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Activated Integrin VLA-4 Localizes to the Lamellipodia and Mediates T Cell Migration on VCAM-1

Young-Min Hyun,* Hung-Li Chung,† James L. McGrath,† Richard E. Waugh,† and Minsoo Kim²

Lymphocyte migration from blood into lymphoid tissues or to sites of inflammation occurs through interactions between cell surface integrins and their ligands expressed on the vascular endothelium and the extracellular matrix. VLA-4 (α4β1) is a key integrin in the effective trafficking of lymphocytes. Although it has been well established that integrins undergo functionally significant conformational changes to mediate cell adhesion, there is no mechanistic information that explains how these are dynamically and spatially regulated during lymphocyte polarization and migration. Using dynamic fluorescence resonance energy transfer analysis of a novel VLA-4 FRET sensor under total internal reflection fluorescence microscopy, we show that VLA-4 activation localizes to the lamellipodium in living cells. During T cell migration on VCAM-1, VLA-4 activation concurs with spatial redistribution of chemokine receptor and active Rap1 at the leading edge. Selective inhibition of the activated VLA-4 at the leading edge with a small molecule inhibitor is sufficient to block T cell migration. These data suggest that a subpopulation of activated VLA-4 is mainly localized to the leading edge of polarized human T cells and is critical for T cell migration on VCAM-1. The Journal of Immunology, 2009, 183: 359–369.

In response to extracellular signals such as chemokines and chemotactic cytokines, migrating cells show a distinct polarized morphology with a flattened leading edge followed by a trailing edge or uropod. Cell migration consists of the iterative cyclic process of cell polarization by projection of lamellipodia or filopodia at the leading edge, adhesion to the extracellular matrix or adjacent cells, and retraction at the rear of the cell (1, 2). During lymphocyte locomotion (3) and transendothelial migration (4), cellular lamellipodia bind to the endothelial cell surface through integrin-ligand interactions and assemble adhesion complexes at the front to generate contractile force (5, 6).

Integrin VLA-4 is a critical component in lymphocyte differentiation and homing, as well as in tissue-specific migration during inflammation. VLA-4 is also involved in the pathogenesis of autoimmune diseases and chronic inflammations such as multiple sclerosis (7), Crohn’s disease (8), asthma (9), stroke (10), rheumatoid arthritis (11), and inflammatory bowel disease (12). Therefore VLA-4 has been an important therapeutic target to treat inflammatory diseases. For instance, a mAb to the α4 subunit that blocks the binding of VLA-4 to VCAM-1 on brain-infiltrating T411 cells has been used as a monotherapy treatment of multiple sclerosis (13, 14).

Although it has been well established that conformational changes regulate integrin affinity to its ligands, it is still not known how these are dynamically and spatially regulated during lymphocyte polarization and migration. In this study, we establish an experimental system utilizing dynamic fluorescence resonance energy transfer (FRET)³ measurement in total internal reflection fluorescence (TIRF) microscopy to selectively monitor integrin activation at the plasma membrane proximal cell-substrate contact zone. TIRF microscopy exploits an optical phenomenon called “evanescent wave” that penetrates ~80 nm into the cell. The plasma membrane of a mammalian cell has a thickness of 5–10 nm and the size of integrin extracellular domains is <20–15 nm in the extended form. This translates to near exclusive illumination of integrin activation proximal to the cell membrane by TIRF microscopy. In this study, our data demonstrate that activated VLA-4 localizes to the leading edge at the lamellipodium during T cell migration and is spatially overlapped with localized distribution of chemokine receptor CXCR4 and Rap1 activation. In addition, we show that selective inhibition of the activated VLA-4 at the leading edge is sufficient to block T cell migration on VCAM-1.

Materials and Methods

Reagents and Abs

Chinese hamster ovary cell lines expressing human VCAM-1-Ig fusion protein was a generous gift from M. Ginsberg (University of California, San Diego, CA). The protein was purified from Chinese hamster ovary cell supernatants (15). A small molecule VLA-4 inhibitor BIO1211 was synthesized as the form conjugated with FITC and 4-(N-2-methylphenyl)ureido-phenylacetyl-LDVPAAK(FITC)-OH (Commonwealth Biotechnologies). Pertussis toxin (PTX) was from Calbiochem. Anti-α4 Ab 9F10 (eBiosience), anti-β1, Ab M106 (Santa Cruz Biotechnology), anti-β1 Ab B44 and anti-β1 Ab P4C10 (Chemicon International), anti-GFP Ab (Molecular Probes) and mAbs TS2/16 and KIM127 (prepared from hybridoma American Type Culture Collection) and anti-CXCR4 Ab (BioLegend) were used.

Cell culture and transfection

T cells were prepared from human PBMC (16). GD25 cells and 293T cells were cultured in DMEM (HyClone) containing 10% FBS and 100 U/ml

³Abbreviations used in this paper: FRET, fluorescence resonance energy transfer; TIRF, total internal reflection fluorescence; PLL, poly-l-lysine; LIBS, ligand-induced binding site; wt, wild type; PTX, pertussis toxin; DIC, differential interference contrast; PRA, protein kinase A; RIAM, Rap1-GTP-interacting molecule.

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penicillin and 100 µg/ml streptomycin (Life Technologies). For transient transfections of T cells and GD25 cells, Amaxa nucleofector kits were used. 293T cells were transfected with α4-mCFP or β1-mYFP by Lipo-ductamine (Invitrogen). Stable transfection of GD25 cells with wild-type (wt) α4 and β1 subunits of VLA-4 was performed using electroporation. Anti-α4 Ab 9F10 was used for selection of VLA-4-expressing cells during cell sortings.

DNA plasmids and constructs

The Rap1 FRET sensor was a generous gift from M. Matsuda (Osaka University, Osaka, Japan) and is a genetically encoded single-chain fusion protein of Rap1 and its effecter Raf, as well as CFP and YFP to visualize cell sortings.

The PCR product was the mYFP region with a 15-aa linker of

\[ \text{AGGACCTGTCGCCACCATG-3} \]

stream primer 5

\[ \text{GPVPTAAPEKGPVAT}. \]

The PCR product was the mYFP region with a 15-aa linker of

\[ \text{cell sortings.} \]

more PCR extension for mCFP was conducted using

\[ \text{Age I/H11032 CAACCAATTTT-3} \]

in the wt

\[ \text{5'-TATATACCGGTTCCAACTGCAGCTCCGGAGAA TATATACTCGAGGCCACCATGTTCCCC-3' containing the Sac II site. product} \]

were grown in delta T dishes overnight. After treatment with 1 mM MnCl2, 105 parental GD25, wt VLA-4/GD25, or VLA-4 FRET sensor/GD25 cells were added to the delta T dish. DIC images were acquired every 10 s under a ×10 objective lens at 37°C. In the case of VLA-4 FRET sensor/GD25 cells, DIC images were acquired every 10 s and CFP and YFP images every 5 min using a ×60 objective lens.

Soluble VCA-M-1-binding assay

Parental GD25, wt VLA-4/GD25, and VLA-4 FRET sensor/GD25 cells were grown in delta T dishes overnight. After treatment with 1 mM MnCl2 for 1 h to fully activate VLA-4, cells were incubated with human VCAM-1-Ig fusion protein (100 µg/ml) at 37°C for 15 min. Cells were fixed and subsequently labeled with Alexa Fluor 647-goat anti-human IgG (Invitrogen) for 30 min. After a thorough washing, DIC, Alexa Fluor 647, CFP, and YFP images were acquired using a ×60 objective lens.

Photobleaching FRET analysis

Photobleaching FRET imaging was conducted with a Nikon Eclipse TE2000-E microscope using a ×60 objective lens coupled to a Cool- SNAP HQ charge-coupled device camera (Roper Scientific). All image processing was performed with Nikon NIS software. α4-mCFP and β1-mYFP were transiently expressed in GD25 cells. Transfected cells were grown overnight on delta T dishes. Cells were incubated for 10 min at 37°C in a FCS2 live-cell imaging chamber (Biophotex). In the case of FRET measurement of activated VLA-4, MnCl2 and anti-β1-activating Ab TSA2/16 were individually or simultaneously added to transfected cells in the chamber for a final concentration of 1 mM MnCl2 and 10 ng/ml TSA2/16. Cells were incubated at 37°C for 10 min before taking CFP and YFP images. Both CFP and YFP images were acquired before and after acceptor (YFP) photobleaching without a ND filter for 3 min. For fluorescence image acquisition, cells were exposed for 0.5 s with an one-eighth ND filter and 2 × 2 binning using a 120-W mercury arc lamp of the X-Cite 120 XL system (EXFO Photonic Solutions). For pre- and postphotobleaching image sets of CFP, the same cell membrane region of interest was selected and the background was subtracted. Then, FRET efficiency (E) was calculated as

\[ E = 1 - (\frac{F_{\text{CFP}}(d_{\text{pre}})}{F_{\text{CFP}}(d_{\text{post}})})^{20}, \]

where \( F_{\text{CFP}}(d_{\text{pre}}) \) and \( F_{\text{CFP}}(d_{\text{post}}) \) are the mean CFP emission intensity of pre- and postphotobleaching.

Dynamic TIRF/FRET analysis

Dynamic FRET imaging under TIRF microscopy was conducted with a dual-view image splitter (Photometrics) and CFP/YFP dual-band filter set (Chroma) by the sensitized emission method (21). Data analysis was performed with the Autoquant Imaging algorithm of AutoDeblur. The system

\[ \beta_1 \text{ integrin activation assay} \]

The conformation state of VLA-4 was determined on T cells and wt VLA-4/GD25 cells. For T cells, 2 × 106 cells were incubated on coverslips coated with VCAM-1 plus CXCL12 for 30 min at 37°C. For wt VLA-4/GD25 cells, cells were grown on coverslips coated with VCAM-1 or poly-l-lysine (PLL) overnight. After incubation, T cells and GD25 cells were fixed with 3.7% formaldehyde in PBS for 10 min. After blocking with 5% nonfat dry milk (Bio-Rad) for 30 min, cells were labeled simultaneously with 5 µg/ml mouse monoclonal anti-β1 integrin ligand-induced binding site (L15B) Ab, B44, and 2 µg/ml nonfunctional rabit polyclonal anti-β3 integrin M106 for 1 h. The secondary Ab labeling was performed with Cy5-goat anti-mouse IgG (Zymed Laboratories) and Alexa Fluor 488-donkey anti-rabbit IgG (H + L) (Invitrogen) for 1 h. Coverslips were mounted onto glass slides and sealed. Subsequently, TIRF images were acquired to Cy5 for B44 labeling and FITC for M106 labeling.

Cell migration assay

Delta T dishes (Fisher Scientific) were coated with 20 µg/ml protein A (Zymed Laboratories) and 1 µg/ml CCL12 (R&D Systems) in PBS buffer (pH 9) overnight at 4°C and then incubated for 4 h at room temperature. They were then coated with 100 µg/ml human VCAM-1-Ig fusion protein for 2 h at room temperature. Two × 106 T cells were suspended in 1 ml of L15 medium with 2 mg/ml glucose and allowed to adhere to the delta T dish coated with VCA-M-1 and CCL12. Differential interference contrast (DIC) images were acquired every 10 s under a ×10 objective lens at 37°C. To investigate the effect of VLA-4 blockade during T cell migration on VCAM-1, 200 or 400 mM BOD121 was added and then DIC images were acquired.

Cell spreading assay

VCAM-1 was coated on delta T dishes as described above. Approximately 2 × 106 parental GD25, wt VLA-4/GD25, or VLA-4 FRET sensor/GD25 cells were added to the delta T dish. DIC images were acquired every 10 s under a ×20 objective lens at 37°C. In the case of VLA-4 FRET sensor/GD25 cells, DIC images were acquired every 10 s and CFP and YFP images every 5 min using a ×60 objective lens.

Flow cytometry

Two × 106 GD25 cells expressing wt α4/β1 or α4-mCFP/β1-mYFP were incubated with 5 µg/ml anti-α4 Ab 9F10 and anti-β1 PAMC10 for 30 min. Cells were then incubated with PE goat anti-mouse IgG Ab (BioLegend) for 30 min. Mean fluorescence intensity was determined using a BD FACScalibur system and FlowJo software (Tree Star). Cell surface labeling for 30 min. Mean fluorescence intensity was determined using a BD FlowCalibur system and FlowJo software (Tree Star). Cell surface label-

\[ \text{for 30 min. Mean fluorescence intensity was determined using a BD} \]

\[ \text{were incubated with 5 µg/ml anti-α4 Ab 9F10 and anti-β1 PAMC10 for 30 min. Cells} \]

\[ \text{cells were first fixed and subsequently labeled with Alexa Fluor 647-goat anti-human IgG (Invitrogen) for 30 min. After a thorough washing, DIC, Alexa Fluor 647, CFP, and YFP images were acquired using a ×60 objective lens.} \]
was equipped with a perfect focus unit (Nikon) and a vibration isolation system (Technical Manufacturing) to acquire time-lapse images without drift. Twenty-four hours after transfection of the Rap1 FRET sensor and the VLA-4 FRET sensor into T cells and GD25 cells, respectively, cells were resuspended in L15 medium with 2 mg/ml glucose. One milliliter of transfected cells was placed in a delta T dish coated with VCAM-1 plus CXCL12 and equilibrated for 10 min at 37°C. Then each image of CFP, YFP, and DIC of cells was acquired for 0.5 s every 10 s with 2 x 2 binning through a x 100 oil 1.49 aperture immersion objective lens without a ND filter.

**BIO1211-binding assay**

One x 10^6 T cells were transferred into 96-well plates and then incubated at 37°C for 10 min. In case T cells are activated, 1 mM MnCl2 was added before a 10-min incubation. Subsequently, cells were incubated with various concentrations of BIO1211 at 37°C for 10 min and then fixed. The fluorescent intensity of FITC of each sample was measured by flow cytometry. For imaging BIO1211 binding to T cells during migration, 2 x 10^5 T cells were suspended in 1 ml of L15 medium with 2 mg/ml glucose and allowed to adhere to the delta T dish coated with 5 μg/ml human ICAM-1-Ig fusion protein (R&D Systems) and 1 μg/ml CXCL12. Following a 4 nM BIO1211 addition onto T cells on the delta T dish, DIC and FITC images were acquired every 10 s under a x 60 objective lens at 37°C. To investigate how PTX affects BIO1211 binding to T cells on ICAM-1 and CXCL12, T cells were preincubated with 100 ng/ml PTX for 4 h. The cells were incubated with 4 nM BIO1211 for 5 min in the presence or absence of 1 mM MnCl2 and then fixed. DIC and FITC images were acquired.

**Cell migration tracking**

Time-lapse images were acquired under DIC microscopy and binarized into black and white with a user-defined threshold of pixel intensity. The corresponding centroids of each cell in consecutive binarized images were connected to form the migration trajectory. The cells tracked were separated into two populations: migrating and firmly adherent. Firmly adherent cells are defined based on a user-defined threshold of cell displacement (length between initial and final position). The tracking and the identity of the migrating and firmly adherent cells was verified with tracking videos superimposed with the respective trajectories of the migrating and firmly adherent cells. The ratio of migrating cells to the total number of cells tracked was reported as a metric for effective migration across different experimental treatments.

**CXCR4 staining of migrating T cells**

For T cell migration, cells were placed on a delta T dish coated with ICAM-1 (or VCAM-1) and CXCL12 as described above. For CXCR4 staining, migrating T cells were fixed and cells were stained with anti-CXCR4 Ab and then Cy5-labeled secondary Ab. DIC and Cy5 images were acquired under a x 60 objective lens. For both CXCR4 and BIO1211 staining, cells were incubated with 4 nM FITC-BIO1211 for 5 min on ICAM-1 plus CXCL12 before fixation. The cells were stained with anti-CXCR4 Ab as described above. DIC, Cy5, and FITC images were acquired under a x 100 objective lens.

**Data analysis**

Data are presented as mean ± SEM. Statistical significance was computed using one-way ANOVA and pairwise comparisons were performed with the Student t test. Values of p are indicated in the figures or figure legends.

**Results**

**VLA-4 mediates T cell migration**

To investigate VLA-4 activation during T cell migration on VCAM-1, human primary T cells were allowed to migrate on VCAM-1 coated with or without CXCL12. T cells formed lamellipodia at the leading edge and migrated on VCAM-1 plus CXCL12 (Fig. 1A, left panel, and supplemental video 1). In contrast, cells on VCAM-1 alone or PLL plus CXCL12 did not form lamellipodia or migrate (Fig. 1, A, middle and right panels, and supplemental video 1). The necessity of VLA-4 for T cell migration on VCAM-1 was further confirmed in the presence of a highly specific VLA-4 inhibitor (see Fig. 5).

To visualize the activation pattern of the βι subunit of VLA-4 at the interaction between T cell and VCAM-1 during migration, TIRF imaging was performed by labeling βι integrins with a conformation-independent anti-βι Ab, M106, and an anti-βι integrin LIBS Ab, B44. LIBS Ab B44 detects both ligand- and unbound active βι integrin (22). Therefore, the Ab was used as a reporter to recognize βι integrin functionality. In TIRF images, M106 labeling was distributed evenly across the region of contact between migrating T cells and immobilized VCAM-1, but B44 labeling was concentrated at the front of the cells (Fig. 1, B and C). Predominant labeling of B44 at the anterior of the migrating T cells indicates that active βι integrins are localized at the anterior, while βι integrins at the middle and posterior exist primarily in a basal conformation during cell migration on VCAM-1 (Fig. 1C).

In addition to leukocytes, several other cell types also express VLA-4, such as myocytes, cardiac, placental, and fibroblastic cells (23). GD25 is a mouse fibroblastic cell line lacking the integrin βι subunit and does not express the integrin αι subunit (24). To study specific and dynamic VLA-4 activation in live GD25 cells, we stably expressed human VLA-4 in GD25 cells (wt VLA-4/GD25 cells). wt VLA-4/GD25 cells spread rapidly on VCAM-1 forming lamellipodial protrusions (Fig. 1D and supplemental video 2). TIRF imaging of wt VLA-4/GD25 cells stained by both M106 and B44 Abs showed that βι integrin is distributed over the entire cell surface in the presence or absence of VCAM-1, but active βι integrin exists exclusively at the lamellipodium on VCAM-1 (Fig. 1E). These data suggest that VLA-4 is activated at the lamellipodia of spreading cells on VCAM-1 and becomes inactive from the cell edge inward.

**Development of VLA-4 FRET sensor**

Due to the lack of proper biological tools to study VLA-4 activation in living cells, we designed a novel VLA-4 FRET sensor based on the integrin activation model (25) that detects the conformational state of VLA-4 in living cells by reporting the relative distance between the cytoplasmic tails of the αι and βι subunits (Fig. 2A) (26). The VLA-4 FRET sensor was optimized by testing FRET efficiency among four construct pairs designed initially with different linker lengths between CFP/YFP and αι/βι integrin subunits. Constructs containing amino acid linkers between αι and mCFP and between βι and mYFP of 12 and 5 residues, respectively, showed the highest FRET under basal conditions at the acceptor intensity range between 100 and 400 arbitrary fluorescence units in the membrane and were used for most subsequent experiments (Fig. 2B). To investigate whether clustering of neighboring VLA-4 affects FRET efficiency between αι and βι subunits, we transiently transfected αι-mCFP, αι-mYFP, and wt βι into GD25 cells. In the same acceptor intensity range of 100–400, intersubunit FRET efficiency of the VLA-4 FRET sensor (12- to 5-aa linker pair) was 0.21 (Fig. 2B), whereas FRET efficiency of neighboring αι-mCFP/wt βι and αι-mYFP/wt βι was 0.05 (the highlighted area in Fig. 2C). The results demonstrate that the energy transfer of the VLA-4 FRET sensor occurs mainly through intersubunit FRET of the individual heterodimers. Expression of the VLA-4 FRET sensor was confirmed in GD25 cells (VLA-4 FRET sensor/GD25 cells) with flow cytometry (Fig. 2D). Immunoblotting with polyclonal anti-GFP Ab for CFP/YFP confirmed appropriate molecular weights of αι-mCFP and βι-mYFP subunits of the VLA-4 FRET sensor in 293T cells (Fig. 2E). To verify the correlation between FRET efficiency and conformational state of VLA-4, the FRET efficiency in VLA-4 FRET sensor/GD25 cells treated with Mn2+ and/or βι integrin-activating Ab, TS2/16 (27), was determined. Compared with untreated cells, FRET efficiency in Mn2+ - or TS2/16- treated cells was significantly decreased by

4 The online version of this article contains supplemental material.
>50%, and the efficiency in cells treated with both Mn\(^{2+}\) and TS2/16 was decreased by 70% (Fig. 2F). Unlike LFA-1 (26), we observed a decrease in FRET efficiency for the VLA-4 FRET sensor in response to Mn\(^{2+}\) treatment (Fig. 2F). Several integrins, such as LFA-1 and Mac-1, have an \(\alpha\) domain which is the ligand binding site, whereas other integrins, such as VLA-4 and \(\alpha_\beta_5\), that lack an \(\alpha\) domain bind ligand at the \(\beta\)-like domain (25). Thus, our data suggest that the effect of Mn\(^{2+}\) on the conformation of cytoplasmic domains is different in integrins in the presence or absence of an I domain and that Mn\(^{2+}\)-induced activation is an intermediate step during the process of I domain-containing integrin conformational change, while Mn\(^{2+}\) is sufficient to induce full conformational changes in VLA-4. To further investigate this, we measured the mAb B44 epitope exposure after Mn\(^{2+}\) and Mn\(^{3+}\) plusVCAM-1 treatments. No significant increase in B44 staining was observed in Mn\(^{2+}\)+plus VCAM-1-treated T cells compared with cells treated with Mn\(^{2+}\) alone. In contrast, binding of mAb KIM127, which recognizes the active conformation of the \(\beta_2\) integrin, was significantly increased by Mn\(^{2+}\) plus ICAM-1 compared with Mn\(^{2+}\) alone (Fig. 2G).

Consistent with the adhesive function of stable GD25 cells expressing wt VLA-4 (Fig. 1D and supplemental video 2), GD25 cells that were transiently expressing the VLA-4 FRET sensor, but not cells lacking the VLA-4 FRET sensor, showed efficient spreading on VCAM-1 (Fig. 2H and supplemental video 3), indicating that \(\alpha_x\)-mCFP and \(\beta_1\)-mYFP retain the normal ability of VLA-4 to bind and spread on VCAM-1. To further investigate the function of \(\alpha_x\)-mCFP and \(\beta_1\)-mYFP, VLA-4 FRET sensor/GD25 cells were incubated with soluble VCAM-1 in the presence of Mn\(^{2+}\). Fluorescence labeling of cell surface-bound VCAM-1 revealed that only cells expressing both \(\alpha_x\)-mCFP and \(\beta_1\)-mYFP can successfully bind to VCAM-1 (Fig. 2I). When GD25 cells expressed only \(\alpha_x\)-mCFP (Fig. 2I, arrow) or \(\beta_1\)-mYFP (data not shown), they did not bind to VCAM-1. Quantifying the fraction of each cell type showed that parental GD25 cells have no affinity for VCAM-1, while most of wt VLA-4/GD25 and VLA-4 FRET

![Figure 1](https://www.jimmunol.org/)

**FIGURE 1.** VLA-4 is activated at the lamellipodia during cell migration on VCAM-1. A, Human T cells were allowed to adhere to VCAM-1 in the presence or absence of CXCL12 or PLL in the presence of CXCL12. Migrating T cells were tracked over 10 min at 37°C, and videos were generated using DIC images acquired every 10 s (supplemental video 1). The **left corner** of each image is the magnified image of a randomly selected region. Scale bars, 100 \(\mu\)m. B, Human T cells were incubated on VCAM-1 plus CXCL12-coated coverslips for 30 min at 37°C. After fixation, dual-immunofluorescence labeling with M106 and B44 Abs was performed and samples were visualized using TIRF microscopy to detect total and activated VCAM-1 at the contact between cells and immobilized VCAM-1. From a fixed cell during migration, M106- and B44-labeling intensities were measured following the dashed arrow and profiled from the tail to the head for intensity comparison on the **lower panel**. Scale bar, 5 \(\mu\)m. Ratio images were generated by subtracting the background and dividing B44 intensity by M106 intensity. The color bar represents fluorescence intensity ratio (B44:M106). C, From three independent experiments of B, 40 cells were randomly selected and ratio images were generated as above. Cells were carefully analyzed and scored for the presence of B44 staining enriched at the anterior region, the posterior region, or middle based on the ratio images (B). Each bar represents the percentage of cells whose B44 staining was dominant over M106 staining (B44:M106 ratio, >1.0). D, Stable wt VLA-4/GD25 cells were allowed to settle on immobilized VCAM-1 for 10 min at 37°C. Spreading cells were tracked over 10 min and DIC images were acquired every 10 s (supplemental video 2). Time-lapse images of the highlighted region of a cell spreading (**right panels**). The arrow shows spreading direction. Scale bar, 20 \(\mu\)m. E, Stable wt VLA-4/GD25 cells were grown on VCAM-1 plus CXCL12 overnight. Dual-immunofluorescence labeling with M106 and B44 Abs was performed as described in B. M106- and B44-labeling intensities were measured following the dashed arrow (**left panels**) and profiled from an edge to an opposite edge for intensity comparison (**right panels**). Scale bar, 10 \(\mu\)m.
FIGURE 2. Development of VLA-4 FRET sensor. 

A, Schematic representation of VLA-4 activation. In the basal, bent conformation, cytoplasmic domains of $\alpha_4$ and $\beta_1$ subunits of VLA-4 are in close proximity. In this case, energy transfer occurs between CFP ($\alpha_4$-mCFP) and YFP ($\beta_1$-mYFP). In the active, extended conformation, there is low or no FRET because of the distal location of the cytoplasmic domains. Ex, Extracellular domain; TM, transmembrane; Cy, cytoplasmic domain.

B, Intersubunit FRET measurements in GD25 cells transiently expressing VLA-4 FRET construct pairs containing different linker lengths.

C, GD25 cells were transiently transfected with $\alpha_4$-mCFP, $\alpha_4$-mYFP, and wt $\beta_1$. Interheterodimer FRET between neighboring $\alpha_4$ and $\beta_1$ integrins for individual cells (○) was fit to the saturable one-site binding model $E\% = E\%_{max}F/F + K$, where FRET efficiency ($E\%$) is a hyperbolic function of the YFP acceptor intensity ($F$), and $K$ is analogous to a dissociation constant. The nonlinear least-squares regression fits of FRET efficiency between $\alpha_4$-mCFP/wt $\beta_1$ and $\alpha_4$-mYFP/wt $\beta_1$ (red curve) yielded $K = 301$, showing little association.

D, The cell surface expression levels of VLA-4 FRET sensor (transient) and wt VLA-4 (stable) on GD25 were determined by flow cytometry. Parental GD25 cells were used as control.

E, Whole cell lysates of nontransfected control, $\alpha_4$-mCFP-expressing, $\beta_1$-mYFP-expressing, and C/YFP-expressing 293T cells were subjected to SDS-PAGE and immunoblotting with polyclonal anti-GFP Ab that has cross-reactivity to CFP and YFP. The band at $75\,kDa$ is a nonspecific band detected by anti-GFP Ab. We conducted the transfection of 293T cells, since we were unable to detect protein bands from the immunoblotting with GD25 cells that were used as a host cell for transfection for other functional studies.

F, FRET efficiency of the VLA-4 FRET sensor transiently expressed in GD25 cells was measured after stimulation with 1 mM MnCl$_2$, 10$\,\mu$g/ml 1-activating Ab (TS2/16), and both of them.

G, Human T cells were activated with 1 mM MnCl$_2$ in the absence and presence of VCAM-1 or ICAM-1. The cells then were stained with B44 and KIM127 Abs and then measured by flow cytometry to detect activation of VLA-4 and LFA-1, respectively. Control was parental T cells without stimulation. MFI, Mean fluorescence intensity.

H, DIC, CFP, and YFP images were shown every 10 min during spreading of GD25 cells transiently expressing the VLA-4 FRET sensor on immobilized VCAM-1 (supplemental video 3). Scale bar, 20 $\mu$m.

I, VLA-4 FRET sensor/GD25 were treated with 1 mM MnCl$_2$ and incubated with soluble human VCAM-1-Ig fusion protein. Cells were fixed and then labeled with Alexa Fluor 647-goat anti-human IgG. DIC, VCAM-1 (Alexa Fluor 647), CFP, and YFP images were acquired. The arrows represent that a cell expressing only $\alpha_4$-mCFP without $\beta_1$-mYFP does not bind VCAM-1. Scale bar, 10 $\mu$m.

J, From three independent experiments in I and with stable wt VLA-4/GD25 cells, >100 cells were randomly selected and carefully analyzed to count the percentages of cells that bound VCAM-1.
sensor/GD25 cells bound to soluble VCAM-1 (Fig. 2J). These results demonstrate that these fusion constructs of VLA-4 possess normal function.

**Dynamic activation of VLA-4 at the lamellipodia**

Cell protrusion during spreading or migration is coordinated by actin-dependent membrane extension (the lamellipodium) and integrins are involved in lamellipodial extension and periodic contraction (28). With TIRF microscopy, only the shallow contact between the cell surface and the immobilized ligand is visualized (Fig. 3A) (29). Therefore, in TIRF microscopy, the VLA-4 FRET sensor exclusively indicates the presence and activation of VLA-4 proximal to the plasma membrane during cell spreading on VCAM-1 (Fig. 3B).

To measure dynamic FRET, we extended FRET measurements to the sensitized emission FRET, where FRET is measured by the increase in sensitized YFP emission over the decreased intensity in CFP emission by energy transfer; in this manner, dynamic FRET over a time course can be measured. Thus, the sensitized emission FRET is suitable for investigating the dynamic conformational changes of VLA-4 in living cells. For accurate calculation of the sensitized dynamic FRET efficiency without substantial spectral overlap between the donor and the acceptor fluorophores, CFP and YFP cross-talk coefficients were measured (Fig. 3C) and the measurements were used for the sensitized emission FRET calculations. Since our live-cell FRET experiments involve reduction in FRET during activation as the cytoplasmic parts of the $\alpha_4$ and $\beta_1$ subunits separate, photobleaching of CFP and YFP could result in a false-positive signal. To ensure that CFP and YFP intensities remain constant during image acquisition, we confirmed that repeated exposure of cells resulted in minimal photobleaching of both CFP and YFP and, furthermore, average FRET signals remained constant for 30 min (Fig. 3D). In addition, FRET measurements under TIRF microscopy showed that the sensitized emission FRET were in theoretical agreement with those of the acceptor photobleaching FRET (Fig. 3E) (30). Thus, the sensitized emission FRET measurement demonstrated that the VLA-4 FRET sensor is a valid indicator of the activation state of VLA-4.

To visualize the conformational changes in VLA-4 during live-cell spreading on VCAM-1, we performed dynamic FRET analysis under TIRF microscopy. TIRF/FRET analysis revealed that, during cell spreading on VCAM-1, low FRET signal, indicating VLA-4 activation, was detected over a broad region of the leading edge (Fig. 4A, upper panels, and supplemental video 4). In contrast, when cells spread on PLL, the low FRET signal was restricted to a narrow region at the front of the leading edge and an increase in FRET was observed in areas adjacent to the leading edge toward the cell body (Fig. 4A, lower panels, and supplemental video 4). Kymographs of FRET efficiency for spreading cells showed that VLA-4 is activated at the leading edge on both VCAM-1 and PLL (Fig. 4A, right panels). However, active VLA-4 broadly accumulates at the lamellipodium during cell spreading on VCAM-1, whereas VLA-4 rapidly returns to an inactive state as the lamellipodium advances on PLL (Fig. 4A, right panels). Quantitative analysis of spreading cells on VCAM-1 and PLL revealed that accumulation of active VLA-4 at the spreading edge (θ) was >2-fold greater in cells on VCAM-1 (Fig. 4B). Thus, data suggest that once VLA-4 is activated at the leading edge and subsequently binds to VCAM-1, the ligand-bound active conformation is stabilized during lamellipodial protrusion on VCAM-1. It is worth noting that VLA-4 mainly remained inactive at the inner area (Fig. 4A) and the uropod of migrating T cells (Fig. 1), suggesting that VLA-4 at the posterior contact zone mediates cell adhesion without conformational activation, probably through avidity regulation (clustering) (16).

**Selective blocking of active VLA-4 at the leading edge is sufficient to inhibit T cell migration on VCAM-1**

Although GD25, a mouse fibroblastic cell line, has been widely used for study of $\beta_1$ integrins, VLA-4 has been studied primarily in lymphocytes with respect to dynamic regulation of cell migration in response to chemokine stimulations. However, FRET analysis of VLA-4 in primary T cells is not feasible because of the

![FIGURE 3. Dynamic FRET measurement under TIRF microscopy.](http://www.jimmunol.org/)

A. YFP signal from the transient VLA-4 FRET sensor/GD25 cells on VCAM-1 was visualized, and kymographs were generated from the highlighted area of the TIRF images (left two panels) and EPI fluorescence images (right two panels). Scale bars, 5 μm (TIRF image) and 10 μm (EPI image). B. A schematic representation of how the FRET signal is measured in TIRF microscopy (1); evanescent wave transmitting through the contact between cell surface and immobilized VCAM-1 (2); and glass surface coated with VCAM-1 (3). C. Objective lens; the critical angle for a change in refractive index. C. $\alpha_4$-mCFP/wt $\beta_1$ or $\alpha_4$-mYFP/wt $\beta_1$, were transiently expressed, and CFP and YFP cross-talk with the FRET filter set (CFPex/YFPEm) were calculated for the sensitized emission FRET measurement. D. CFP and YFP intensities of transient VLA-4 FRET sensor/GD25 cells were measured every 10 s for 30 min on randomly selected regions of interest. FRET efficiency was also calculated based on CFP and YFP intensities. E. From individual regions of interest selected on VLA-4 FRET sensor/GD25 cell, CFP and YFP images were acquired with a CFP/YFP dual filter for 3 min for FRET calculation by the sensitized emission method, and then CFP images were acquired before and after permanent photobleaching of YFP for FRET calculation by the acceptor photobleaching method. The dashed line depicts the theoretical correlation between these two FRET measurement methods.
abundant expression of endogenous α4 and β1 integrin subunits. Therefore, to investigate the dynamic distribution of active VLA-4 in live migrating T cells, we have made use of a VLA-4 antagonist, BIO1211. BIO1211 binds to activated VLA-4 with 200-fold greater selectivity than to the resting VLA-4 (31). It was reported that the Kd of FITC-conjugated BIO1211 for inactive VLA-4 is 12 nM, but the Kd is no more than 0.4 nM for VLA-4 activated by Mn2+ and/or TS2/16 (32). Consistently, at concentrations of 4 and 10 nM BIO1211, which are lower than its Kd for inactive VLA-4 (12 nM), no significant BIO1211 binding was observed on the resting T cell surface (Fig. 5A). However, when T cells were stimulated with Mn2+, 4 nM BIO1211 was sufficient to increase T cell surface binding by >50% compared with control (Fig. 5A). In contrast, incubation of T cells with excessive concentrations (200 and 400 nM BIO1211) resulted in greatly enhanced binding of BIO1211 even without VLA-4 activation with Mn2+. Therefore, these data suggest that BIO1211 selectively binds to active VLA-4 at 4 nM.

To test whether selective inhibition of activated VLA-4 is sufficient to block T cell migration on VCAM-1, we measured the migration trajectory of T cells in the presence of various concentrations of BIO1211 (Fig. 5B). The percentages of migrating cells, normalized to the control condition, are shown under different blocking conditions in B. The data are produced from at least 100 cells of each condition.
soluble BIO1211. As hypothesized, T cell migration on VCAM-1 plus CXCL12 was readily decreased at 4 nM BIO1211 (Fig. 5B, upper middle panel). Cell migration trajectory at 400 nM BIO1211 was similar to that at 4 nM BIO1211 (Fig. 5B, upper right panel). Almost no migration was observed on VCAM-1 alone or PLL plus CXCL12 (Figs. 1A and 5B, lower panels). Percentages of total T cell migration affected by 4 nM and 400 nM BIO1211 were similar (Fig. 5C). Treatment of human T cells with BIO1211 for 1 h did not affect the viability (data not shown).

To further investigate whether activated VLA-4 localized at the leading edge of migrating T cells and to test whether BIO1211 inhibits cell migration by selectively targeting the subpopulation of active VLA-4, we visualized binding of fluorescent BIO1211 on migrating T cells. On ICAM-1 plus CXCL12, binding of BIO1211 was highly localized at the leading edge of migrating T cells (Fig. 6A and supplemental video 5). PTX, an inhibitor of Gαi hetero-meric G proteins, blocks chemokine-induced T cell adhesion and migration both on ICAM-1 and VCAM-1 (33, 34). Consistent with previous studies, pretreatment of T cells with PTX abolished cell migration and BIO1211 binding, whereas Mn2+ treatment induced BIO1211 staining to PTX-treated cells (Fig. 6B). Therefore, our data suggest that activated VLA-4 is predominantly localized at the leading edge of migrating T cells and selective inhibition of the active VLA-4 at the front is sufficient to inhibit T cell migration.

CXCR4 and active Rap1 are localized at the leading edge of migrating T cells

Chemokines presented on the endothelium in lymphoid and inflamed tissues rapidly enhance the adhesiveness of leukocyte integrins, enabling integrin-dependent firm adhesion, induce cell polarization, and facilitate migration. Chemokine-dependent redistribution of chemokine receptors to the leading edge has been observed previously (35–37). Consistently, CXCR4 was highly localized at the leading edge of migrating T cells on VCAM-1 plus CXCL12 (Fig. 7A). Active VLA-4, stained with fluorescent BIO1211, was also localized at the leading edge in >54% of migrating T cells (Fig. 7B), suggesting an important role for chemokine signals in the distribution of active VLA-4. Dynamic colocalization of active VLA-4 and CXCR4 at the scale of molecular interactions was not possible due to the resolution limit of our imaging system and sequential labeling of BIO1211 before fixation, and then CXCR4 after fixation (see Materials and Methods).

Recent evidence indicates that Rap1, a member of the Ras family of small GTPases, is a key molecule that regulates chemokine-mediated LFA-1 and VLA-4 activation during lymphocyte migration (38, 39). To investigate dynamic activation of Rap1 during T cell migration, we used a genetically encoded, single-chain

![FIGURE 6. VLA-4 activation at the leading edge of migrating human T cells. A. FITC-conjugated BIO1211 stained the leading edge of T cells during migration on ICAM-1 plus CXCL12 (supplemental video 5). ICAM-1 was used to trigger cell migration through LFA-1-ICAM-1 interaction. Under these conditions, active VLA-4, which is not occupied by VCAM-1, can be detected by BIO1211 binding. B. PTX inhibited most of BIO1211 staining of migrating T cells on ICAM-1 plus CXCL12, while 1 mM MnCl2 induced BIO1211 staining to T cells. Scale bars, 10 μm.](http://www.jimmunol.org/)

![FIGURE 7. CXCR4 and active Rap1 are localized at the leading edge of migrating human T cells. A. Anti-CXCR4 Ab staining showed that CXCR4 is localized mainly at the leading edge of migrating T cells on VCAM-1 plus CXCL12. Dynamic FRET images are shown every 2 min (supplemental Video 6). YFP intensity is shown in gray and FRET efficiency is shown in rainbow colors (highest (red) to lowest (blue)). Scale bars, 10 μm. B, During migration on ICAM-1 plus CXCL12, T cells were incubated with BIO1211 for 5 min and cells were fixed. Following CXCR4 Ab staining, BIO1211 and CXCR4 staining was visualized. Scale bar, 10 μm. C, T cells expressing the Rap1 FRET sensor were tracked during migration on VCAM-1 plus CXCL12. Dynamic FRET images are shown every 2 min (supplemental Video 6). YFP intensity is shown in gray and FRET efficiency is shown in rainbow colors (highest (red) to lowest (blue)). Scale bars, 10 μm. D, FRET efficiency (red) and YFP intensity (blue) of a T cell expressing the Rap1 FRET sensor from an image in C (time = 8 min) were profiled along the dashed arrow. E, A kymograph was generated from the highlighted region of the left panel from the time-lapse images of migrating T cells (supplemental video 6). D, Spreading direction; T, time. Scale bar, 10 μm.)
intramolecular FRET sensor to visualize Rap1 activation and its distribution during T cell migration. This biosensor is a fusion protein containing Rap1 and the Rap1-binding sequence of its effector, Raf, as well as CFP and YFP, which serve as the FRET donor and acceptor, respectively. In numerous studies, this FRET sensor successfully reported Rap1 activation in living cells (17, 40, 41). For live-cell FRET imaging in TIRF microscopy, we transfected primary human T cells with the Rap1 FRET sensor. Activation of Rap1 at the region of contact between T cells and VCAM-1 was observed consistently at the leading edge (Fig. 7C and supplemental video 6). The profile of FRET efficiency and YFP intensity along the arrow from the tail to the head (Fig. 7C, 8 min) showed that Rap1 activation (FRET efficiency depicted in red line) is mainly localized at the leading edge of the migrating cell, whereas the Rap1 FRET sensor (depicted in blue line) is evenly distributed throughout the cell (Fig. 7D). Localized Rap1 at the cell leading edge is shown in the kymograph of FRET images (Fig. 7E). The results in Fig. 7 suggest that the chemokine receptor and Rap1 activation are colocalized with VLA-4 activation at the leading edge during T cell migration.

Discussion
In leukocyte trafficking from the blood to peripheral tissues during normal immune surveillance and host defense, integrin activation should be spatially and temporally regulated (42). Like many other cellular proteins, integrins are not fixed in a particular conformation. Instead, they reversibly equilibrate between the bent, low-affinity and the extended, high-affinity conformation (43, 44). Recently, dramatic advances in defining the structure of integrins by crystallography, nuclear magnetic resonance, and electron microscopy have demonstrated important molecular mechanisms for integrin activation and ligand binding and provided useful information in drug discovery. Despite recent progress in defining the structure of integrins, a key question that remains unanswered is whether the conformational changes in integrins actually occur in living cells during migration and, if so, how they are regulated. Based primarily on studies with mAbs that preferentially bind to active integrins, it was hypothesized that high-affinity integrins are localized at the leading edge of spreading and migrating cells (45). In this study, we have been able to directly test this hypothesis in live cells using a fluorescent VLA-4 peptide inhibitor and an activation-dependent mAb in human T cells and our novel TIRF/FRET-based assay for VLA-4 activation in GD25 cells.

Since FRET is the only biological assay that measures the dynamics of protein structure in living cells, our novel FRET system should be considered complementary techniques. TIRF microscopy is advantageous in studying the molecular mechanisms involved in cell migration, since the technique allows us to monitor optical events within 100 nm of the plasma membrane, where cell-substrate traction occurs. Thus, by combining FRET with TIRF microscopy, we were able to investigate the dynamic activation of VLA-4 at the cell-substrate interface during cell migration on VCAM-1.

The data presented here demonstrate that the activated form of VLA-4 is dynamically distributed to the leading edge of migrating cells and the spatial distribution of active VLA-4 is critical for T cell migration on VCAM-1. The low FRET signal of the VLA-4 FRET sensor at the leading edge indicates the separation of the α and β cytoplasmic domains of VLA-4. During lamellipodial protrusion at the leading edge, VLA-4 is activated at the extending front of the cell and subsequently maintains the active and extended conformation only when it encounters VCAM-1, suggesting that the active form of VLA-4 is induced by intracellular signaling and then further stabilized by ligand binding. However, at the inner region of the cell contact zone and more distal region from the leading edge, VLA-4 returns to a low affinity state, as it exhibits a lack of LIBS Ab staining on human T cells and higher FRET signals on GD25 cells. Consistent with our study, α4 subunit phosphorylation by protein kinase A (PKA) inhibits paxillin binding and paxillin-free phosphorylated α4 is mainly localized at the leading edge and promotes cell migration (5). Recent evidence demonstrates that AKAP-Lbc, a specific A kinase-anchoring protein, is critical for generating a PKA activity gradient at the leading edge (46), although regulation of AKAP-Lbc distribution and PKA activity gradients by chemokine stimulation is not known. Interestingly, the α4 subunit cytoplasmic domain can function as a type I PKA-specific AKAP and the association is critical for α4 phosphorylation and persistent directional cell migration (47). Our data indicate that chemokine receptor CXCR4 and Rap1 activation are restricted to the front of migrating T cells. Active Rap1 has been shown to interact with RIAM (Rap1-GTP-interacting molecule) and this Rap1-RIAM complex can target talin to the plasma membrane to activate integrins (48). Therefore, it is likely that, when the α4 subunit of VLA-4 is phosphorylated by PKA at the leading edge, VLA-4 switches to an intermediated affinity state. After VCAM-1 binding, the β1 subunit of this intermediate-affinity VLA-4 could again associate with the Rap1-RIAM-talin complex and interact with the cytoskeleton. The interaction will then reinforce the interaction of VLA-4 and VCAM-1, thus stabilizing the high-affinity state of VLA-4 at the leading edge and providing traction force for cell migration.

Our studies on VLA-4 do not define the mechanisms of active redistribution of other integrins during T cell migration; nonetheless, evidence indicates that on a given cell, activation of one integrin subset may be regulated by activation or ligation of another, allowing flexibility for specific leukocyte adhesion in different microenvironments. In monocytes, ligation of high-affinity LFA-1 by ICAM-1 results in decreased binding of VLA-4 to VCAM-1 (49). Although it is not known whether the negative cross-talk between LFA-1 and VLA-4 occurs in T cells, activated LFA-1 may not simultaneously localize with high-affinity VLA-4 during migration. Indeed, unlike VLA-4, the high-affinity LFA-1 is excluded from the leading edge and is restricted to the mid-cell focal zone, whereas intermediate-affinity LFA-1 is expressed at the leading edge (50).

Conventional antiadhesion therapy is designed for nonselective inhibition of cell surface integrins by complete saturation. This might cause massive suppression of normal immune reactions and increase susceptibility to infections (51). In addition, relatively high-dose Ab administration is often required to produce clear clinical improvement due to the lack of the specificity or because of rapid consumption after systemic administration. As described in Fig. 5, we found that T cell migration on VCAM-1 plus CXCL12 is inhibited by BIO1211, a selective antagonist against VLA-4. At a concentration of 4 nM, BIO1211 could bind to VLA-4 only when cells were treated with Mn²⁺, suggesting that BIO1211 selectively blocks activated VLA-4 at this concentration.
Interestingly, 4 nM BIO1211 could successfully block T cell migration on VCAM-1 and was as potent as 400 nM, a concentration at which BIO1211 binds to both inactive and active VLA-4. These data suggest that selective blocking of the subpopulation of activated VLA-4 is sufficient to inhibit T cell migration on VCAM-1 and may prove to be an effective therapy for inflammatory diseases.

Our study also addresses the mechanisms of preferential distribution of active VLA-4 at the leading edge during lymphocyte migration in response to chemokine stimulation. Recent evidence demonstrates that chemokines induce redistribution of chemokine receptors to the leading edge of polarized lymphocytes in a PTX-sensitive manner, suggesting that localization of chemokine receptors at the leading edge leads to rapid chemokine receptor activation (35, 44). Alternatively, activation of VLA-4 at the leading edge might be caused by localized activation of Rap1 complex with RIAM/talin at the region. If this is indeed the case, the transportation of active Rap1 to the leading edge might be caused by localized activation of Rap1 complex with RIAM/talin at the region.

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We thank Craig Lefort for critical reading of this manuscript. Rap1 complex with RIAM/talin at the region. If this is indeed the case, the transportation of active Rap1 to the leading edge might be caused by localized activation of Rap1 complex with RIAM/talin at the region.

Disclosures

The authors have no financial conflict of interest.

References


