Association between α4 integrin cytoplasmic tail and non-muscle myosin IIA regulates cell migration

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Summary

α4β₁ integrin regulates cell migration via cytoplasmic interactions. Here, we report an association between the cytoplasmic tail of α4 integrin (α4 tail) and non-muscle myosin IIA (MIIA), demonstrated by co-immunoprecipitation of the MIIA heavy chain (HC) with anti-α4-integrin antibodies and pull-down of MIIA-HC with recombinant α4 tail from cell lysates. The association between the α4 tail and MIIA does not require paxillin binding or phosphorylation at Ser988 in the α4 tail. We found that substituting Glu982 in the α4 tail with alanine (E982A) disrupts the α4–MIIA association without interfering with the paxillin binding or Ser988 phosphorylation. By comparing stably transfected CHO cells, we show that the E982A mutation reduces the ability of α4β₁ integrin to mediate cell spreading and to promote front–back polarization. In addition, we show that E982A impairs shear-flow-induced migration of the α4-integrin-expressing CHO cells by reducing their migration speed and directional persistence. The E982A mutation also leads to defects in the organization of MIIA filament bundles. Furthermore, when cells are plated on fibronectin and simulated with shear flow, α4β₁ integrin forms filament-like patterns that co-align with MIIA filament bundles. These results provide a new mechanism for linking integrins to the actomyosin cytoskeleton and for regulating cell migration by integrins and non-muscle myosin II.

Key words: Integrin, Non-muscle myosin II, Cell migration, Cytoskeleton

Introduction

Integrins are a family of heterodimeric transmembrane glycoproteins composed of non-covalently bound alpha (α) and beta (β) subunits (Hynes, 1992), and are the major class of cell surface receptors that mediate interactions between a cell and the extracellular matrix (ECM). Integrin-mediated signaling pathways regulate many cellular functions including cell proliferation, cell survival, cell differentiation and cell migration. Integrins transduce signals bi-directionally, transmitting information from the ECM into the cells by activating intracellular signaling through outside-in pathways, and, conversely, responding to intracellular signals by changing their ligand-binding activities through inside-out pathways (Ginsberg et al., 2003; Hynes, 2002). The integrin cytoplasmic domains lack enzymatic activities; therefore integrin signaling relies on cytoplasmic adaptor proteins and signaling proteins that form multi-molecular complexes (Giancotti and Tarone, 2003). The cytoplasmic interactions not only facilitate signal transduction but also mediate the physical association of integrins with the actin cytoskeleton, which is crucial for adhesion assembly and transmission of bi-directional force across the plasma membrane (Evans and Calderwood, 2007; Geiger et al., 2001). The associations between integrins and the actin cytoskeleton have largely been attributed to the binding of the cytoplasmic tail of the β-subunits to adaptor proteins, such as talin (Calderwood et al., 1999), kindlin-2 (Shi et al., 2007), kindlin-3 (Moser et al., 2008) and filamin (Kiema et al., 2006). However, the interactions with the cytoplasmic tails of the α-subunits are thought to primarily facilitate the formation of signaling complexes.

The best known example of such a signaling complex is mediated through the binding of paxillin to the cytoplasmic tail of the α4 integrin subunit (referred to as the α4 tail) (Liu et al., 1999). This cytoplasmic interaction allows the formation of a molecular complex that also includes an Arf-GAP, GIT1 (Nishiya et al., 2005). The α4–paxillin–GIT1 complex inhibits Rac activities by regulating Arf6 (Nishiya et al., 2005), and the formation of this complex is negatively regulated by phosphorylation at Ser988 in the α4 tail (Han et al., 2001). This pathway provides a mechanism for α4β₁ integrin to regulate cell migration, in which preferential phosphorylation of the α4 tail at the leading edge restricts Rac activities to this area and promotes polarized lamellipodia formation (Goldfinger et al., 2003; Nishiya et al., 2005).

α4β₁ Integrin has a pivotal role in cell migration in vivo. α4-integrin-deficient mouse embryos have defects in the migration of many progenitor cells, including neural crest cells, epicardial progenitor cells and pericytes (Grazioli et al., 2006; Kil et al., 1998; Pinco et al., 2002). In culture, pericytes migrate in a directionally persistent manner in response to shear flow stimulation, and this migratory behavior is impaired when α4 integrin is knocked out (Grazioli et al., 2006). This phenotype is recapitulated in a CHO model system. In this system, α4 integrin is ectopically expressed in CHO cells that lack endogenous α4 integrin, which promotes the formation of stable front–back polarity and directionally persistent migration in response to a directional shear force (Dikeman et al., 2008; Pinco et al., 2002). In the present study, we used this CHO cell system and identified an association between the α4 tail and non-muscle myosin IIA (MIIA).
Results
Non-muscle myosin IIA associates with the cytoplasmic tail of α4 integrin independently of phosphorylation and paxillin binding at the α4 tail

To identify components in an α4β1-integrin-dependent signaling pathway, we performed mass spectrometry analysis of proteins from cell lysates that were pulled down by an anti-α4-integrin antibody. The lysates were obtained from CHO cell lines (Dikeman et al., 2008; Pinco et al., 2002) that ectopically express α4 integrin with either a pharmacological disruption mutation (Ser988 was substituted with a alanine, referred to as α4S988A) (Han et al., 2001) or a mutation that disrupts paxillin binding (Tyr991 was substituted with an alanine, referred to as α4Y991A) (Liu and Ginsberg, 2000). The parental CHO cells, which do not express α4β1, were used as a negative control. The most prominent bands that were detected by staining the gel with Coomassie Blue were analyzed by mass spectrometry (Fig. 1A). In addition to the integrin subunits α4 and β1, a 200 kDa band was identified as non-muscle myosin IIA heavy chain (referred to as MIIA-HC) (supplementary material Fig. S1), suggesting that this protein co-immunoprecipitates with α4β1 integrin.

To confirm that MIIA-HC can co-immunoprecipitate with α4 integrin specifically, we tested the ability of the anti-α4-integrin antibody to co-immunoprecipitate α4 integrin and MIIA-HC from a lysate of CHO cells that expressed wild-type α4 integrin (referred to as α4WT) (Pinco et al., 2002). The antibody was able to pull down MIIA-HC from the cell lysate in a dosage-dependent manner, whereas an isotype control antibody pulled down a much lower amount of MIIA-HC, which did not correlate with the antibody dosage (Fig. 1B). As an additional negative control, we also showed that normal mouse IgG pulled down only background levels of MIIA-HC from CHO-α4WT lysates (Fig. 2A, Fig. 3C). These results strongly suggest that MIIA-HC can co-immunoprecipitate specifically with α4 integrin. To verify that the co-immunoprecipitation of MIIA-HC with α4 integrin was not an artifact as a result of ectopically expressing α4 integrin in CHO cells, we tested B16-F0 mouse melanoma cells and A375 human melanoma cells that endogenously express this integrin. We also tested antibodies against α5 integrin, another fibronectin-binding integrin that is associated with β1 integrin. MIIA-HC co-immunoprecipitated with α4 integrin, but not with α5 integrin, from these melanoma cell lines (Fig. 1C,D). We conclude that non-muscle MIIA can physically associate with α4β1, likely via the cytoplasmic domain of the α4 integrin subunit. To test if the α4 tail can associate with MIIA, pull-down assays were performed using a recombinant GST-α4 tail fusion protein. GST-α4-tail pulled down MIIA-HC from CHO cell lysate (Fig. 1E, Fig. 2B, Fig. 3B), whereas GST-β1-tail (Fig. 1E) or GST-α5-tail (Fig. 3B) only pulled down background amount of MIIA-HC. Therefore, the association between α4β1 integrin and MIIA is mediated by the α4-tail.

The results from the mass spectrometry study (Fig. 1A) suggested that MIIA can associate with α4 integrin in the absence of paxillin binding or Ser988 phosphorylation at the α4 tail. This was confirmed by additional co-immunoprecipitation studies showing that MIIA-HC was co-immunoprecipitated with α4 integrin from the lysate of CHO-α4Y991A (Fig. 2A) and CHO-α4S988A cells (Fig. 2A, Fig. 3C). Similar results were obtained in pull-down assays using GST-α4-tail fusion proteins that carried the Y991A or S988A mutation (Fig. 2B). Neither of these mutations abolished the ability of the GST-α4-tail to pull down MIIA-HC from CHO cell lysate, indicating that Ser988 phosphorylation and paxillin binding were not required for α4–MIIA association. In addition, we found that actin did not co-immunoprecipitate with α4 integrin and MIIA-HC as judged by immunoblotting of the eluates with an anti-actin antibody (Fig. 3C), suggesting that actin-binding is also not required for α4–MIIA association.

Because we have previously shown that the Y991A/S988A double mutation (referred to as α4DM) has an inhibitory effect on the ability of α4β1 integrin to promote directionally persistent cell migration, whereas neither of the single mutations have this inhibitory effect (Dikeman et al., 2008), we also tested the ability of the doubly mutated α4 integrin to associate with MIIA. The amount of MIIA-HC that co-immunoprecipitated with α4DM in CHO-α4DM cells or was pulled down with GST-α4DM was markedly less than that with wild-type α4 integrin or GST-α4-tail (P<0.01) (Fig. 2A,B). One interpretation is that both Ser988
Fig. 2. α4–MIIA association occurs when paxillin binding or Ser988 phosphorylation is disrupted, or when Ser988 of α4 is phosphorylated. (A) The lysate of CHO and CHO-α4WT (WT), α4Y991A (Y/A), α4S988A (S/A) or α4DM (DM) cells was used for immunoprecipitation with an anti-α4-integrin antibody followed by immunoblotting with antibodies against MIIA-HC and GFP (for GFP-tagged α4 integrin). The dot plot shows normalized ratios of MIIA-HC and α4 integrin, in which the solid lines represent the means (n=5). *P=0.0013, compared with WT; s.e.m.=0.11. (B) CHO cell lysate was used to pull down MIIA-HC with GST–α4WT (WT), GST–α4Y991A (Y/A), GST–α4S988A (S/A) or GST–α4DM (DM) tail, followed by immunoblotting with anti-MIIA-HC and ponceau S staining to visualize the GST fusion proteins. The dot plot shows normalized ratios of MIIA-HC and GST fusion proteins, in which the solid lines represent the means (n=3). *P=0.0075, compared with WT; s.e.m.=0.072. (C) The lysate of CHO-α4WT cells (L) was used for immunoprecipitation with an anti-α4-integrin antibody (α4), an antibody that specifically recognizes phosphorylated Ser988 in the α4 tail (P-α4), mouse IgG (m-IgG) or rabbit IgG (r-IgG). The immunoprecipitation was followed by immunoblotting with antibodies against MIIA-HC and GFP (for GFP-tagged α4 integrin). Note that MIIA co-immunoprecipitated with Ser988-phosphorylated α4 integrin.

Phosphorylation of Tyr991 reside in a region in the α4 tail that is responsible for the α4–MIIA association; although disruption of one of the residues does not impair α4–MIIA association, disruption of both residues has a synergistic effect. Another possibility is that the double mutation might synergistically perturb the secondary structure of the α4 tail, thus inhibiting α4–MIIA association.

If Ser988 is part of the α4 integrin region that is responsible for the association of α4 integrin with MIIA, phosphorylation at this site might have an inhibitory effect on the association. To test this possibility, we performed a co-immunoprecipitation experiment using an antibody that specifically recognizes Ser988-phosphorylated α4 integrin [the specificity of this antibody has been reported (Dikeman et al., 2008) and is shown in Fig. 3D]. The data showed that this antibody was able to pull down MIIA from the lysates of CHO-α4WT cells (Fig. 2C), indicating that Ser988-phosphorylated α4 integrin can associate with MIIA. This result, however, does not rule out the possibility that Ser988 phosphorylation could regulate α4–MIIA association that was not detected in this assay.

Specifically disrupting α4–MIIA association by a point mutation in the α4 tail causes a migration defect

To determine the in vivo function of α4–MIIA association, it is crucial to disrupt this association without disrupting paxillin binding and Ser988 phosphorylation. We tested several highly conserved residues in the α4 tail that have been reported to reside outside the region responsible for paxillin binding and Ser988 phosphorylation (Fig. 3A). These residues were mutated by substitution with alanine. GST–α4-tail fusion proteins carrying each mutation were tested for their ability to pull down MIIA-HC from CHO cell lysates. The mutation that substituted Glu982 with an alanine (referred to as E982A) had a marked inhibitory effect (P<0.001) (Fig. 3B). We generated CHO cell lines that stably expressed α4 integrin carrying E982A (CHO-α4E982A), and selected those with a surface expression level of α4 integrin equivalent to that of CHO-α4WT cells for our studies (supplementary material Fig. S2). E982A inhibited the co-immunoprecipitation of MIIA-HC and α4 integrin from the lysates of the CHO-α4E982A cells (P<0.001) (Fig. 3C), whereas Ser988 phosphorylation of the α4 tail in these cells was not affected (Fig. 3D). It has been reported that the E982A mutation does not interfere with paxillin binding (Liu and Ginsberg, 2000). We confirmed this result by showing that a recombinant α4E982A tail protein pulled down paxillin from CHO cell lysate in a similar manner as did α4WT tail protein (Fig. 3E). These results indicate that E982A disrupts α4–MIIA association without interfering with paxillin binding or Ser988 phosphorylation.

The CHO-α4E982A cells were then compared with CHO-α4WT cells to determine the effect of disrupting α4–MIIA association on the adhesion, spreading and migration functions of α4β1 integrin, using a recombinant fibronectin fragment that contains the CS-1 region (an α4β1 integrin-specific ligand, referred to as CS-1) as the substrate. Our data showed that the two cell types had similar adhesive properties as indicated by similar resistance to shear flow-induced detachment (P>0.6) (Fig. 4A) and similar static adhesion (Fig. 4B). As a negative control, CHO cells failed to adhere to CS-1. Although there was no difference in the adhesive properties between the cell types, they did exhibit differences in their ability to spread. CHO-α4E982A cells had a spreading rate that was significantly lower than that of CHO-α4WT cells within the first 15 minutes after plating on CS-1 (P<0.05). Beyond 15 minutes, the spreading rates of the two cell types became similar (P>0.1) as the spreading activities leveled off (Fig. 4C; supplementary material Fig. S3). At the steady state on CS-1, the mean spreading area of CHO-α4E982A cells was reduced by 52% compared with that of CHO-α4WT cells (P<0.02) (Fig. 4D).

To assess the role of α4β1 integrin in cell migration, we have previously shown that, when plated on fibronectin or CS-1 and stimulated with a directional shear flow, CHO-α4WT cells form a fan shape with broad leading edge lamellipodia and flattened trailing edges and migrate in a directionally persistent manner similar to that of fish epidermal cells (Euteneuer and Schliwa, 1984; Dikeman et al., 2008). By contrast, CHO cells that lack α4β1 integrin do not have this ‘fanning’ activity, neither do the CHO-α4WT cells when plated on a recombinant FNI11-11 fragment of fibronectin that lacks the α4β1 binding site (Dikeman et al., 2008). Consistent with this finding, we found that a majority of CHO-α4WT cells (81±15%, n=3) had the fanning activity (Fig. 5A; supplementary material Movie 1) when plated on CS-1 and...
stimulated with shear flow. In response to shear flow, the cells began to migrate in the direction of the shear flow although most of these cells initially had no front–back polarity (in the ‘random’ stage). The cells then polarized by protruding lamellipodia at the leading edges (in the ‘polarized’ stage). After 60 minutes of shear flow stimulation, the cells formed a fan shape by flattening their trailing edges (in the ‘fan’ stage). By contrast, none of the CHO-α4E982A cells formed a fan shape under the same conditions (Fig. 5A; supplementary material Movie 2), among which 32±11% (n=3) were able to polarize in shape, 68±11% remained in the random stage and were never polarized, and these ‘random’ cells appeared not to migrate in a directionally persistent manner. These results show that the α4–MIIA association contributed to the ability of α4β1 to promote front–back polarization, leading to the morphological feature of fanning.

To gain further insight to the motile activities of the cell types in response to shear flow, we also quantified the directional persistence and migration speed of the cells. To quantify directional persistence, we determined the ratio of the shortest, linear distance from the starting point of a time-lapse recording to the end point (\(D/T\)) compared with the total distance traveled by the cell (\(T\)) (Pankov et al., 2005). The closer the \(D/T\) ratio was to 1, the more directionally persistent the cells migrated. We first measured the overall \(D/T\) ratio for each cell in every movie. The overall mean \(D/T\) ratio of CHO-α4WT cells (0.87±0.038, \(P=0.002\)) (Fig. 5B). Similar results were obtained when the cells were plated on fibronectin, although the reduction was slightly less (supplementary material Fig. S4, Movies 3 and 4). We next calculated the \(D/T\) ratios of each cell in the ‘random’, ‘polarized’ and ‘fan’ stages...
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Fig. 4. Effect of disrupting α4–MIIA association on cell adhesion and spreading. CHO-α4E982A and CHO-α4WT cells were compared for detachment (A), adhesion (B), spreading rate (C) and steady state spreading area (D) when plated on CS-1-coated surface. Data represent means ± s.e.m. for each cell type (n=3). In each of the detachment experiments (A), at least 95 cells were initially attached to the CS-1-coated surface. Note that there were no significant differences between CHO-4WT and CHO-α4E982A cells in A and B (P>0.6), as a negative control few CHO cells adhered to CS-1. In each of the spreading rate assays in C, at least 13 cells were analyzed. For the spreading area measurements in D, the first frames (Time 0 for shear flow) of the movies described in Fig. 5 were used, in which the cells had been plated on CS-1 for 2 hours. Means ± s.e.m. were calculated from the mean areas of the cells in three independent movies for each cell type. WT, CHO-α4WT cells; E982A, CHO-α4E982A cells; *P<0.05 compared with CHO-α4WT cells.

Fig. 5. Effect of disrupting α4–MIIA association on shear-flow-induced motile activities. (A) Time-lapse images of cells migrating under shear flow. CHO-α4WT and CHO-α4E982A cells on CS-1 under shear flow (4 dynes/cm²) were imaged at 30 second intervals for 5 hours by time-lapse microscopy. Three frames from designated time points (indicated on the left) from an area of a typical movie sequence are presented. The arrows underneath the time points show the direction of the shear flow. Scale bars: 50 μm. (B–E) D/T ratios (B,C) and migration velocities (D,E) are shown, where B and D were calculated from the entire migration path; C and E were calculated from fan (black), polarized (dark grey) or random (light grey) stages. The data represent the means ± s.e.m. (from three independent trials; each trial had 25 cells). WT, CHO-α4WT; E982A, CHO-α4E982A; *P<0.04; **P<0.1.

when plated on CS-1 and stimulated with shear flow (Fig. 5C). The mean D/T ratios of CHO-α4WT cells at all three stages were above 0.7 with no statistically significant differences (P=0.2), suggesting that α4β1 integrin begins to promote directional persistent migration before the cells proceed to the polarized stage. However, many CHO-α4E982A cells (~68%) remained at the ‘random’ stage and had a reduced mean D/T ratio (0.42±0.022) compared with CHO-α4WT cells (P<0.002). Some CHO-α4E982A cells (~32%) proceeded to the polarized stage and had a similar mean D/T ratio (0.85±0.019) as CHO-α4WT cells (P>0.2). Similar results were obtained from two independent CHO-α4E982A cell lines. These data suggest that the overall reduction in the directional persistence of the CHO-α4E982A cells was due to the contribution by those that never proceeded to the polarized stage.

We also calculated the overall mean migration speed and the mean migration speed at the three stages. The overall mean migration speed of CHO-α4E982A cells was decreased by 41% (P<0.02) compared with CHO-α4WT cells (Fig. 5D). In contrast to directional persistence, the CHO-α4WT and CHO-α4E982A cells in random and polarized stages (among which P>0.1) migrated more slowly than the CHO-α4WT cells in the fan stage (P<0.04) (Fig. 5E). These data suggest that the reduced overall migration speed of CHO-α4E982A cells was due to their failure to proceed to the fan stage. When the cells were plated on fibronectin, however, there was no statistically significant difference between CHO-α4WT and α4E982A cells for the overall mean migration speed (P>0.1) (supplementary material Fig. S4, Movies 3 and 4), suggesting that the migration speed could be compensated by other integrins.

In summary, these data show that α4–MIIA association has important roles in regulating directional persistence and migration speed, possibly by facilitating front–back polarization and fan-shape formation, respectively.

α4–MIIA association has an important role in MIIA filament organization

To understand how α4–MIIA association functions in cell migration, we studied the spatial distributions of MIIA that was close to the ventral surfaces of CHO-α4WT and CHO-α4E982A cells, using immunofluorescence and confocal microscopy. The cells were plated on fibronectin (Fig. 6) or CS-1 (Fig. 7; supplementary material Fig. S5) and stimulated with shear flow. The distribution patterns of MIIA were similar on either of the substrates, on which the MIIA patterns varied at different time points of shear flow (Figs 6, 7; supplementary material Fig. S5). In the absence of shear flow and at the 15 minute time point, CHO-
α4WT and CHO-α4E982A cells both had a condensed staining pattern at the cell cortex. At 30 minute and later time points, however, the MIIA patterns of the two cell types were quite different. In CHO-α4WT cells, MIIA no longer had condensed cortex staining. Instead, MIIA formed prominent parallel arrays of filament bundles. In CHO-α4E982A cells, the MIIA filament bundles were also formed, but failed to arrange into parallel arrays. The MIIA bundles were disorganized and displayed condensed staining patterns, which we referred to as ‘plaque-like’ MIIA structures. The plaque-like MIIA structures were also observed in some CHO-α4WT cells, but to a much lesser extent than CHO-α4E982A cells (Figs 6, 7). To test whether this MIIA defect was due to reduced spreading and/or a difference in cell shape, we examined a few CHO-α4WT cells that had similar spreading area and shape as CHO-α4WT cells. The MIIA organization defect was also evident in these cells (supplementary material Fig. S5).

To quantify this ‘plaque’ phenotype, the cells were analyzed with ImageJ software, in which fixed threshold and minimum particle size were set to identify and measure the regions with plaque-like MIIA structures. Our data showed that when the cells were stimulated with shear flow for 30 minutes or longer the CHO-α4E982A cells had a significantly greater area occupied by the plaque-like MIIA structures than observed in CHO-α4WT cells (*P<0.04). The difference of the two cell types in their MIIA organization was observed when plating the cells on either fibronectin (Fig. 6) or CS-1 (Fig. 7; supplementary material Fig. S5). Furthermore, a similar plaque phenotype was also observed in CHO cells that lack α4β1 integrin (Fig. 6). Therefore, ectopically expressing α4β1 in CHO cells facilitates the organization of MIIA bundles, and this function is inhibited when α4–MIIA association is disrupted. We conclude that α4β1 integrin contributes to some aspects of MIIA filament organization through its association with MIIA.

Filament-like patterns of α4 integrin co-align with MIIA bundles

To determine whether α4β1 integrin could be directly involved in organizing MIIA filament bundles, we performed dual-color
immunofluorescence confocal analysis on CHO-α4WT cells. The cells were plated on fibronectin and stimulated with shear flow for 1 hour, fixed and stained with antibodies against α4 integrin and MIIA-HC. As reported previously, at low magnification, α4 integrin was seen throughout the cell surface (Dikeman et al., 2008; Pinco et al., 2002). At a higher magnification, in areas with prominent MIIA filament bundles, α4 integrin displayed a filament-like pattern that co-aligned with many MIIA bundles (Fig. 8A; supplementary material Fig. S6A). Two controls were performed to show that there was no bleed-through from the channel for MIIA to the α4-tail channel. First, CHO cells, which lack α4 integrin, were stained with antibodies against MIIA-HC and α4 integrin. Although strong MIIA staining was observed in the red channel, there was no α4 integrin staining at all in the green channel (supplementary material Fig. S6B). Second, CHO-α4WT cells were single-stained with an antibody against α4 integrin. α4 integrin showed filament-like patterns in the absence of MIIA staining (supplementary material Fig. S6C). The filament-like pattern of α4 integrin (referred to as α4 filament pattern) was also observed in CHO-α4E982A cells (Fig. 8A; supplementary material S6A), suggesting that this pattern can form independently of α4–MIIA association. However, the percentage of MIIA filaments that co-aligned with the α4 filament pattern was significantly reduced in CHO-α4E982A cells compared with CHO-α4WT cells (Fig. 8B). These data suggest that the α4–MIIA association partially contributes to the formation of the α4 filament pattern. An alternative interpretation is that α4E982A does not completely disrupt α4–MIIA association in vivo, although biochemically we have observed a drastic reduction of α4–MIIA association by this mutation. In either case, we can conclude that α4–MIIA association contributes, at least in part, to the formation of the α4 filament pattern.

Discussion

In this study, we report an association between the cytoplasmic tail of α4 integrin and MIIA. MIIA is one of the isoforms of non-muscle myosin II, which are actin-based molecular motors. A myosin II monomer is composed of two myosin heavy chains, two regulatory myosin light chains and two essential myosin light chains. The C-terminal rod domain of the two heavy chains form a long α-helical coiled coil, which allows the myosin II monomers to assemble into bipolar filaments in which the monomers are oriented with the heads pointing in opposite directions at the ends of the filament. The myosin filaments are further packed with F-actin and other proteins into higher-order bundles (Langanger et al., 1986). The F-actin in these bundles form anti-parallel stress fibers (Langanger et al., 1986) or graded polarity filaments (Cramer et al., 1997). There are three non-muscle myosin II isoforms, MIIA, MIIB and MIIC, of which MIIA has been shown to have unique roles in cell motility and cell adhesion (Cai et al., 2006; Even-Ram et al., 2007; Vicente-Manzanares et al., 2007). Here, we provide evidence that α4β1-integrin-promoted motile activities require an association between the cytoplasmic domain of the α4 integrin subunit and MIIA.

The α4–MIIA association was demonstrated by co-immunoprecipitation of MIIA-HC with α4 integrin and pull-down of MIIA-HC with α4-tail proteins. Although it remains unclear whether the α4–MIIA association is due to direct binding or through other proteins, this association clearly does not involve the α4–paxillin complex. Given that actin does not co-immunoprecipitate with MIIA-HC and α4 integrin, it is also possible that α4–MIIA association does not require actin binding.

In our co-immunoprecipitation experiments, the lysis buffer contained 300 mM NaCl, and the cell lysates were pre-cleared with centrifugation of at least 30,000 g. Under these conditions, the concentration of actin in the lysate could have been reduced as a result of polymerization and sedimentation of F-actin, therefore preventing co-immunoprecipitation of actin with myosin II from the lysate (Blanchon et al., 1995). We found that the high-salt condition was required for detecting α4–MIIA association in our co-immunoprecipitation and pull-down studies (supplementary material Fig. S7). It has been reported that ionic strength influences the conformation and assembly state of non-muscle myosin II (Trybus and Lowey, 1984). At physiological salt concentration (150 mM), non-muscle myosin II switches between two conformations, a 6S extended conformation and a 10S folded conformation. In the 10S conformation, myosin forms a hairpin in which the tail folds over with the regulatory light chain (RLC) of the myosin head, which sequesters myosin away from filament assembly and is soluble, remaining in the supernatant after high-speed centrifugation. When the RLC is phosphorylated, myosin is unfolded into the 6S conformation, which is assembled into filaments and becomes insoluble,
sedimenting into the pellet after high-speed centrifugation. At high salt concentration (300–400 mM), myosin is forced into a soluble 6S monomeric state (Trybus and Lowey, 1984). This ionic strength effect was initially demonstrated using purified myosin (Trybus and Lowey, 1984) and later supported by studies using cell lysates (Breenkridge et al., 2009). Based on these studies, we propose that the association of α4 integrin and MIIA depends on the conformation and assembly state of MIIA where an intermediate extended monomeric state of MIIA is required for the association. Although we do not have sufficient data to demonstrate that the α4–MIIA association occurs in vitro under physiological conditions, our observation that disruption of the α4–MIIA association in cultured cells leads to functional defects and reduces co-alignment of α4 integrin with MIIA bundles provides strong evidence that this association occurs in vivo and has important physiological roles.

We show that a point mutation E982A, which disrupts α4–MIIA association without interfering with paxillin binding and Ser988 phosphorylation in the α4 tail, inhibits cell spreading and impairs the ability of α4β1 integrin to promote directionally persistent cell migration and to organize MIIA filament bundles. The mechanism by which myosin II filaments are organized into actomyosin bundles is poorly understood, making it difficult to envision how α4β1 integrin is involved in this process. One possible role of α4β1 integrin is to regulate bundle assembly. It is known that the assembly of actomyosin stress fibers depends on focal adhesions, which anchors the ends of stress fibers via integrins (Naumanen et al., 2008). α4β1 integrin, however, is not detected in focal adhesions in our CHO cell system when the cells are plated on fibronectin (Picco et al., 2002). We found that α4 integrin forms filament patterns that co-align with MIIA bundles, and this co-alignment is partially impaired by disrupting α4–MIIA association. Based on this observation, we propose that, instead of being recruited to focal adhesions, α4β1 integrin clusters along the MIIA bundles; this clustering event is cooperatively mediated by α4–MIIA association and other α4–MIIA-association-independent pathways, such as a β1-mediated pathway. Once in the vicinity of MIIA bundles, the α4–MIIA association influences the dynamics and organization of the MIIA bundles. This unique mode of integrin–cytoskeleton interaction might facilitate the response of the cells to external signals through α4β1 integrin during cell spreading and migration.

In response to shear flow stimulation, α4β1 integrin promotes front–back polarization and directionally persistent cell migration. α4β1 integrin facilitates front–back polarity in part by spatially regulating the formation of an α4–paxillin–GIT1 complex, which is preferentially localized to the sides flanking the protruding leading edge (Dikeman et al., 2008; Goldfinger et al., 2003), where this complex inhibits Rac activities (Nishiyama et al., 2005). At the leading edge, the α4–paxillin–GIT1 complex is negatively regulated by Ser988 phosphorylation of α4 integrin (Han et al., 2001). In contrast to this complex, we did not observe any apparent spatial regulation of α4–MIIA association. Since the CHO-α4E982A cells have a reduced ability to spread, it is possible that the reduced spreading is the primary defect that leads to reduced front–back polarization and directional persistence. Alternatively, α4–MIIA association might contribute to cell spreading and front–back polarization via separate pathways, which is supported by our observation that a small population of CHO-α4E982A cells were able to polarize in shape with reduced spreading ability compared with CHO-α4WT cells, suggesting that cell spreading and front–back polarization are independent phenotypes.

Although disruption of α4–MIIA association has an inhibitory effect on cell spreading, knockdown of MIIA enhances cell spreading (Cai et al., 2006). It is possible that α4–MIIA association somehow inhibits MIIA-dependent contractility, probably by inhibiting MIIA assembly or facilitating MIIA disassembly, thus enhancing the ability of the cells to spread. Another function of α4–MIIA association revealed in our studies involves cell migration speed. Disrupting α4–MIIA association reduces migration velocity, and again, knockdown of MIIA had an opposite effect (Even-Ram et al., 2007). It is possible that α4–MIIA association facilitates faster cell migration and cell spreading by a similar mechanism that involves inhibiting MIIA-driven contractility.

α4β1 integrin might not be the only integrin that can associate with MIIA through the α-integrin subunit. LFA-1 and αvβ3 have also been reported to co-immunoprecipitate with MIIA, and associations of these integrins with MIIA were implicated in T-lymphocyte migration and vascular healing, respectively (Morin et al., 2008; Sajid et al., 2000). These studies, however, did not clarify whether the α-subunits of these integrins are responsible for the associations. It will be desirable to determine whether the cytoplasmic domain of αv and αL subunits, as well as other α-subunits, can associate with MIIA.

In summary, we have provided evidence that the cytoplasmic tail of α4 integrin associates with MIIA and that this association has an important role in cell migration. This work provides a new mechanism for linking integrins to the actomyosin cytoskeleton and for regulating cell migration by integrins and non-muscle myosin II.

**Materials and Methods**

### Antibodies and Reagents

Anti-integrin antibodies that recognize the extracellular domains were used for co-immunoprecipitation studies: anti-human α4 integrin clones PJJ1 and P1H4, from Sigma and Chemicon, respectively; anti-mouse α4 integrin (clone R1-2), anti-mouse CD49e (α5, clone MFR5) and anti-human CD49d (α6, clone IIA1), Pharmingen/BD Biosciences.

Other antibodies: anti-GFP, Molecular Probes; FITC-conjugated anti-GFP, Abcam; anti-MIIA-HC, Sigma; anti-actin, Millipore; anti-paxillin, BD Transduction; APC-conjugated anti-mouse IgG, Santa Cruz Biotechnology; Alexa-Fluor-546-conjugated anti-mouse IgG and Cy5-conjugated anti-rabbit IgG, Invitrogen. Anti-phospho-α4β1 integrin was generated as described (Han et al., 2001), pre-absorbed with recombinant wild-type α4 tail protein and characterized for specificity (Dikeman et al., 2008). Immunopure streptavidin-HRP, Pierce.

### Surface biotinylation and immunoprecipitation

Surface biotinylation was performed by incubating confluent cells with 100 μg/ml of EZ-link sulfo-NHS-LC-biotin (Pierce) in wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM CaCl2, 5 mM MgCl2) for 1 hour at room temperature. For immunoprecipitation, all steps were performed at 4°C. Cells confluent in 10 cm plates were lysed in 240 μl lysis buffer [25 mM Tris-HCl, pH 7.2, 300 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.05% Tween-20, and protease inhibitors (Sigma)], 30 minutes at 4°C. Lysates were clarified by centrifugation at 11,000 g for 30 minutes, the supernatants were further clarified by dialysis of the lysate in 5 mM PIPES, pH 6.5, 20 mM NaCl, 10 mM MgCl2 followed by centrifugation at 112,000 g, dialysis of the supernatant in 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, and clarification by centrifugation at 112,000 g. The supernatants were then pre-cleared with protein G PLUS agarose beads (pG-beads, Santa Cruz Biotechnology) for 30–45 minutes. Lysate with approximately 225 μg total protein was incubated with 2 μg primary antibody overnight, followed by addition of 30 μl pG-beads. After incubation for 30 minutes, the beads were harvested by centrifugation followed by three washes with the high-salt buffer. The eluates were resolved by SDS-PAGE and analyzed by immunoblotting or visualized by SimplyBlue™ SafeStain (a Coomassie stain from Invitrogen). The bands on the immunoblots were measured using Quantity One software (Bio-Rad).

### Construction of plasmids, transfection and cell culture

pQ4NE982AG for expressing α4 integrin with an E982A mutation (tagged with GFP at the C-terminus) was made by site-directed mutagenesis using pQN4G (Sengbusch et al., 2002) as a template and a QuickChange kit (Stratagene). The
plasmids for expressing recombinant GST-tagged fusion proteins and integrin tail model proteins were constructed by PCR amplification of the cDNAs encoding various integrin tail domains and insertion of the PCR products into pGEX-1X (GE Healthcare) for GST fusion proteins or into pET-15b for integrin tail model proteins. pET-15b was a gift from Sue Craig (Johns Hopkins University, Baltimore, MD) who constructed the plasmid using a design report (Lad et al., 2007).

pQNE982AG was transfected into CHO cells using FuGENE 6 reagent (Roche) following the manufacturer’s instructions. Stable transfectants were selected in 0.8 mg/ml G418 and clones were obtained by fluorescence-activated cell sorting (FACS) using an anti-ω4-integrin antibody (Chemicon) and an APC-conjugated secondary antibody. The wild-type and mutant ω4-integrin-expressing CHO cell lines (Dikeman et al., 2008; Pino et al., 2002) were maintained in F-12 containing 10% FBS, L-glutamine, antibiotics and 0.4 mg/ml G418. CHO, B16-F0 mouse melanoma cells and A375 human melanoma cells were cultured in DMEM supplemented with 10% FBS, L-glutamine and antibiotics.

Integrin tail proteins and binding assays
Recombinant GST fusion proteins were purified from E. coli using glutathione–agarose beads (Pierce). All steps were performed at 4°C. Approximately 50 μg GST-tagged proteins were bound to glutathione-agarose beads for 2 hours, and washed three times with lysis buffer (25 mM Tris- HCl, pH 7.2, 1% NP-40, 300 mM NaCl). For each sample, two confluent plates of CHO cells were lysed in 600 μl lysis buffer with protease inhibitors (Sigma) for 30 minutes. The lysates were clarified as described for immunoprecipitation with the additional mammalian assembly-disassembly steps. Lysate with approximately 300 μg total protein was incubated with GST-tagged protein-loaded beads overnight. The beads were harvested by centrifugation. Bound complexes to GST-tagged proteins were washed twice with lysis buffer, then once each with lysis buffer plus 0.1% SDS, lysis buffer plus 0.5 M NaCl, and TBS (25 mM Tris-HCl, pH 7.2 and 120 mM or 300 mM NaCl). The eluates were resolved by SDS-PAGE and analyzed by immunoblotting. The bands on the immunoblots were measured using Quantity One software (Bio-Rad).

For testing ω4 integrin and paxillin binding, wild-type and mutant ω4 integrin tail model proteins were purified using nickel beads (Ni-NTA His-Bind Resin, Novagen) and used in pull-down as described (Lad et al., 2007).

Integrin ligands
Rat plasma fibronectin was purchased from Calbiochem. Plasmids pGIF-H12-V120-15 (expressing FNIII12-V120-15 containing the CS-1 region of fibronectin, referred to as CS-1), which was derived from pGH.F12 (Bloom et al., 1999), was provided by Richard Hynes (MIT, Cambridge, MA).

Static cell adhesion
Static cell adhesion assay was performed as described (Rose et al., 2003). Briefly, 96-well plates were coated with 10 μg/ml CS-1 for 2 hours and blocked with 2 mg/ml heat-inactivated BSA for 1 hour, followed by plating 5×103 cells per well for 15 minutes. After 15 minutes of incubation at 37°C, non-adherent cells were removed by submerging the plate in PBS and shaking off the cells. The remaining cells were stained with 0.5% crystal violet (Sigma) in 20% methanol for 15 minutes, and the cell-incorporated crystal violet was solubilized with 10% acetic acid and measured in a Fluostar Omega microplate reader (BMG Labtech).

Spreading, detachment and shear flow migration assays
Spreading, detachment and shear flow migration assays were performed using a Zeiss Axiovert 200M microscope equipped with a Hamamatsu IEE1394 digital CCD camera, Velocity acquisition software (Improvision) and a microscope stage that is enclosed in a 37°C incubator (supplied with 5% CO2 in the spreading assay). The cells were imaged under a 10×/0.30 NA objective for initial analysis, and imaging under a 63×/1.4 NA oil objective to observe the most ventral actin fibers and focal contacts in cultured cells via the heparin-binding site in repeat G151-C159.

Immunofluorescence, confocal microscopy and image analyses
Cells were plated on coverslips coated with 10 μg/ml fibronectin or CS-1, stimulated with shear flow as described in the shear flow assay for various time periods, fixed in 3% PFA in PBS for 15 minutes and permeabilized in 0.5% Triton X-100 in PBS for 3 minutes. Immunofluorescence staining was performed using the following antibodies: rabbit anti-MIIA-HC, 1:200; FITC goat anti-GFP, 1:200; Cy5 goat anti-rabbit, 1:100. Confocal images were taken using a Zeiss LSM510 Meta confocal microscope. To quantify plaque-like MIIA fluorescence staining, we randomly picked any cells that were spread as visualized by the fluorescence of ω4 integrin staining. Most ventral MIIA fluorescence images (confocal slice closest to coverslip) were analyzed with ImageJ, where fixed threshold level and minimum particle size were set to select objectively regions with plaque-like MIIA fluorescence. The percent area occupied by the selected regions was calculated. Total ventral surface area of each cell was measured with ImageJ using ω4 integrin fluorescence images.

Statistical analysis
Statistical analyses were performed using ANOVA (KaleidaGraph) or the t-test function in R (R Development Core team, http://www.R-project.org), using a two-tailed t-test and 95% confidence interval.

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