et al. (2002) have also noted the lack of colocalization of α4 and paxillin in the leading edge of migrating cells. However, at the lateral edges, where the α4 is not phosphorylated, there was strong colocalization of α4 and paxillin. Consequently, α4 phosphorylation is likely to be required to prevent the α4-paxillin association at the anterior of the cell. Indeed, enforced association of paxillin with the α4 tail leads to the similar inhibition of migration observed in the α4(S988A) mutant. The enforced association of paxillin with α4 did not impair α4 phosphorylation, indicating that irreversible paxillin association inhibits migration even when α4 can become phosphorylated. Conversely, α4 phosphorylation or a phosphorylation-mimetic Asp substitution at Ser^{988} blocks paxillin binding (Han et al., 2001) and prevents colocalization of paxillin with α4 in cells. Paxillin binding to α4 is required for efficient migration and when α4 phosphorylation is not localized, it inhibits migration (unpublished data). Thus, de-phosphorylation of α4 at the lateral edge of cells is required for both paxillin association and resulting optimal migration. Consequently, the spatial regulation of α4 phosphorylation controls the topographic localization of paxillin binding to α4, leading to enhanced cell migration.

Enforced association of paxillin with the α4 tail may block formation of stable lamellipodia by interfering with signaling by the small GTPase, Rac. Rac initiates lamellipodia by promoting Arp2/3-dependent actin polymerization via Scar/WAVE (Eden et al., 2002). Therefore, a reduction in Rac activation could provide a biochemical explanation for the effects of enforced α4-paxillin association on cell spreading and on lamellipodial extension. Rac activation is preferentially localized in the leading edge of migrating cells (Kraynov et al., 2000) and in pseudopodia (Cho and Klemke, 2002), and paxillin binds many potential regulators of Rac activation including the ARF-GAP p95PKL (Turner et al., 1999), PTP-PEST (Sastry et al., 2002), and Csk (Sabe et al., 1994). Indeed, West et al. (2001) showed that displacement of paxillin from adhesion sites by a ΔLD4 mutant leads to persistent lamellipodia and enhanced Rac activation. Thus, α4 phosphorylation at the front prevents paxillin binding and, therefore, could permit Rac activation and lamellipodial extension. Conversely, efficient cell migration requires suppression of lamellipodia at the sides and rear of cells. In these regions, the binding of paxillin to dephosphorylated α4 could inhibit Rac activation, thus, suppressing lamellipodia, thereby promoting migration.

Phosphorylation of several integrin cytoplasmic domains can contribute to cell migration; however, the present studies now define the importance of regional control of integrin phosphorylation and show how the phosphorylation controls a specific protein–protein interaction, thereby spatially defining the signaling capacity of the integrin. Previous studies identified a role for PKC-mediated phosphory-

lation of the α3 tail in cell motility (Zhang et al., 2001), but did not define the biochemical consequences of that phosphorylation. On the other hand, interaction of the intermediate filament cytoskeletal adaptor protein IFAP300/plectin with α6β4 integrins in epithelial cells is negatively regulated by serine phosphorylation of the α6 subunit, possibly mediated by PKCδ (Baker et al., 1997; Alt et al., 2001). Furthermore, β tail tyrosine phosphorylation at conserved NPxY motifs can regulate the binding of talin (Tapley et al., 1989; Pfaff et al., 1998; Calderwood et al., 1999) or Shc (Cowan et al., 2000) and is important in cell migration (Sakai et al., 2001). However, the relationships of effects on Shc and talin binding to migration have not been established.

The present results permit us to propose a scheme to explain the importance of α4 phosphorylation in integrin-mediated directional migration. α4 integrin is expressed around the perimeter of cells, but is selectively phosphorylated by PKA at the leading edge. Phosphorylation negatively regulates paxillin binding, so that paxillin is bound to α4 integrin along the sides of the cell, not at the leading edge. Binding of paxillin to α4 leads to localized inhibition of lamellipodia at the sides and rear of the cell. At the leading edge, α4 phosphorylation displaces paxillin, permitting formation and stabilization of the lamellipodium in the direction of migration. Consequently, efficient α4-mediated cell migration requires spatio-temporal regulation of paxillin–α4 interaction by α4 phosphorylation to maintain position-specific lamellipodial extension at the leading edge.

Materials and methods

Antibodies and reagents

HP2/1 anti-α4 mAb was purchased from Immunotech. Antipaxillin antibody (clone 349) was purchased from Transduction Laboratories. Rb038 rabbit polyclonal antibodies raised against the cytoplasmic tail of α4 have been described previously (Han et al., 2001). Purification of the human CS-1 region of fibronectin fused to GST has been described previously (Jongewaard et al., 1996), using cDNA which was provided by J.W. Smith (Burnham Institute, La Jolla, CA). cDNA encoding the 3Fn(9–11) fibronectin fragment was a gift from J.W. Ramos (Rutgers University, New Brunswick, NJ) and was purified as described previously (Ramos and DeSimone, 1996). Soluble VCAM-1-human IgG1 heavy chain fusion protein (Jakubowski et al., 1995) was generated from cDNA as described previously (Rose et al., 2000) at the National Cell Culture Center.

Construction of α4–COOH-terminal fusions

For construction of a mammalian expression vector (pCDNA3; Invitrogen) encoding an α4-paxillin chimera, pCDNA3.1(−)α4 was modified replacing the stop codon with a KpnI site, GTGGGC encoding Val-Gly). KpnI-XbaI fragment of full-length human paxillin α was subcloned into the modified pCDNA3.1(−)α4 resulting in a Val-Gly spacer between the COOH terminus of α4 and the NH_2 terminus of paxillin. For construction of an α4-TAP fusion, the stop codon in a full-length α4 construct (American Type Culture Collection) in pcDNA3.1(−) was deleted by PCR mutagenesis and replaced with a KpnI-NcoI fragment containing a 3-Gly spacer at the COOH terminus. A 500-bp NcoI-EcoRV fragment containing the complete TAP tag (Rigaut et al., 1999) was removed from a cDNA provided by Bertrand Séraphin (European Molecular Biology Laboratory, Heidelberg, Germany) and inserted into the corresponding sites in the mutant α4 construct.

Generation of phospho-specific anti-α4 mAb

The peptide $RDS^{988}WSYINSK$ was synthesized with or without phosphorylation at Ser^{988}. Both peptides were purified by reversed-phase HPLC and their sequences were confirmed by mass spectrometry. The synthetic peptide ($RDS^{988}WSYINSK$ with phosphorylation at Ser^{988}) was coupled to keyhole limpet hemocyanin with glutaraldehyde as the coupling reagent and injected into mice. Splenocytes were isolated and fused and individual hybridoma clones were screened by immunoblotting. The specificity of the monoclonal anti–phospho-α4 was established by ELISA and then by immunoblotting and immunofluorescence as described in Results. The mAbs were purified by protein G affinity chromatography from tissue culture supernatants.

Cell culture and transfection

CHO cells and A7r5 rat smooth muscle cells (American Type Culture Collection) were maintained in DME supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin sulfate, and 1% nonessential amino acids. Cells were transfected using LipofectAMINE reagent (Invitrogen) following the manufacturer's instructions, and selected in DME containing the appropriate antibiotic at 500 μg/ml. Single cell sorting for CHOα4 transfectants was performed on HP2/1-labeled cells at the Scripps Research Institute Cell Sorting Facility. Expression was confirmed by flow cytometry on FACScan® (Becton Dickinson), and cells were maintained in DME with 250 μg/ml selective antibiotic.

In vitro phosphorylation of integrin α4 and Western blotting

Integrin tail mimic proteins were generated and purified as described previously (Pfaff et al., 1998; Liu et al., 1999). For in vitro phosphorylation assays, 1 μg of recombinant tail model proteins bound to nickel agarose was incubated with purified recombinant PKA (Sigma-Aldrich; 50 μg total protein) in kinase buffer (20 mM Hepes, pH 7.0, 2 mM $MgCl_2$, 40 μM ATP, 2 μg/ml aprotinin, 40 μg/ml bestatin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.5 mM Pefabloc, 20 mM glycerophosphate, 50 μM sodium vanadate, 1 mM NaF, and 10 mM *p*-nitrophenol phosphate) containing γ-[^{32}P]ATP (6,000 Ci/mmol) for 20 min at 30°C. The bead-bound recombinant tail was washed several times with kinase buffer without ATP, boiled in SDS-PAGE sample buffer, and resolved by 4–20% SDS-PAGE under reducing conditions. ^{32}P-Labeled recombinant tail mimic proteins were visualized after autoradiography. For Western blotting, protein samples were separated by SDS-PAGE and processed by the method of Laemmli (1970). Membranes were incubated with either primary antibody followed by goat anti–mouse HRP conjugate (Biosource International), or biotinylated proteins were detected by Vectastain (Vector Laboratories). Labeled proteins were detected using Supersignal chemiluminescent substrate (Pierce Chemical Co.).

Monocyte isolation

Monocytes were isolated from human peripheral blood obtained from healthy adult donors from the Scripps Clinic (La Jolla, CA). Whole blood containing anticoagulant was centrifuged and the buffy coat was transferred to a fresh container, and washed three times in PBS(−), without Ca^{2+} or Mg^{2+}. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Amersham Pharmacia Biotech) and washed three times in PBS(−). Monocytes were isolated by plating washed cells in tissue culture flasks for 1 h at 37°C. Adherent cells were preserved and maintained in RPMI 1640, supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin sulfate, and 1% nonessential amino acids. Monocytes were later suspended with trypsin/EDTA, replated on ECM-coated glass coverslips in tissue culture wells, and stimulated with 15 ng/ml stromal cell-derived growth factor–1α (R&D Systems) in a modified Dunn chamber (Zicha et al., 2003). Cells were washed, fixed, and processed for immunocytochemistry as described in the next paragraph.

Immunocytochemistry

Sterile glass coverslips were coated with ECM proteins (CS-1 and 3Fn(9–11) fragments of fibronectin) at 2–10 μg/ml overnight at 4°C, then blocked for 1 h with 1 mg/ml BSA. Cells were suspended with trypsin/EDTA and plated onto coated coverslips and treated as described below in Scratch wound and random migration assays. Cells were fixed for 5 min in 3.7% formaldehyde (Sigma-Aldrich) in TBS (0.1 M Tris-HCl, pH 7.4, and 150 mM NaCl) at room temperature, in some cases containing phosphatase inhibitors: 20 mM β-glycerophosphate, 50 μM sodium vanadate, and 1 mM NaF. Cells were permeabilized in 0.1% Triton X-100 in TBS containing phosphatase inhibitors, for 3 min at room temperature. Fixed, permeabilized cultures were blocked with 1 mg/ml BSA for 1 h at 37°C. Coverslips were labeled with appropriate antibodies for 1 h at 37°C in the presence of normal goat serum. Coverslips were washed in TBS, and goat anti–mouse IgG FITC conjugate (Biosource International) or goat anti–rabbit IgG rhodamine conjugate (Santa Cruz Biotechnology, Inc.) were added at 5 μg/ml and 20 μg/ml, respectively, for an additional 45 min at 37°C. Cells were washed and mounted onto glass slides with Immuno Floure mounting medium (ICN Biomedicals). Cells were viewed on a confocal micro-

scope (model MRC 1024; Bio-Rad Laboratories) and images were captured and analyzed using Lasersharp (Bio-Rad Laboratories) and ImageProPlus (Media Cybernetics) software applications. Percent colocalization was calculated as the ratio of pixels containing both red and green color above a threshold level to the total number of red and green pixels over the threshold level in a defined area.

Pseudopod purification

Pseudopod purification was performed as described previously (Cho and Klemke, 2002). In brief, serum-starved cells were trypsinized and allowed to attach and spread for 2 h on FN-coated, 3-μm pore polycarbonate membranes in Costar Transwell inserts. 10 ng/ml PDGF-BB chemoattractant (Upstate Biotechnology) was added to the lower chamber and cells were allowed to extend processes through the membrane for 1 h. Inserts were rapidly washed in PBS, cell bodies were removed from the upper surface with cotton swabs, and pseudopodia on the underside were scraped into lysis buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, phosphatase and protease inhibitors [Boehringer], and 1% Triton X-100). Alternatively, pseudopodia were removed with cotton swabs and cell bodies on the upper surface were scraped into lysis buffer. Lysates were clarified by centrifugation (16,110 *g* for 15 min at 4°C) and equal amounts of soluble protein (determined by BCA assay; Pierce Chemical Co.) were used for immunoprecipitation.

Scratch wound and random migration assays

Tissue culture dishes (Falcon) or glass coverslips were coated with 2 μg/ml GST-CS-1 or GST-9–11 overnight at 4°C. For scratch wound assays, cells were suspended with trypsin/EDTA and plated onto coated dishes at a density of 10^6 cells/35-mm dish. After 2 h, cell cultures were scratched with a single pass of a pipet tip, and incubated at 37°C for various times. In some cases, cultures media was supplemented with either 100 μM cAMP, Rp-isomer TEA (Calbiochem) or 30 μM H-89 (BIOMOL Research Laboratories, Inc.). Cultures were washed twice with PBS and fixed in 3.7% formaldehyde for 10 min. Fixed cultures were either viewed on a phase-contrast microscope (model IX70; Olympus) and photographed with a camera (model CoolSnapPro CCD; Media Cybernetics) and ImageProPlus, or were processed for immunocytochemistry as described above in Immunocytochemistry. For random migration assays, glass coverslips were fitted with silicone grease onto the underside of 35-mm tissue culture dishes that had been bored through with a heated metal cylinder. The inner glass surface was coated with ECM proteins as described above in Immunocytochemistry, and 3×10^4 cells were plated into each dish for 1 h. Cells were placed in an open chamber with atmospheric and temperature controls (Schwartz, 1993) and viewed on a phase-contrast microscope (model IX70; Olympus). Images were photographed and processed using ImageProPlus software.

Online supplemental materials

Fig. S1 shows α4(S988A) and α4 COOH-terminal paxillin fusion do not perturb α4 integrin–dependent adhesion in CHO cells. Fig. S2 shows that paxillin colocalizes with nonphosphorylated α4 at the lateral and trailing edges but not with phospho-α4 at the leading edge in wounded confluent monolayers. CHO cells expressing α4wt (Video 1), α4(S988A) (Video 2), or an α4-paxillin chimera (Video 3) were plated onto 2 μg/ml CS-1 for 1 h, and viewed by phase-contrast microscopy as above in random migration assays for a total of 6 h at 37°C. Images were captured every 10 min, 1 min, or 10 s, as indicated in the figure legends. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200304031/DC1.

We gratefully acknowledge Dr. David Rose for assistance with monocyte isolation and maintenance, Nima Yousefi for technical assistance, and Dr. Sanford Shattil for critical review of the manuscript.

Funding for this work was provided by grants from the National Institutes of Health (M.H. Ginsberg). L.E. Goldfinger is a postdoctoral fellow of the American Cancer Society; J. Han is a postdoctoral fellow of the Multiple Sclerosis Society. This is publication #15469VB from the Scripps Research Institute.

Submitted: 7 April 2003
Accepted: 26 June 2003

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