Lipid raft regulates the initial spreading of melanoma A375 cells by modulating β1 integrin clustering

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Cell adhesion and spreading require integrins-mediated cell–extracellular matrix interaction. Integrins function through binding to extracellular matrix and subsequent clustering to initiate focal adhesion formation and actin cytoskeleton rearrangement. Lipid raft, a liquid ordered plasma membrane microdomain, has been reported to play major roles in membrane motility by regulating cell surface receptor function. Here, we identified that lipid raft integrity was required for β1 integrin-mediated initial spreading of melanoma A375 cells on fibronectin. We found that lipid raft disruption with methyl-β-cyclodextrin led to the inability of focal adhesion formation and actin cytoskeleton rearrangement by preventing β1 integrin clustering. Furthermore, we explored the possible mechanism by which lipid raft regulates β1 integrin clustering and demonstrated that intact lipid raft could recruit and modify some adaptor proteins, such as talin, α-actinin, vinculin, paxillin and FAK. Lipid raft could regulate the location of these proteins in lipid raft fractions and facilitate their binding to β1 integrin, which may be crucial for β1 integrin clustering. We also showed that lipid raft disruption impaired A375 cell migration in both transwell and wound healing models. Together, these findings provide a new insight for the relationship between lipid raft and the regulation of integrins.

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1. Introduction

Cell migration plays a central role in a wide variety of biological phenomena, including embryogenesis, inflammatory response, wound healing and cancer metastasis (Lauffenburger and Horwitz, 1996). In vivo, studies suggest that in order to migrate to a new tissue site, various cells, such as leukocyte and cancer cell, must cross through the connective tissue barriers, which requires cell–extracellular matrix (ECM) interaction (Gehe et al., 2005; Werr et al., 1998). In vitro, studies suggest that cell migration on two-dimensional substrate requires cell adhesion and spreading in response to ECM, which depends on the best-studied adhesion receptors – integrins (Li et al., 2010).

Integrins, composed of both α and β subunit, play a crucial role in cell-fate decision such as cell differentiation, proliferation and migration (Streuli, 2009). During cell adhesion, individual integrin recognizes and binds to unique ECM. Upon ligand binding, integrins undergo conformational changes leading to outside-in signaling and triggering the recruitment of cytoskeletal adaptor proteins (Hynes, 2002). On the other side, the signals within the cell can also propagate through integrins and provide reversible regulations over integrin-ligand binding affinity (Liu et al., 2000). During cell spreading, integrins cluster laterally within the membrane and then associate with numerous proteins to form focal adhesion. Depending on focal adhesion formation, the cell cytoskeleton is physically linked to the ECM, multiple signaling events are initiated, and cell shape is changed from a round, spheroid morphology to an irregular flattened shape (Holly et al., 2000). As spreading on the ECM, cells start the migration process. At present, although integrin clustering has been shown to play an important role in focal adhesion formation and cell spreading, the mechanisms responsible for integrin clustering remain poorly understood.

Early studies suggested that integrin affinity for ECM ligand influences its clustering (Docić et al., 1998). More recently,
evidences have shown that besides ECM ligand, the recruitment of numerous cytoplasmic proteins, such as adaptors and kinases, to the integrin cytoplasmic tail is also required for integrin clustering at the cell surface (Cluzel et al., 2005; Welf et al., 2012). Interestingly, by binding to membrane lipid, some adaptors can increase their affinity for integrin and induce integrin conformational change (Cluzel et al., 2005; Legate and Fassler, 2009). Some membrane molecules, such as cholesterol and glycosphingolipids, have also been shown to be involved in the regulation of integrins (Pandé, 2000). All these researches imply that membrane or membrane-proximal molecules may play an important role in controlling integrin function.

Lipid raft is a liquid ordered plasma membrane microdomain, enriched in cholesterol and sphingolipid, and is believed to be involved in forming dynamic platforms within the bilayer (Simons and Toomre, 2000). Although the existence of lipid raft has been controversial in the past few years, recent data place them at the forefront of cell biology and biomembrane research (Bodin et al., 2005). Lipid raft is in principle well suited to play major roles in regulating membrane motility by excluding or including proteins selectively (Golub et al., 2004; Simons and Toomre, 2000). The role of lipid raft in regulating the function of cell surface receptor, such as EGFR, PDGFR and IGF, has also been proposed in various cell types (Baron et al., 2003; Huo et al., 2003). Recent evidences show that integrins may be localized to lipid raft and the disruption of lipid raft inhibits integrin function (Lee et al., 2008; Runz et al., 2008). Despite the recent significant advances, the question of whether lipid raft regulates integrin clustering and cell spreading remains unclear.

A375 cells, a highly malignant melanoma cell line, have been proved to express β1 and β3 integrins abundantly on their surfaces (Oikawa et al., 2011). Here, we investigated the role of lipid raft in adhesion and spreading of A375 cells. We show that methyl-β-cyclodextrin (MβCD) treatment, which over the years has been demonstrated to disrupt the integrity of lipid raft by effectively depleting cholesterol, prevents β1 integrin-mediated initial spreading of A375 cells on fibronectin. We found that the disruption of lipid raft inhibits β1 integrin clustering which is a prerequisite for focal adhesion formation and rearrangement of actin cytoskeleton in cell spreading process. Furthermore, lipid raft regulates β1 integrin clustering by modulating the interaction of the adaptor molecules and β1 integrin via concentrating and modifying the adaptor proteins. These results have extended our understanding of the relationship between lipid raft and β1 integrin clustering, and underscores the importance of lipid raft integrity in β1 integrin clustering and cancer cell migration.

2. Materials and methods

2.1. Cell culture

A375 cells were purchased from the cell bank of type culture collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS).

2.2. Antibodies and agents

4-Morpholineethanesulfonic acid (MES), MβCD, cholesterol oxidase (C8649), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, M2128), HRP-conjugated cholera toxin subunit B (CTXB, C3741), mAbs to phosphorytrosine (PY20), actin (AC-40), tubulin (T4026) and vinculin (V4505) were purchased from Sigma Aldrich. Human fibronectin, mAbs to αvβ3 integrin (LM609), β1 integrin (TDM29) and active β1 integrin (HUTS-4) were obtained from Millipore. mAb to active β3 integrin (APS) was a generous gift from Peter J. Newman (Medical College of Wisconsin, Milwaukee, USA). Nystatin (CAS 34786-70-4), mAbs to β1 integrin (J818) and flotillin-2 (8-6), and polyclonal antibodies to α-actinin (H-300), p-FAK (Tyr 397-R) and p-FAK (Tyr 925) were purchased from Santa Cruz Biotechnology. Polyclonal antibodies to FAK (Ab-925), paxillin (phospho-tyr31), β3 integrin (Ab-785) and paxillin (Ab-88) were purchased from Signalway Antibody. mAb to talin (MCA 725S) was purchased from Serotech. Polyclonal antibody to paxillin (Y-118) was purchased from Cell Signaling Technology. Rhodamine-conjugated phalloidin and Alexa Fluor® 488-conjugated CTXB were obtained from Molecular Probe. TRITC or FITC-conjugated goat anti-mouse IgG Ab, FITC-conjugated anti-rabbit IgG Ab and horseradish-peroxidase-conjugated secondary Abs were obtained from Jackson Immunoresearch Laboratories. ECL Plus Western blotting detection reagents (RPN2232) were purchased from GE Healthcare.

2.3. Flow cytometry

Cells (1 × 10⁶) were resuspended in DMEM with or without 5 mM MβCD at 37 °C. After 30 min, cells were fixed, incubated with 2 μg of isotype IgG or specific antibodies for 60 min, washed with PBS and then stained with FITC-conjugated secondary antibody. The labeled cells were washed again and then detected by a FACScan (Beckman-Couter, USA).

2.4. Small interference RNA (siRNA)

We used siRNA approaches to knockdown endogenous β1 and β3 integrins. The sense and antisense sequences of β3 integrin siRNA oligoribonucleotides were 5’-CAAGCCUGUGUUCACCAUAC-3’ and 5’-GUAUGUGACACAGGCUUGC-3’ (Monferran et al., 2008), and those of β1 integrin siRNA oligoribonucleotides were 5’-GCCCAUACUGGAAUUUG-3’ and 5’-CAAUUUCGACAAUGCGC-3’ (Cordes et al., 2006), respectively. SiRNA duplex oligoribonucleotides were synthesized by GenePharma (China). Transfection of siRNA duplexes was performed using the Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer’s instructions. Experiments were performed 48 h after transfection.

2.5. Cell adhesion and spreading assays

Cell suspension (200 μL/well, 5 × 10⁵ cells/ml) was added to glass coverslips coated with fibronectin (10 μg/ml) in 24-well flat bottom tissue culture plates. After incubation for 10 min at 37 °C, adherent cells were fixed. Five fields were imaged using phase-contrast microscope (Nikon, Japan). The number of adherent cells was counted and presented as attachment percentage (100% attachment corresponds to the attachment of untreated cells exposed to fibronectin). In cell spreading assays, A375 cells were allowed to spread for 10 min, 30 min, 60 min and 120 min, respectively. In blocking experiments, cells were preincubated with 10 mM EDTA or 10 μg/ml of blocking mAbS for 60 min at 22 °C and subjected to adhesion or spreading assay. In siRNA experiments, 48 h after transfection with β1 or β3 integrin siRNA oligoribonucleotides, the cells were collected and subjected to adhesion or spreading assay. To disrupt lipid raft, cells were preincubated with 5 mM MβCD for 30 min at 37 °C.

2.6. Immunofluorescence

Cells were incubated with or without 5 mM MβCD and allowed to spread for the indicated times prior to fixation. The fixed cells were permeabilised with 0.1% Triton X-100 for 3 min, blocked
with 3% BSA for 60 min and then stained with primary antibodies for 60 min at 22 °C, followed by secondary antibodies and/or Rhodamine-coupled phalloidin for 45 min at 22 °C. The cells were then washed for three times and mounted on the stage of a confocal microscope (Olympus, Japan). Images were collected and analyzed using the Olympus confocal software. For ganglioside GM1 labeling, cells were not permeabilized. The number of focal adhesion was counted using Image J software. The colocalization between GM1 and β1 integrin was calculated using Pearson-Spearmen correlation (PSC) plugin in Image J (French et al., 2008). A subselection as a region of interest was set up around the plasma membrane using the Selection Brush with a width of 20 pixels.

2.7. Membrane raft preparation and dot blotting

Membrane raft preparation was performed by density gradient centrifugation at 4 °C. Briefly, A375 cells (2 × 10⁷) were suspended in DMEM with or without 5 mM MJCD at 37 °C for 30 min, centrifugated and lysed by adding 1.2 ml of lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 50 mM sodium fluoride, 10 mM sodium pyrophosphate and 1 mM sodium vanadate. For membrane raft preparation of the spreading cells, cells with or without 5 mM MJCD treatment were added to 10 cm cell culture plate coated with fibronectin (10 μg/ml) at 37 °C for 30 min and then lysed on ice. The lysates were homogenized with 10 strokes in a Dounce homogenizer and then repeated passage through a 22-gauge needle (30 times). To generate the density gradients for centrifugation, the homogenate (1 ml) was mixed with an equal volume of 80% sucrose in MNE buffer (25 mM MES, pH 6.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 1 mM PMSF, 1 μg/μl of aprotinin), and then overlaid with 2 ml of 30% sucrose followed by 1 ml of 5% sucrose. The gradients were ultracentrifugated (200,000 × g at 4 °C for 18 h) using a Beckman MLCS50 rotor. Twelve fractions (400 μl/fraction) were obtained from the top to bottom. Each fraction (2 μl) was dot-blotted onto nitrocellulose membranes, and the membranes were blocked with 1% BSA in TBST for 60 min and then the lipid raft marker GM1 was detected using HRP-conjugated CTXB.

2.8. Immunoprecipitations

Cells were resuspended in DMEM with or without 5 mM MJCD at 37 °C. After 30 min, cells were lysed on ice using lysis buffer either after centrifugation or directly after spreading on fibronectin-coated (10 μg/ml) cell culture plate for 30 min. The lysates were subjected to centrifugation at 12,000 × g for 30 min. The supernatant was precleared with 20 μl of protein G-Sepharose beads (Millipore) at 4 °C for 3 h, and incubated with the indicated antibodies at 4 °C overnight prior to incubation with 30 μl of protein G-Sepharose beads at 4 °C for 3 h. The beads were washed three times with lysis buffer. Bound proteins were eluted by boiling the beads in SDS sample buffer for 10 min.

2.9. Western blotting

Briefly, lipid raft fractions, immunoprecipitates or lysates from A375 cells were separated on 10% SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% fat-free milk powder or 3% BSA for 45 min, and then incubated with the primary antibodies for 60 min prior to detection with secondary antibodies. Chemiluminescent detection was performed by using ECL Plus Western blotting reagents.

2.10. Transwell assay

Cell migration was assayed using transwell chamber inserts (polycarbonate membrane, 8 μm pore size, Costar). The underside of the membrane was coated with 10 μg/ml fibronectin for 90 min. Cells (2 × 10⁵) were resuspended in 200 μl of serum-free DMEM containing 5 mM MJCD or 10 μg/ml β1 integrin function-blocking antibody for 30 min at 37 °C, added to the upper surface of the membrane and incubated at 37 °C for 16 h. The adherent cells at the upper surface were mechanically removed, and membranes were fixed and stained with crystal violet. Then cells were imaged using an inverted microscope. Crystal violet was solubilized in acetic acid to measure absorbance (495 nm) with a microplate reader (Bio Tek Instruments, USA).

2.11. Wound healing assay

A375 cells were cultured just to confluence in 24-well culture plate coated with fibronectin and then scratch-wounded using a 10 μl pipette tip. The cells were washed twice with PBS, refed with fresh 2% FBS/DMEM with or without 5 mM MJCD and then incubated at 37 °C. Cells were then imaged under phase contrast microscope at 0 h, 12 h and 24 h, respectively. Area of the wound closure was calculated by T-Scratch software.

3. Results

3.1. The initial spreading of A375 cells on fibronectin is mainly β1 integrin-dependent

β1 and β3 integrins are thought to be the two most important integrins on malignant cells and confer different cell adhesive properties, particularly with respect to direct and random cell migration (Huveneers and Danen, 2009). Using FACs analysis, we confirmed the presence and constitutive activation of β1 and β3 integrins on A375 cell surface (Fig. 1A). To test the functional differences of the two molecules, we performed cell adhesion and spreading experiments. Results from adhesion experiments indicated that EDTA (an effective inhibitor of integrin-ligand binding), β3 integrin blocking, β1+β3 integrin blocking and β3 integrin knockdown reduced A375 cell adhesion on fibronectin to 7%, 26%, 14% and 36%, respectively. Whereas, the β1 integrin blocking and knockdown only reduced cell adhesion to 67% and 74%, respectively (Supplementary Fig. S1; Fig. 1B and C), suggesting that blocking or knockdown of β3 integrin, but not β1 integrin, can severely impair the adhesion of A375 cell on fibronectin. Next we investigated the roles of β1 and β3 integrins in cell spreading after adhesion. The results from spreading experiments showed that both control and β1 integrin blocking or knockdown cells on glass coverslips coated with fibronectin attached but remained rounded for the first 10 min. Interestingly, the control cells acquired ‘fried-egg’ and flattened shapes respectively at 30 min and 60 min, and by 120 min, almost all control cells exhibited more flattened shape, indicating that the spreading of control cells was time-dependent. In contrast, β1 integrin blocking cells exhibited abnormal spreading at 30 min, 60 min and 120 min, and was not flattened enough (Fig. 1D). Quantitative analysis showed that compared with control group, the number of spreading cells in β1 integrin blocking group was reduced by 2.2-fold, 2.3-fold, and 2.3-fold respectively at 30 min, 60 min and 120 min (Fig. 1E). Further analysis indicated that although spreading area at 10 min was almost indistinguishable for control cells and β1 integrin blocking cells, the difference between control and β1 integrin blocking group in spreading area at 30 min, 60 min and 120 min was statistically significant (P < 0.05, Fig. 1F). Similar to β1 blocking group cells, the number and area of spreading cells in β1 integrin knockdown group were also dramatically reduced (Fig. 1D–F). We also detected the effect of β3 integrin blocking or knockdown on A375 cell spreading. As shown in Fig. 1D–F, morphology changes similar to control cells were also observed in β3 integrin blocking or knockdown cells, although blocking or knockdown of β3 integrin led to a slight reduction in the spreading area of the cells. All these data indicate that β1 and β3 integrins play different roles in A375 cell adhesion and spreading, and that the initial spreading of A375 cells on fibronectin is mainly β1 integrin dependent.

3.2. Lipid raft integrity is required for β1 integrin-mediated initial spreading of A375 cells

To determine the roles of lipid raft in A375 cell adhesion and spreading on fibronectin, isolated lipid raft as described in Section 2, and explored the effects of different concentrations (1 mM, 2.5 mM and 5 mM) of MJCD on lipid raft integrity. We found that 5 mM MJCD treatment, but not others, resulted in the relocation of GM1, a lipid raft marker, to non-raft fractions from lipid raft fractions (Fig. 2A),
Fig. 1. β1 integrin is necessary for the initial spreading of A375 cells on fibronectin. (A) A375 cells were stained with specific mAbs for total β3 integrin, active β3 integrin, total β1 integrin and active β1 integrin or normal mouse IgG as negative control. Expression and activation of β1 and β3 integrins on A375 cell surface were determined by FACS analysis. (B) A375 cell adhesion experiments were performed as described in Section 2. Images of adherent cells were recorded by phase-contrast microscopy (10×). − represents negative control (coverslip coated without fibronectin), + represents positive control (coverslip coated with fibronectin). (C) Cells adhering to fibronectin in B were counted. *Significant differences in the number of adherent cells in positive control group versus another group (P<0.05). (D) Control cells, integrin blocking cells and integrin knockdown cells were used for spreading assays. Images of cell spreading at indicated time frames were recorded by phase-contrast microscopy (20×). Magnified views of the arrowed cells are shown in the inserts. (E) Quantitative analysis of the number of spreading cells in (D). (F) Quantitative analysis of spreading area of cells in (D) by Image J software. *P<0.05. Bars represent means ± SD from three independent experiments. Statistical analysis was performed using Student’s t-test.
indicating that 5 mM MβCD treatment effectively disrupted the lipid raft. We next examined the effect of lipid raft disruption on A375 cell adhesion and spreading. The results from adhesion experiments showed that the disruption of lipid raft did not severely impair the adhesion of A375 cells on fibronectin (Fig. 2B and C). Interestingly, the results from spreading experiments showed that the control cells exhibited normal shape-change behavior in a time-dependent manner as described in Fig. 1, but the MβCD-treated cells only displayed slight changes in their shape at 30 min, 60 min and 120 min (Fig. 2D). Quantitative analysis showed that MβCD treatment resulted in the reduction of spreading cell numbers by 2.2-fold, 2.2-fold and 2.6-fold respectively at 30 min, 60 min and 120 min when compared with the control cells (Fig. 2E). Further analysis indicated that the area of spreading cells at 10 min was almost similar between the control cells and the treated cells, whereas the differences at 30 min, 60 min and 120 min were statistically significant (*P<0.05, Fig. 2F). To exclude possible nonspecific effects of MβCD, we also tested the influence of lipid raft disruption on the adhesion and initial spreading of A375 cells on fibronectin by using nystatin (the cholesterol-sequestering drug) and cholesterol oxidase (the cholesterol-oxidizing drug). Similar to 5 mM MβCD treatment, 100 μg/ml nystatin or 2 U/ml cholesterol oxidase could effectively impair the initial spreading of A375 cells (Supplementary Fig. S2). All these data clearly indicate that lipid raft integrity is required for β1 integrin-mediated initial spreading of A375 cells.

3.3. Lipid raft disruption inhibits focal adhesion formation and rearrangement of actin cytoskeleton by preventing β1 integrin clustering

The binding of integrins to ECM mediates the focal adhesion formation, the dynamic rearrangement of actin cytoskeleton and the connection of the two structures, which are necessary for cell spreading and migration (Holly et al., 2000). Thus we next examined whether lipid raft regulates β1 integrin-mediated focal adhesion formation and actin cytoskeleton rearrangements. Using confocal microscopy, we found that at 10 min after cell adhesion to fibronectin, vinculin-containing focal adhesion was hardly detectable and F-actin was organized into a cortical ring in control, β3 or β1 integrin blocking cells (Fig. 3A, a1–a3; b1–b3; c1–c3). Focal adhesion

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**Fig. 2.** Lipid raft integrity is indispensable for β1 integrin-mediated A375 cell spreading on fibronectin. (A) Suspension A375 cells were incubated for 30 min at 37 °C with various concentrations of MβCD. Lipid raft fractions were prepared as described in Section 2. Twelve fractions or total lysate of equal volume (2 μl) were subjected to dot blotting analysis by using HRP-CTXB that binds to GM1. (B) A375 cell adhesion experiments were performed as described in Section 2. Images of adherent cells were recorded by phase-contrast microscopy (10×). Magnified fields of view are shown in the inserts. (C) Cells adhering to fibronectin in 8 were counted. (D) Cells with or without MβCD treatment were replated on the fibronectin coated glass coverslips, incubated at 37 °C for different times and then fixed. Images of spreading cells at indicated times were recorded by phase-contrast microscopy (20×). Magnified views of the arrowed cells representing different shape are shown in the inserts. (E) Quantitative analysis of the number of spreading cells. (F) Quantitative analysis of spreading cell area from control and MβCD-treated A375 cells by using Image J software. *P<0.05. Bars represent mean ± SD from three independent experiments. Statistical analysis was performed using Student’s t-test.
assembly was visualized at 30 min in control cells, which was mainly localized in the cell peripheral protrusions and colocalized with the finger-like actin fiber (Fig. 3A, e1–e3), and by 60 min, mature focal adhesion was found, which was localized to cell protrusions and colocalized with the tip of F-actin stress fiber (Fig. 3A, i1–i3). After 120 min, mature focal adhesion depicted a more elongated shape and connected to the end of F-actin stress fiber, which reflected further maturation of focal adhesion and the stability of cell spreading (Fig. 3A, m1–m3). Compared with the control cells, β1 integrin blocking cells did not exhibit significant change in the average number of focal adhesions although they displayed differences in focal adhesion shape at 30 min, 60 min and 120 min (Fig. 3A, f1–f3; j1–j3; n1–n3). However, most cells in β1 integrin blocking group and 5 mM MJCD-treated group displayed hardly detectable focal adhesion at 30 min, 60 min and 120 min (Fig. 3A, g1, k1 and o1; h1, l1, and p1). Furthermore, β1 integrin blocking cells showed irregular F-actin structure around cell periphery at indicated times (Fig. 3A, g2, k2 and o2), whereas 5 mM MJCD-treated cells maintained a cortical F-actin ring (Fig. 3A, h2, l2 and p2), suggesting that lipid raft may regulate the rearrangement of actin cytoskeleton largely (not solely) by modulating β1 integrin in A375 cells. Quantitative analysis showed that the average number of focal adhesion in β1 integrin blocking cells was dramatically decreased at 30 min, 60 min and 120 min compared with control cells, and 5 mM MJCD-treated cells presented even less focal adhesion number at the indicated time points (Fig. 3B). From these findings, we conclude that lipid raft is crucial for β1 integrin-mediated focal adhesion formation and rearrangement of actin cytoskeleton during the initial spreading of A375 cells.

Integrin clustering is an essential event in the early assembly of focal adhesion. It has also been shown that lipid raft has an active role in asymmetric redistribution of membrane proteins during cell polarization (Görgens et al., 2012; Viola and Gupta, 2007). Thus we hypothesized that the action of lipid raft in focal adhesion formation is carried out by regulating β1 integrin clustering. We plated cells with or without 5 mM MJCD treatment on glass coverslip coated with fibronectin for indicated time intervals, and then labeled β1 integrin and paxillin. As shown in Fig. 4, β1 integrin scattered evenly at control cell margins at 10 min (Fig. 4a1), distinct aggregates (clustering) were easily visible at 30 min (Fig. 4c1), and large clustering was observed around cell periphery at 60 min and 120 min (Fig. 4e1 and g1). However, the cells treated with 5 mM MJCD demonstrated an even distribution of β1 integrin around cell margins at time points of 10 min, 30 min, 60 min and 120 min (Fig. 4b1, d1, f1 and h1). Furthermore, paxillin was enriched in discrete foci at cell protrusions at 30 min, 60 min and 120 min (Fig. 4c2, e2 and g2) and exhibited obvious colocalization with β1 integrin clustering in control cells (Fig. 4c3, e3 and g3; c4, e4 and g4). Whereas 5 mM MJCD treated cells presented a more diffuse staining of paxillin (Fig. 4d2, f2 and h2) and no obvious colocalization of paxillin and β1 integrin (Fig. 4d3, f3

Fig. 3. Lipid raft disruption inhibits β1 integrin-mediated focal adhesion formation and rearrangement of actin cytoskeleton. (A) In serum-free medium, control, β3 integrin blocking, β1 integrin blocking or 5 mM MJCD-treated A375 cells were added to glass coverslips coated with fibronectin. After incubation at 37°C for indicated times, cells were fixed, permeabilized, then stained for vinculin (green), a focal adhesion marker, and F-actin (red). The images were obtained by confocal microscopy (60×). The merged images show the connection of focal adhesion and F-actin. Scale bar = 5 μm. (B) The average numbers of focal adhesion in control cells, β3 blocking cells, β1 blocking cells and 5 mM MJCD-treated cells were counted. *P<0.05. Bars represent means ± SD from three independent experiments. Statistical analysis was performed using Student’s t-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
and h3; d4, f4 and h4), suggesting that focal adhesion formation depends on lipid raft-regulated β1 integrin clustering. Taken together, all these results suggest that lipid raft disruption inhibits focal adhesion formation and rearrangement of actin cytoskeleton by preventing β1 integrin clustering.

3.4. Lipid raft is required for β1 integrin to recruit adaptor molecules

To further explore the relationship between lipid raft and β1 integrin clustering, we labeled GM1 and β1 integrin. After plating on fibronectin for 10 min, both control and 5 mM MβCD treated cells exhibited even distribution of GM1 and β1 integrin in the cell margins, whereas only the control cells showed obvious colocalization of GM1 and β1 integrin (Fig. 5A, a1–a4; b1–b4). After 30 min, lipid raft (GM1) aggregated at sites of membrane protrusion (Fig. 5A, c1), and β1 integrin effectively clustered (Fig. 5A, c2) and co-patched with GM1 in multiple cell extensions (Fig. 5A, c3 and c4). In contrast, 5 mM MβCD treatment dramatically inhibited protrusion formation and reduced the co-patching of β1 integrin clustering and GM1 (Fig. 5A, d1–d4). At 60 min and 120 min, lipid raft formed big patches within cell membrane protrusion and β1 integrin showed similar phenomenon (Fig. 5A, e1 and e2; g1 and g2). The two structures were colocalized in the membrane protrusion around the entire periphery of the control cells (Fig. 5A, e3 and e4; g3 and g4), but the disruption of lipid raft integrity by 5 mM MβCD treatment resulted in the inability of lipid raft patch formation and significantly prevented β1 integrin clustering (Fig. 5A, f1–f4; h1–h4). We also quantified colocalization between GM1 and β1 integrin by calculating the linear Pearson correlation coefficient (rP). As shown in Fig. 5B, the values of rP in cells treated with 5 mM MβCD for different time periods were significantly lower than that in control cells, indicating that lipid raft disruption reduces β1 integrin localization and clustering in lipid raft. All these results suggest that lipid raft integrity is a prerequisite for β1 integrin clustering.

At present, several adaptors that bind to β1 integrin cytoplasmic tail in suspension or spreading cells by interacting with lipids, have been determined to regulate β1 integrin clustering (Legate and Fässler, 2009). To investigate whether lipid raft is involved in the process and the possible mechanism, we detected the interaction of β1 integrin with talin, vinculin, FAK, paxillin and α-actinin, which are well correlated with β1 integrin clustering (Cruzel et al., 2005; Dibya et al., 2009; Humphries et al., 2007; Pichard et al., 2001; Vicente-Manzanares and Horwitz, 2011). The results from immunoprecipitation and Western blotting demonstrated that under suspension...
Fig. 6. Lipid raft is required for β1 integrin to recruit the adaptor molecules. (A) Total cell lysate was prepared under suspension and spreading conditions, and subjected to coimmunoprecipitation using β1 integrin antibody as described in Section 2. Samples were separated by SDS–PAGE, transferred onto Immobilon TM–NC membranes and then Western blotting was performed. (B) A375 cells in suspension were treated with or without 5 mM MβCD for 30 min. The cells were lysed and ultracentrifuged prior to Western blotting experiments. (C) A375 cells with or without 5 mM MβCD treatment were replated on fibronectin. After 30 min, cells were lysed, ultracentrifuged and Western blotting experiments were performed. (D) After 5 mM MβCD treatment, A375 cells in suspension were directly lysed or spread on fibronectin for 30 min and then lysed. Samples were subjected to SDS–PAGE and assessed by Western blotting using antibody PY20 against phosphorysorosine. (E) A375 cells with or without 5 mM MβCD treatment were replated on fibronectin for 30 min and then lysed. Phosphorylation of FAK at tyr-397 and 925, and phosphorylation of paxillin at tyr-31 and 118 were assessed by Western blotting.

condition, talin, vinculin and α-actinin were found in β1 integrin immunoprecipitated complex and 5 mM MβCD treatment resulted in the dissociation of the three proteins from β1 integrin (Fig. 6A, left). Under spreading condition, in addition to the three proteins, FAK and paxillin were also found in β1 integrin immunoprecipitated complex. 5 mM MβCD treatment dramatically decreased these associations (Fig. 6A, right). These data suggest that lipid raft integrity is necessary for β1 integrin to recruit the adaptors.

Lipid raft is thought to be a scaffold and signaling platform. So we speculated that it might regulate the interaction between the adaptors and β1 integrin cytoplasmic tail by spatially concentrating and temporally modifying the adaptors. Thus we next prepared lipid raft fractions under suspension or spreading condition and examined the association of β1 integrin and the adaptors in lipid raft fractions. As shown in Fig. 6B and C, flotillin-2 (a protein known to reside in lipid raft fractions), but not tubulin (a protein known to reside in non-lipid raft fractions), was found in lipid raft fractions. 5 mM MβCD treatment resulted in the redistribution of flotillin-2 from lipid raft fractions to non-lipid raft fractions, indicating that lipid raft fractions were isolated successfully. We found that β1 integrin, α-actinin, talin and vinculin resided together in lipid raft fractions under suspension and spreading conditions, and 5 mM MβCD treatment resulted in their delocalization from lipid raft fractions (Fig. 6B and C). However, paxillin and FAK were shown to reside in lipid raft fractions only in spreading condition, and 5 mM MβCD treatment caused their dissociation from lipid raft fractions (Fig. 6B and C). These results imply that intact lipid raft provides a possible space for the close interaction of the adaptors and β1 integrin.

We next detected the influence of 5 mM MβCD treatment on the phosphorylation of the adaptors by Western blotting. We found that under suspension condition, vinculin, α-actinin, FAK, talin and paxillin had no obvious phosphorylation in both control and treated cells. Interestingly, in spreading condition, FAK and paxillin, not vinculin, α-actinin and talin, were heavily phosphorylated in control cells, and the phosphorylation levels of the two proteins were dramatically reduced in 5 mM MβCD-treated cells (Fig. 6D). Previous reports showed that phosphorylation of FAK at tyr-397 and 925, and phosphorylation of paxillin at tyr-31 and 118 are dependent on the interaction of integrin and fibronectin (Deakin and Turner, 2008; Meng et al., 2009). Consistent with these reports, our results showed that after 30 min of spreading, control cells presented strong phosphorylation at tyr-397 and 925 of FAK, and tyr-31 and 118 of paxillin. However, 5 mM MβCD-treated cells exhibited near-absent phosphorylation at these sites. These results suggest that intact lipid raft could regulate the phosphorylation of some adaptors, which may facilitate their recruitment to β1 integrin. Taken together, all these data imply that lipid raft can concentrate and modify some adaptor molecules, especially under spreading condition, which is important for integrin to recruit them.

3.5. Lipid raft disruption attenuates A375 cell migration

It has been reported that the disruption of lipid raft by MβCD results in inability of intestinal epithelial cells and non-small cell lung cancer cells to migrate (Jeon et al., 2010; Vassileva et al., 2008). Based on our results, we were interested
to investigate whether lipid raft is involved in A375 cell migration. First, we
determined the cytotoxic effects of different concentrations of MβCD by MTT
assay. As shown in Table S1, 5 mM MβCD did not exhibit dramatic toxicity toward
A375 cells at 12 h, 16 h and 24 h. Next, we performed the transwell and wound
healing experiments. The results from transwell experiments showed that control
cells migrated onto the undersurface of the membrane within 16 h, but β1 integrin
blocking or 5 mM MβCD treatment significantly decreased the cell migration
(Fig. 7A). The relative number of cells transmigrated onto the undersurface of the
membrane was also determined by the solubilizing crystal violet in acetic acid to
measure the absorbance value. As shown in Fig. 7B, compared with the control
cells, the migration of β1 integrin blocking cells and 5 mM MβCD-treated cells was
reduced to approximately 98% and 27%, respectively. Consistent with above results,
our wound healing experiments showed that control A375 cells briskly migrated
into the wound area and almost closed the wound within 24 h, whereas 5 mM
MβCD treatment caused the reduction in cell migration (Fig. 7C). Quantitative
analysis showed that, at 12 h and 24 h, migration of control cells reduced the
wound area to 33% and 9% respectively, but migration of 5 mM MβCD-treated cells
only reduced the wound area to 91% and 70% respectively (Fig. 7D). All these data
clearly show that the disruption of lipid raft attenuates the ability of A375 cell
migration.

4. Discussion

Previous researches showed that integrin-mediated cell adhesion
and spreading are modulated by multiple factors such as ECM,
cellular signal proteins and membrane lipids (Cavalcanti-Adam
et al., 2007; Cluzel et al., 2005; Pande, 2000). Recently, lipid raft,
a microdomain of plasma membrane, has received considerable
attention in various cellular processes, including cell migration
(Jeon et al., 2010; Lee et al., 2008; Park et al., 2009; Yanagisawa et al.,
2004). Thus, it is extremely necessary to investigate whether lipid
raft is involved in integrin-mediated cell adhesion and spreading.
Previous studies showed that lipid raft disruption by MβCD inhibits
cell adhesion and stimulates cell detachment from ECM (Park et al.,
2009; Yanagisawa et al., 2004). However, we found that MβCD
treatment only slightly prevented A375 cell adhesion on fibronectin
(Fig. 2B and C). This may be due to the fact that after cholesterol
extraction, the two integrins (β1 and β3) expression and activity
level on A375 cell surface still remained high (Supplementary Fig.
S3). Using flow adhesion assays, we further confirmed that MβCD
treatment had no significant effect on the adhesion of A375 cells
even at the flow rates of 8 cm/min (Supplementary Fig. S4A and
B). Interestingly, our results indicated that the initial spreading of
A375 cells on fibronectin is mainly β1 integrin-dependent and
MβCD treatment dramatically inhibited the process (Fig. 2). This
prompted us to consider that lipid raft may play an important role in
β1 integrin-mediated initial spreading of A375 cells on fibronectin.

Cell spreading requires integrin-mediated focal adhesion for-
mation and actin cytoskeleton rearrangement. Our confocal
microscopy data indicated that both β1 integrin blocking and
MβCD treatment effectively decreased focal adhesion formation
and inhibited actin cytoskeleton rearrangement during cell spreading
(Fig. 3A and B). Also, our study indicated that MβCD treatment
resulted in a dramatic decrease of β1 integrin clustering, the
fundamental event underlying focal adhesion formation (Fig. 4),
and inhibited the copatching of β1 integrin and lipid raft (Fig. 5).
This suggests that lipid raft may regulate focal adhesion formation
and rearrangement of actin cytoskeleton through modulating β1 inte-
grin clustering. Pande G. proposed that membrane lipid molecules
might be crucial for the formation of focal adhesion (Pande, 2000).
However, little is currently known about the role of lipid raft in focal
adhesion formation. Our study provides a possible explanation for
the relationship between lipid raft and focal adhesion formation.
That is, lipid raft may regulate early focal adhesion formation by
modulating β1 integrin clustering in cell spreading.

It has been reported that several adaptor molecules can bind
to the cytoplasmic tails of β1 integrin and regulate β1 integrin
clustering (Cluzel et al., 2005; Dibya et al., 2009; Humphries et al.,
2007; Ithychanda et al., 2009). Interestingly, through phosphoty-
rosine binding domain–lignands interaction, some adaptors, such as
talin, generate long range conformational changes that increase
their affinity for the β integrin subunit (Legate and Fassler, 2009).
Moreover, Phosphatidylinositol 4, 5-Diphosphate, cholesterol and
ganglioside GM3 of lipid raft fractions have also been shown to be
involved in the regulation of integrins (Cluzel et al., 2005; Ohkawa
et al., 2010; Pande, 2000). These researches imply that lipid raft
may regulate the interaction between adaptor molecules and β1 inte-
grin. We selected some of these important adaptors to test this
hypothesis. Our results showed that in suspension cells, talin, vin-
culin and α-actinin, not FAK and paxillin, were found in β1 integrin
immunoprecipitated complex, whereas MβCD treatment caused
the adaptors to break away from the complex (Fig. 6A). In spreading
cells, FAK and paxillin were present in the complex together with
talin, vinculin and α-actinin. MβCD treatment led to the adaptors
dissociation from the complex. All these data suggest that intact
lipid raft causes the recruitment of some adaptors to β1 integrin,
especially under spreading condition, which may be important for
β1 integrin clustering.

Lipid raft possesses two of the most important properties,
(1) concentrating or excluding some proteins to facilitate spatial
interaction of protein–protein or protein–lipid; (2) modifying the
phosphorylation state of some recruited proteins by local kinase and phosphatase to cause temporal increase or decrease of downstream signal transduction (Golub et al., 2004; Simons and Toomre, 2000). Thus we wondered whether lipid raft can concentrate and modify the adaptors. Our results demonstrated that vinculin, talin and α-actinin coexisted with β1 integrin in the lipid raft both in suspension and spreading cells, but FAK and paxillin were present in the lipid raft only in spreading cells. Following MBCD treatment, both suspension and spreading cells displayed the absence of vinculin, talin, α-actinin, FAK and paxillin in lipid raft fractions (Fig. 6B and C), suggesting that intact lipid raft is necessary for the spatial interaction of adaptors and β1 integrin. The phosphorylation of some adaptors, such as tensin, paxillin and FAK, play an important role in integrin function and are tightly linked to integrin-mediated cell spreading and migration (Bockholt and Burridge, 1993). Our results indicated that paxillin and FAK, not talin, vinculin and α-actinin, can be heavily phosphorylated in spreading cells, but not in suspension cells. Interestingly, we found that lipid raft disruption-induced spreading-deficient cells showed significant decrease in phosphorylation of paxillin and FAK (Fig. 6D). Since it has been reported that the phosphorylation of FAK and paxillin is important for the two proteins to target to plasma membrane and interact with β1 integrin (Chen et al., 2000), our results raise the possibility that lipid raft regulates the interaction of β1 integrin with FAK and paxillin by phosphorylating the two proteins.

Consistent with previous researches (Jeon et al., 2010; Vassilieva et al., 2008), we indicated that the disruption of lipid raft dramatically reduced A375 cell migration (Fig. 7), suggesting that lipid raft-integrin interaction may be tightly linked to cancer cell migration. Interestingly, lipid raft has also been documented to regulate epithelial-mesenchymal transition (EMT) and matrix metalloproteases (MMPs) activity which are two crucial events in cancer cell migration and invasion process. For example, lipid raft is essential for podoplanin-mediated EMT in Madin-Darby canine kidney cells and TGF-β-induced EMT in the human keratinocyte HaCaT cells (Fernández-Muñoz et al., 2011; Zuo and Chen, 2009); lipid raft is also a key regulator of the location and activity of MMPs in various cancer cells (Mira et al., 2004; Park et al., 2012; Raghlu et al., 2010). Investigating whether lipid raft-integrin interaction is involved in the regulation of EMT and MMP activity of cancer cells and the potential mechanisms will be an interesting and challenging issue.

Cell spreading, an essential step in cell migration on ECM, requires integrin clustering. Although researchers have paid much attention to answering the question of how integrin clustering occurs, it is still not fully understood. Our study reveals that lipid raft plays a critical role in mediating focal adhesion formation and actin cytoskeleton rearrangement by regulating β1 integrin clustering. Lipid raft modulates β1 integrin clustering probably by concentrating and modifying the adaptors to facilitate their binding to β1 integrin. Our findings provide a new insight for the relationship between lipid raft and the regulation of integrins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.04.031.

References


Welf ES, Naik UP, Ogunnaikie BA. A spatial model for integrin clustering as a result of feedback between integrin activation and integrin binding. Biophysical Journal 2012;103:1379–89.

