

The Critical Cytoplasmic Regions of the α L/ β 2 Integrin in Rap1-induced Adhesion and Migration[□]

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Rap1 is a potent inside-out signal that increases LFA-1 adhesive activity. In this study, we have defined the cytoplasmic region of the α L and β 2 integrin that are required for Rap1-stimulated adhesion and subsequent migration on ICAM-1. Human LFA-1 bearing truncated and point-mutated α L and β 2 cytoplasmic regions were reconstituted in mouse IL-3–dependent proB cells, BAF/3. Truncation of the α L, but not β 2 subunit cytoplasmic region, abolished Rap1V12-dependent adhesion to ICAM-1. The alanine substitution of two lysine residues (K1097/K1099) in the α L subunit was found to be critical in adhesion induced by Rap1V12, but not PMA. This mutation suppressed Rap1V12-induced LFA-1 conformation changes and ligand-binding affinity. The K1097/K1099 mutation also impaired binding to ICAM-1 induced by TCR cross-linking or SDF-1. In contrast, the alanine substitution for tyrosine in the β 2 subunit endocytosis motif inhibited internalization of LFA-1, and severely impaired detachment at the cell rear, which resulted in long-elongated cell shapes. This result demonstrates that internalization of LFA-1 is a critical step in the deadhesion process. Our study revealed novel requirements of amino acid residues of the LFA-1 cytoplasmic region in the response to the inside-out signaling and the subsequent deadhesion process.

INTRODUCTION

The integrin leukocyte-function-associated antigen-1 (LFA-1) is a surface glycoprotein, comprised of an α subunit (α L or CD11a) and common β 2 subunit (CD18), which is shared with α M, α X, and α D (Springer, 1990; Van der Vieren *et al.*, 1995). LFA-1 is expressed in leukocytes, their precursor cells, and related cells derived from bone marrow and mediates cell adhesion in inflammatory and immune responses through binding to immunoglobulin superfamily members, intercellular adhesion molecule (ICAM)-1, -2, and -3 (Marlin

and Springer, 1987; Staunton *et al.*, 1989; de Fougères *et al.*, 1994).

Adhesive interactions of LFA-1 to ICAMs require an activation process termed “inside-out signaling” (Dustin and Springer, 1989), triggered by antigen, chemokines, or cytokines, which generate intracellular second messengers that ultimately bring about changes of conformation and ligand binding activity of integrins (van Kooyk and Figdor, 2000). Rapid upregulation of integrin adhesiveness by inside-out signals enables circulating leukocytes to interact avidly with the endothelium and antigen-presenting cells (APC; Springer, 1995; Grakoui *et al.*, 1999).

We previously reported that phosphatidylinositol-3 kinase, PKC, and Rap1 were inside-out signaling molecules that are capable of activating LFA-1 to bind ICAM-1 with distinct effects on LFA-1 conformation and ligand-binding affinity (Katagiri *et al.*, 2000). Rap1 was reported to be involved in activation of LFA-1 by antibody cross-linking of CD31 (Reedquist *et al.*, 2000) and CD98 (Suga *et al.*, 2001). Rap1 was activated in T cells stimulated with TCR cross-linking or antigen-loaded APC and regulated adhesive strength in T cell-APC interactions (Katagiri *et al.*, 2002). A transgenic mice study showed that Rap1V12 induced LFA-1

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Abbreviations used: APC, antigen-presenting cells; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte-function-associated antigen-1; SDF-1, stromal-derived factor-1; TCR, T-cell receptor.

clustering and stimulated lymphocytes adhesion (Sebzda *et al.*, 2002), suggesting Rap1 functions through regulation of adhesive interactions *in vivo*, too.

Many lines of evidence indicate that the integrin cytoplasmic regions modulate cell adhesion. In the case of LFA-1, truncation of the β 2 cytoplasmic region (Hibbs *et al.*, 1991b) and the T758TT/AAA mutation in the β 2 cytoplasmic region decreased binding to ICAM-1 in COS cells (Hibbs *et al.*, 1991a) and PMA-stimulated EBV-transformed B lymphoblastoid cells (Weber *et al.*, 1997a). The F754A mutation of the β 2 cytoplasmic region also inhibited adhesion to ICAM-1 in CHO cells (Fabbri *et al.*, 1999). The mutation of the TTT motif also affected cell spreading and cytoskeletal interactions in PMA-induced adhesion of CHO cells (Peter and O'Toole, 1995). Regarding the role of the α L cytoplasmic region, deletion of the α L cytoplasmic region 12 amino acids after the conserved GFFKR motif in COS cells did not affect ICAM-1 binding (Hibbs *et al.*, 1991b). However, the deletion just after the GFFKR motif inhibited PMA-stimulated adhesion in Jurkat β 2.7 cells (Weber *et al.*, 1997a). The relative contribution of the α L and β 2 cytoplasmic regions for modulation of adhesive activity is difficult to compare in different cell contexts. Postadhesion events such as cell spreading also enhance adhesion indirectly, making distinction of inside-out and outside-in signals, unclear especially in adherent cells. So far, it is unclear which parts of the LFA-1 cytoplasmic region play a critical role to modulate LFA-1 adhesive activity in response to the specific inside-out signal molecules, such as recently identified Rap1, as well as physiological stimulation by chemokines and TCR.

Leukocytes move at 10–15 μ m/min, \sim 10-fold faster than fibroblasts. Cell migration requires a coordination of front adhesion and rear deadhesion (Sanchez-Madrid and del Pozo, 1999). However, the regulatory process of deadhesion of β 2 integrins in migrating leukocytes is unclear. It is unknown whether the cytoplasmic regions of leukocyte integrins play any role to mediate detachment from the substrate, specifically, whether the site responsible for detachment is the same or distinct from that to modulate integrin adhesive activity. In neutrophils, detachment was dependent on a transient increase of intracellular free calcium and calcineurin followed by endocytosis of this integrin (Lawson and Maxfield, 1995). However, it is uncertain at present whether this is a general detachment mechanism also applicable to β 2 integrins.

Here, we examined the LFA-1 cytoplasmic regions that are required for Rap1-dependent adhesion and migration. Our study shows the critical region of the α L cytoplasmic region in the response to the inside-out signaling and the tyrosine-based endocytosis motif of the β 2 in the subsequent deadhesion event.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies used in this study were purified TS1/18 (blocking anti- β 2 mAb) and TS1/22 (blocking anti- α L mAb; Sanchez-Madrid *et al.*, 1983), TS2/4 (nonblocking anti- α L mAb; Sanchez-Madrid *et al.*, 1982), CBR-LFA1/2 (activating anti- β 2 mAb; Petruzzelli *et al.*, 1995) and RR1/1 (blocking anti-ICAM-1 mAb; Rothlein and Springer, 1986). The anti- α L antibody (clone27; Transduction Laboratories, Lexington, NY) and anti- β 2 polyclonal antibodies (Sc-6623; Santa

Cruz Biotechnology, Inc., Santa Cruz, CA) were used for Western blotting. NKI-L16 and mAb24, which recognize epitopes of LFA-1, were kind gifts from C. Figdor and N. Hogg, respectively. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) F(ab')₂ fragments were purchased from Cappel (Durham, NC). HRP-conjugated goat anti-mouse and anti-rabbit IgG were from Cell Signaling (Beverly, MA). HRP-conjugated streptavidin, protein G- and protein A-Sepharose 4B were purchased from Amersham Biosciences Corp. (Piscataway, NJ). Anti-T7 epitope antibody was from Novagen (Madison, WI) used for immunoblotting. NHS-Biotin was from Pierce (Rockford, IL). Recombinant human ICAM-1-Fc (rhICAM-1-Fc), a fusion protein of an extracellular ICAM-1 consisting of the first to fifth domains with the human IgG1 Fc portion, was purified with protein A-Sepharose from the culture supernatant of CHO cells producing human ICAM-1-Fc (a kind gift from T. Takashi, Daiichi Pharmaceutical, Tokyo, Japan).

Site-directed Mutagenesis and Transfection

α L Δ 1095, Δ 1107 (Lu and Springer, 1997), β 2 Δ 731, Δ 744 (Hibbs *et al.*, 1991b), β 2Y735A, F754A (Fabbri *et al.*, 1999), T758TTAAA (Hibbs *et al.*, 1991a) were previously described. The site-directed mutagenesis by PCR was used for alanine substitutions of K1097, K1097/1099, and K1097EKME. In brief, overlap extension PCR (Horton *et al.*, 1993) was used to amplify two fragments between *Stu*I 5' primer (5'-3214GATCGAGGCCTCTTCCATGTTCAGCCTCTG-3'), or *Avr*II 3' primer (5'-ACTCCTAGTCCAGTCCCTTGCCACCACACTCTC3578-3'); the number indicates the α L subunit nucleotide position) and the specific mutation primer of the appropriate sense or antisense strand. The sense strand sequences of the mutation primers are 3446-AACCTGGCGGAGAAGATGGAGGCTGGC for K1097, 3446-AACCTGGCGGAGGCGATGGAGGCTGGC for KK1097/1099, 3446-AACCTGGCGGCTGCGGCGGCAGCTGGC for K1097EKME encoding alanine in the place for lysine, glutamic acid, and methionine. Two fragments were combined by the PCR with the *Stu*I 5' primer and *Avr*II 3' primer and digested with *Stu*I and *Avr*II. The *Stu*I-*Avr*II fragment was inserted between the *Stu*I and *Avr*II sites of α L in Bluescript (Stratagene, La Jolla, CA). Mutations were verified by sequencing both strands (ABI PRISM Cycle Sequencing FS Ready Reaction Kit, Applied Biosystems, Foster City, CA). The wild-type and mutant α L and β 2 were subcloned in the expression vectors as follows: pEFpuro -wild-type α L, - α L Δ 1095, - α L Δ 1107, pcDNA4/TO (Invitrogen, Carlsbad, CA) - α L K1097A, -KK1097/1099AA, and -K1097EKME/AAAAA (A5), pcDNA3.1/Hygro (Invitrogen) -wild β 2, - β 2 Δ 731, - β 2 Δ 744, - β 2Y735A, - β 2F754A, - β 2T758TT/AAA.

Both α L and β 2 cDNA were introduced into BAF cells by electroporation and selected with 1 μ g/ml puromycin (Sigma Chemical Co., St. Louis, MO) for pEFpuro, 0.5 mg/ml zeocin (Invitrogen) for pcDNA4/TO, or hygromycin B (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for pcDNA3.1/Hygro vector, and maintained in RPMI 1640 medium (Sigma) containing 8% FCS, 50 μ M β -mercaptoethanol, and IL-3. Rap1V12 or *Spa*I was introduced further by retrovirus and selected with 1 mg/ml G418 (Invitrogen) as described (Katagiri *et al.*, 2002). J β 2.7, an α L-deficient Jurkat cell line (Weber *et al.*, 1997b) maintained in RPMI medium containing 8% FCS, was introduced by electroporation with pEFpuro-wild-type α L or - α L Δ 1095, pcDNA3.1/Hygro -KK1097/1099AA, or -K1097EKME/AAAAA (A5) as described above. LFA-1 expression was examined by FACS analysis after cell surface staining with TS1/22 and TS1/18 followed by FITC-labeled anti-mouse IgG F(ab')₂. Expressions of Rap1V12 and *Spa*I were confirmed by Western blotting with the anti-T7 or anti-flag epitope antibody.

Immunoprecipitation and Western Blotting

Transfectants (1 \times 10⁷ cells) were surface-labeled with 2 ml of 0.5 mg/ml NHS-biotin at 4°C for 30 min. Labeled cells were washed

with PBS (pH 8) and resuspended in 1 ml of lysis buffer (1% Triton X-100, 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). After centrifugation, the cell lysate was precleared with control mouse IgG2a antibody and protein G-Sepharose 4B at 4°C for 1 h. The precleared lysate was immunoprecipitated with TS2/4 and protein G-Sepharose 4B at 4°C for 3 h. The beads were washed three times with lysis buffer and subjected to Western blotting.

Cell lysates or immunoprecipitated proteins were separated in a SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham) as described (Katagiri *et al.*, 2000). The membrane was incubated for 1 h with HRP-streptavidin (Amersham) for biotin-labeling samples, or anti-T7 epitope antibody followed by HRP-conjugated goat anti-mouse IgG for 30 min for Rap1V12. ECL (Amersham) was utilized for detection. The membranes were re-probed with anti-human αL (clone27) or β2 polyclonal antibodies (Sc-6623; Santa Cruz).

Cell Adhesion Assays

Adhesion assays were performed as described (Katagiri *et al.*, 2002) using rhICAM-1-Fc. To coat rhICAM-1-Fc, 96-well polystyrene plates (Linbro-Flow, Chantilly, VA) were first coated overnight with rabbit anti-human IgG (1 μg/well) in order to efficiently capture ICAM-1-Fc. The plate was washed and blocked with 1% BSA for 30 min. The plate was incubated further with rhICAM-1-Fc (0.01 μg/well) for 2 h at room temperature. For adhesion assays with Jβ2.7 cells and with CBR-LFA1/2, rhICAM-1-Fc was directly coated with 0.5 μg/well in 96-well plates overnight in order to stimulate cells with anti-CD3 antibody or CBR-LFA1/2. Cells were labeled with 2',7'-bis-(2-carboxyethyl)-5 (and-6) carboxyfluorescein (Molecular Probes, Inc., Eugene, OR) and resuspended with RPMI 1640 containing 10 mM HEPES (pH 7.4) and 5% FCS. Labeled cells (5 × 10⁴/well) were transferred into coated plates alone or with PMA (10 ng/ml), OKT3 (10 μg/ml), or CBR-LFA1/2 (10 μg/ml) and then incubated at 37°C for 30 min. Nonadherent cells were removed with four 21-gauge needle aspirations. Input and bound cells were measured in the 96-well plate using a fluorescence multiwell plate reader (CytoFluor4000; Applied Biosystems). For antibody inhibition, the coated plates were preincubated for 30 min with 20 μg/ml RR1/1 or cells were incubated with TS1/22. The plate was blocked before antibody treatment with human IgG (1 mg/ml; Cappel) in case of indirect coating.

Flow Cytometric Analysis

Cells were incubated with staining buffer (HBSS containing 3% FCS and 10 mM HEPES, pH 7.4) containing 10 μg/ml antibodies indicated (TS1/22, TS1/18, TS2/4, OKT3, or NKI-L16) on ice for 30 min. The cells were then washed twice with staining buffer and further incubated with 1 μg/ml FITC-conjugated F(ab')₂ fragments of goat anti-mouse IgG and subjected to flow-cytometric analysis with FACS Calibur (Becton Dickinson, San Jose, CA).

Measurement of Soluble ICAM-1 Binding

Measurement of the binding of human ICAM-1-Fc to BAF cells was performed as described (Katagiri *et al.*, 2000). Cells were suspended in 50 μl of RPMI 1640 containing 10 mM HEPES (pH 7.4) and 5% FCS and incubated at 2 × 10⁵ cells/50 μl with human ICAM-1-Fc (1 mg/ml). In some cases, CBR-LFA1/2 (10 μg/ml) or PMA (10 ng/ml) was also included. After the incubation for 30 min at 37°C, the cells were washed twice and then incubated with 10 μg/ml FITC-conjugated goat anti-human IgG Fc-specific antibody (Cappel) for 20 min on ice. When stimulated with CBR-LFA1/2, ICAM-1-Fc was detected with FITC-conjugated mouse monoclonal anti-human IgG Fc antibody (Zymed, South San Francisco, CA). Unbound secondary antibody was washed off, and mean fluorescence intensities were measured using a FACS Calibur.

Immunostaining

Rap1V12-introduced BAF cells expressing wild-type β2 and Y735A mutant were incubated on ICAM-1-coated culture slides (Becton Dickinson) at 37°C for 30 min and then fixed with 3.3% PFA for 15 min at room temperature, followed by blocking with 10% goat serum for 20 min. Cells were stained with mAb24 (1/100 dilution with 10% goat serum) for 1 h. After washing five times with PBS/0.1% BSA, cells were incubated 1 h with Alexa Fluor488-conjugated goat anti-mouse IgG (1/400 dilution with 10% goat serum; Molecular Probes). After washing five times with PBS/0.1% BSA, cells were mounted with antifade containing glycerol/PBS (SlowFade; Molecular Probes) and examined with a confocal laser-scanning microscope (LSM510; Zeiss, Oberkochen, Germany).

Internalization Assay

LFA-1 internalization was performed essentially as described (Gao *et al.*, 2000). Briefly, cell surface LFA-1 in BAF cells expressing wild-type β2, and the Y735A mutant was labeled by incubation for 1 h with 10 μg/ml TS2/4 on ice. After washing with ice-cold HBSS, cells were resuspended with RPMI 1640 containing 20 mM HEPES, pH 7.4, and incubated at 37°C for the indicated time. After washing twice with ice-cold HBSS, cells were further incubated with 1 μg/ml FITC-conjugated F(ab')₂ fragments of goat anti-mouse IgG. After washing and resuspending with ice-cold HBSS, cells were immediately subjected to flow-cytometric analysis. T lymphoblasts were prepared by culture of human peripheral blood mononuclear cells with phytohemagglutinin (2 μg/ml) for 3–4 d and subjected for internalization assays as above.

For microscopic analysis of internalized LFA-1, cells were incubated as above with TS2/4-conjugated AlexaFluor488 (Molecular Probes), at 37°C for 15 min. After washing once with PBS, cells were fixed with 3.3% paraformaldehyde and transferred onto PLL-coated slides. Cells were mounted and examined with a confocal laser-scanning microscope (LSM510; Zeiss).

Adhesion Assays under Shear Flow

SDF-1-induced adhesion to ICAM-1 was measured at 37°C under shear flow in a parallel plate flow chamber (FCS2 system, Biopetech Inc., Butler, PA). rhICAM-1-Fc (0.1 μg/ml) was coated on polystyrene disks as described above. The flow chamber was mounted on the stage of an inverted phase-contrast microscope (IX70; Olympus, Tokyo, Japan) equipped with a CCD camera (C2741; Hamamatsu Photonics, Japan). Shear stress was generated with an automated syringe pump (Harvard Apparatus, Natick, MA) attached to the flow chamber. Cells (1 × 10⁶ cells) suspended in 500 μl of Leibovitz's L-15 medium (Invitrogen), containing 0.5% BSA, were loaded with or without SDF-1 (20 nM; R&D Systems, Minneapolis, MN) into a flow chamber (250-μm gap). Cells were incubated for 10 min before applying shear stress for 1 min at 2 dyn/cm². The incubation time and shear stress was determined to achieve the maximum attachment with low backgrounds. Attachment was <1% on BSA-coated disks. Images were recorded in every second and processed to count cells using ImagePro (Media Cybernetics, Silver Spring, MD).

Online Supplemental Material

Time-lapse video of cell migration of BAF transfectants on ICAM-1 are available on-line as follows: video 1: Rap1V12-expressing BAF cells/wild-type LFA-1; video 2: Rap1V12-expressing BAF cells/Y735A; video 3: BAF cells/wild-type LFA-1 stimulated by SDF-1; and video 4: BAF cells/Y735A stimulated by SDF-1.

Random cell migration of BAF cells of wild-type LFA-1 and αL/β2Y735A cells on ICAM-1 was recorded at 37°C with a culture dish system for live-cell microscopy (ΔT culture dish system, Biopetech Inc.). ICAM-1 was coated at 0.1 μg/ml as described above for BAF cells. Images were taken every 30 s. Time-lapse movies

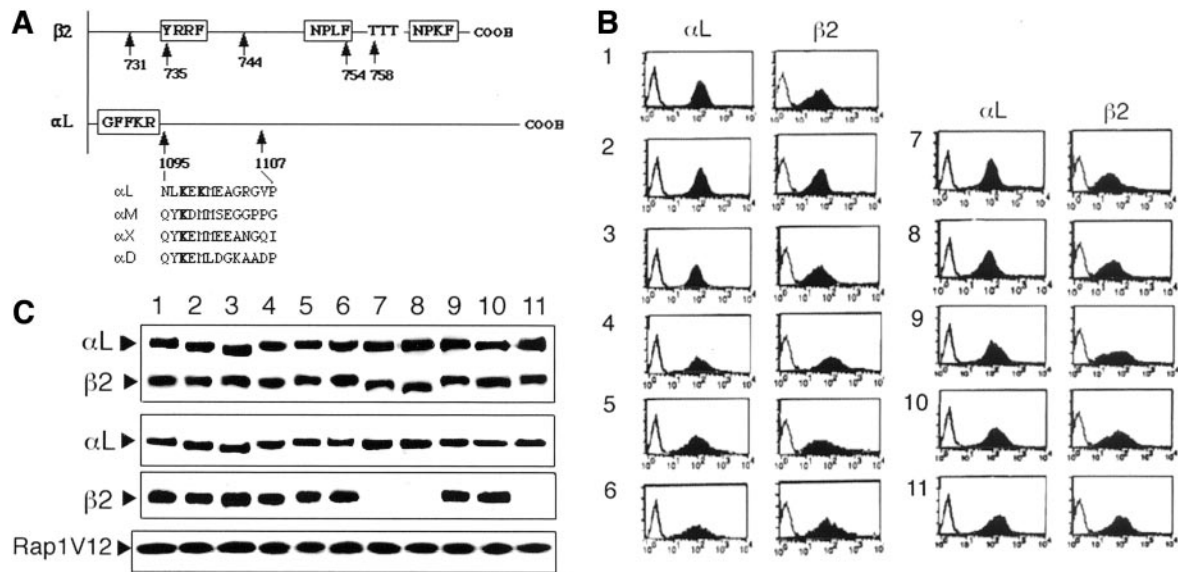


Figure 1. Expressions of a series of mutant α L β 2 in BAF cells. (A) Schematic representation of the cytoplasmic regions of human α L and β 2 subunits. The numbers indicate amino acid residues. The amino acid sequence between 1095 and 1107 in human α L and the corresponding region of α M (human), α X (human), and α D (rat) are shown. (B) FACS analysis of transfected LFA-1. BAF transfectants were stained with anti-human α L antibody (TS1/22) or anti-human β 2 antibody (TS1/18), followed by FITC-labeled anti-mouse IgG F(ab')₂ fragments (filled) or were stained only with the second antibody (open). 1, wild-type α L β 2; 2, α L Δ 1107/ β 2; 3, α L Δ 1095/ β 2; 4, α LK1097A/ β 2; 5, α LK1097A/K1099A/ β 2; 6, α LK1097EKME/AAAAA(A5)/ β 2; 7, α L/ β 2 Δ 744; 8, α L/ β 2 Δ 731; 9, α L/ β 2Y735A; 10, α L/ β 2F754A; 11, α L/ β 2T758TT/AAA. (C) Immunoprecipitation with anti- α L (TS2/4) of surface-labeled BAF transfectants, as described in MATERIALS AND METHODS. The positions corresponding to α L and β 2 are indicated. The same membrane was stripped and probed with anti- α L mAb (α L) or anti- β 2 antibody (β 2). Rap1V12 expression introduced into BAF transfectants is also shown (Rap1V12). The lane numbers are identical to those in B.

were created at 10 frames/s using QuickTime Pro (Apple Computer Inc., Cupertino, CA).

RESULTS

Establishment of Stable Cell Lines Expressing Wild-type and Mutant LFA-1

To define the cytoplasmic region of LFA-1 responsible for Rap1V12-dependent adhesion, we used an IL-3-dependent mouse proB cell line, BAF cells, for reconstitution of LFA-1 because BAF cells expressing wild-type human LFA-1 are able to bind to human ICAM-1 in response to inside-out signaling molecules including Rap1 (Katagiri *et al.*, 2000). Stable BAF cells expressing wild-type and truncated and point-mutated α L and β 2 subunits (Figure 1A) were isolated and selected for comparable expression on the cell surface (Figure 1B). Stable heterodimer formation of introduced α L and β 2 was confirmed by immunoprecipitation of labeled cell surface proteins with anti-human LFA-1 (TS2/4; Figure 1C, top panel). The size of truncated mutants of α L and β 2 is slightly decreased in accordance with the deletion. Immunoprecipitated bands were also probed for α L and β 2 with monoclonal anti-human α L (MAb27), which recognizes the extracellular region of α L, and with polyclonal anti- β 2 antibody, which recognizes the β 2 cytoplasmic region (Figure 1C, second and third panel). It should be noted that this antibody failed to recognize β 2 with the T758AATTT mutation and those with mutations deleting this site (Δ 747, Δ 731;

Figure 1C). To examine the effects of mutations on Rap1V12-induced adhesion, established cell lines were further introduced with Rap1V12 via retrovirus (Figure 1C, lowest panel).

The α L Cytoplasmic Region Is Critical for Rap1V12-induced Adhesion

As we showed previously (Katagiri *et al.*, 2000), BAF cells expressing wild-type LFA-1 adhered to ICAM-1 upon introduction of constitutively active Rap1 (Rap1V12; Figure 2A); however, the parent BAF cells failed to adhere. The adhesion was inhibited either by blocking mAb to human LFA-1 (TS1/22) or ICAM-1 (RR1/1). Then, we examined a series of mutants of the α L cytoplasmic region (Figure 2A). Deletion to the amino acid residue 1102 (our unpublished results) or 1107 from the C-terminal of the α L subunit did not affect both basal and Rap1V12-induced adhesion. However, deletion to the 1095 abolished Rap1V12-induced adhesion. To identify critical amino acid residues, we introduced a mutation of lysine at 1097, which is conserved in α M, α X, and α D. A substitution of lysine for alanine (K1097A) did not affect basal adhesion levels, but reduced Rap1V12-induced adhesion to less than a half when compared with the wild type. Because α L has another lysine residue at 1099, an additional alanine substitution for this lysine was introduced. K1097A/K1099A further decreased Rap1V12-induced adhesion near to basal levels. Alanine substitutions of all amino acids from 1097 to 1101 (α LA5) gave a similar result. In contrast, direct

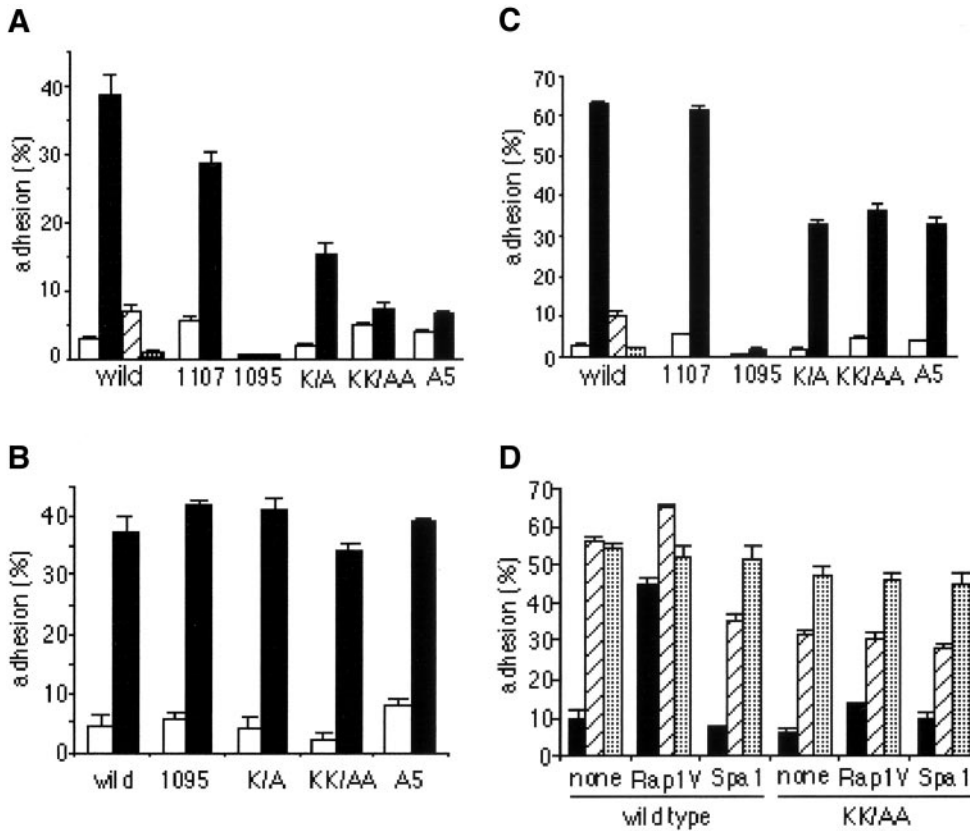


Figure 2. Adhesion of BAF transfectants to ICAM-1. (A) Adhesion to ICAM-1 of BAF transfectants of wild-type LFA-1 (wild), α L Δ 1107/ β 2 (1107), α L Δ 1095/ β 2 (1095), α LK1097A/ β 2 (K/A), α LK1097A/K1099A/ β 2 (KK/AA), or α LA5/ β 2 (A5) with (closed bar) or without (open bar) introduction of Rap1V12 was measured as described in MATERIALS AND METHODS. Adhesion to ICAM-1 in the presence of anti-LFA-1 mAb (TS1/22; shaded bar) or anti-ICAM-1 mAb (RR1/1; dotted bar) is also shown for BAF cells expressing wild-type LFA-1. (B) Adhesion of BAF transfectants to ICAM-1, as shown in A with (closed) or without (open) CBR-LFA1/2 antibody. (C) Adhesion of BAF transfectants to ICAM-1 with (closed) or without (open) PMA. (D) Adhesion to ICAM-1 of BAF cells/wild-type α L β 2 (wild-type) and α L K1097A/K1099A/ β 2 (KK/AA). BAF transfectants were further introduced with Rap1V12 and *Spa1*, as indicated. Adhesion assays were performed without (closed) or with PMA (shaded) or CBR-LFA1/2 (dotted). Means and SEs of triplicate determinations are shown.

activation of α L mutants by an activating antibody, CBR-LFA1/2, resulted in adhesion levels comparable to that of wild-type LFA-1-expressing cells (Figure 2B), indicating that the lysine residues at 1097 and 1099 are critical amino acids in responding to the inside-out signal of Rap1V12, but not to external stimuli.

To compare the Rap1V12 responsive site to that of PMA, we examined PMA-stimulated adhesion of BAF cells with the same set of mutations (Figure 2C). PMA responsiveness was intact upon truncation up to 1107, but was abrogated by deletion up to 1095. Alanine substitution of K1097 decreased PMA-induced adhesion to about a half. However, neither α L K1097A/K1099A nor α L A5 failed to further decrease PMA-stimulated adhesion to ICAM-1. Thus, the alanine substitution of K1097/K1099 abrogated the responsiveness of LFA-1 to Rap1, but partially affected that to PMA.

The partial inhibition of PMA-stimulated adhesion by the K1097/K1099 may reflect that PMA stimulates Rap1 (Reedquist and Bos, 1998) as well as PKC. Indeed, *Spa1*, a Rap1-specific GAP (Kurachi *et al.*, 1997) partially inhibited PMA-stimulated adhesion, the level of which was comparable to that in K1097A/K1099A (Figure 2D). PMA-stimulated adhesion of the K1097A/K1099A was not further affected either by *Spa1* or Rap1V12 expression. *Spa1* or Rap1V12 did not affect adhesion by CBR-LFA1/2 in cells expressing wild-type and K1097/K1099 (Figure 2D). The expression levels of Rap1V12 or *Spa1* were similar in wild type and K1097A/K1099A (our unpublished results). These results support the idea that PMA stimulates adhesion at least in part by Rap1.

On the other hand, truncations to the amino acid residue 744 and 731 in the β 2 subunit did not affect Rap1V12- and PMA-induced adhesion, but rather tended to augment basal levels of adhesion (Figure 3). The point mutation of tyrosine735 (Y735A) in the tyrosine-based endocytosis motif

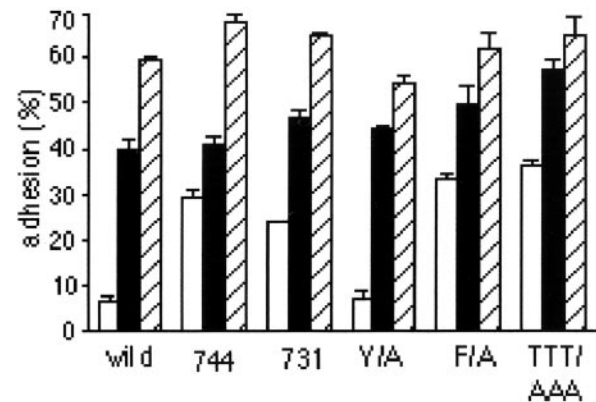


Figure 3. Adhesion to ICAM-1 of BAF transfectants of β 2 mutants. LFA-1 (wild), α L/ β 2 Δ 744 (744), α L/ β 2 Δ 731 (731), α L/ β 2Y735A (Y/A), α L/ β 2F754A (F/A), and α L/ β 2T758TT/AAA (TTT/AAA) with Rap1V12 expression (closed), PMA (shaded), or without stimulation (open). Means and SEs of triplicate determinations are shown.

(Fabbri *et al.*, 1999) did not have any effect on Rap1V12- and PMA-induced adhesion. Point mutations that were previously shown to be important in ligand- and PMA-induced adhesion, such as the T758TTAAA and F754A, did not affect adhesion induced by either Rap1V12 or PMA. These mutations again tended to augment basal adhesion levels. These results demonstrate that the α L cytoplasmic region is critical in responding to the inside-out signal of Rap1V12 and PMA in our system, whereas the β 2 cytoplasmic region may play a role in suppressing basal adhesion levels. Taken together, these results indicate that K1097/K1099 in the α L subunit constitutes the Rap1-responsive site of LFA-1.

K1097/K1099 Mutation Suppresses Rap1V12-induced Active Conformation and Ligand-binding Activity of LFA-1

We previously demonstrated that Rap1V12 induced LFA-1 conformation epitope detected by NKI-L16 and also augmented ligand-binding activity (Katagiri *et al.*, 2000). We examined whether the α L-K1097A/K1099A mutation affects Rap1V12-induced augmentation of NKI-L16 epitope expressions and LFA-1 ligand binding activity. BAF cells expressing wild-type LFA-1 express low levels of the NKI-L16 epitope (Figure 4A). Introduction of Rap1V12 increased NKI-16 expression levels (3.6 ± 0.67 -fold, $n = 3$). In contrast, the basal expression level of the NKI-L16 epitope was decreased in cells expressing the α L-K1097A/K1099A mutant and was not significantly increased by Rap1V12 (1.2 ± 0.16 -fold, $n = 3$; Figure 4A). Therefore, K1097/K1099 is required for both basal and Rap1V12-stimulated NKI-L16 expression.

Soluble ICAM-1-Fc was used to measure ligand-binding activity of LFA-1. Introduction of Rap1V12 into cells expressing wild-type LFA-1 augmented soluble ICAM-1 binding ~ 1.6 -fold (Figure 4B). Cells expressing the K1097/K1099 mutant did not show a significant increase of the binding of soluble ICAM-1 upon introduction of Rap1V12 (Figure 4B). In contrast, CBR-LFA1/2 increased soluble ICAM-1 bindings of both wild type and K1097/K1099 (Figure 4B). Other α L mutants (Δ 1095, K1097A, A5) also failed to increase soluble ICAM-1 bindings by Rap1V12 (Table 1). These results showed that the K1097/K1099 mutation abrogated ligand-binding affinity change of LFA-1 induced by Rap1V12, but not by the external activation.

We also examined soluble ligand bindings of β 2 mutants (Table 1). Although some of β 2 mutants increased basal adhesion levels, we could not detect significant increases of ligand-binding activity of β 2 mutants. In contrast, all of β 2 mutants increased ligand-binding affinity by Rap1V12 expression, indicating that the β 2 cytoplasmic region examined here is not required for affinity modulation of LFA-1 by Rap1. Taken together, these results suggest that the K1097/K1099 is critical for Rap1V12-induced adhesion via conformation and affinity modulation of LFA-1.

Defective Adhesive Responses of the α L Mutant to TCR Cross-linking and SDF-1

We then investigated the importance of the α L cytoplasmic region in the response to physiologically relevant stimuli. Previously, we demonstrated that Rap1 is the major inside-out signal for LFA-1 triggered by TCR (Katagiri *et al.*, 2002). To examine whether the Rap1V12 respon-

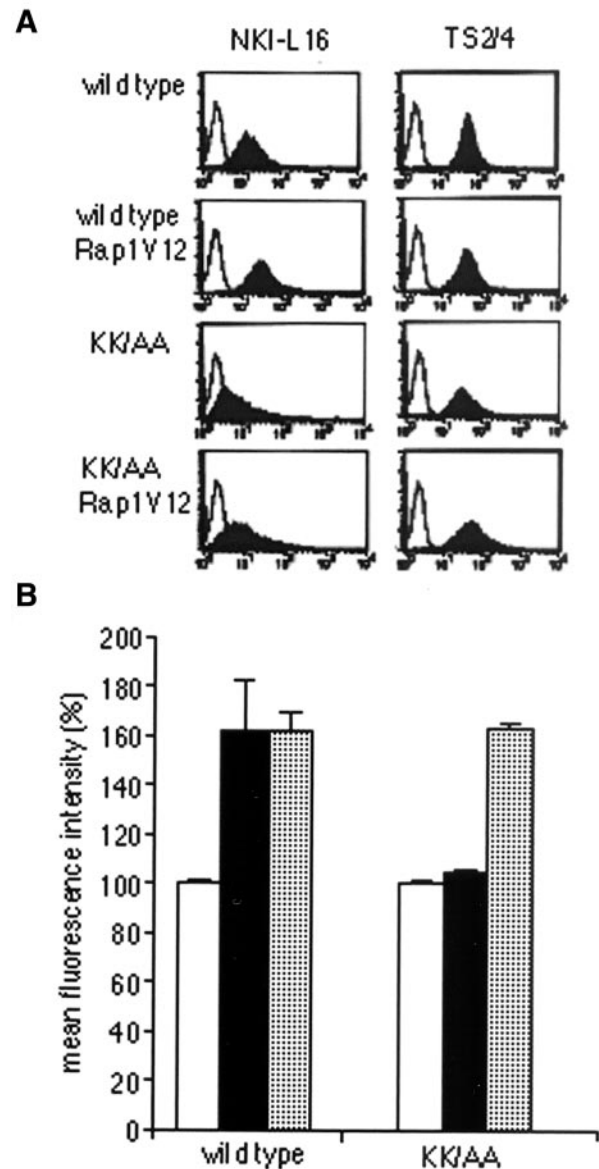


Figure 4. Effects of the K1097A/K1099A mutation on conformation and ligand-binding activity of LFA-1. (A) Expressions of the NKI-L16 epitope (NKI-L16) and total LFA-1 (TS2/4) in wild-type α L β 2 (wild-type) and α L-KK1097/1099AA/ β 2 (KK/AA) with or without Rap1V12 as indicated. The expression levels of the clustering-associated epitope and total surface LFA-1 were examined by staining with NKI-L16 and TS2/4 mAb, respectively. (B) Binding of soluble ICAM-1-Fc to wild-type LFA-1 and α L-K1097A/K1099A/ β 2 (KK/AA) with Rap1V12 expression (closed bar), or CBR-LFA1/2 (dotted), or without stimulation (open) was measured by FACS, as described in MATERIALS AND METHODS. Mean fluorescence intensities of soluble ICAM-1 binding were normalized to the mean fluorescence of TS2/4 (total LFA-1 expression) and presented in percentages to that of wild-type LFA-1 without stimulation. The mean \pm SD of three experiments are shown.

sive site is also important in TCR-stimulated adhesion to ICAM-1, we used a mutant Jurkat cells (J β 2.7) that are deficient in the endogenous α L subunit (Weber *et al.*,

Table 1. Soluble ligand-binding activity of LFA-1 mutants

LFA-1 mutants	None	Rap1V12
αL mutants		
Wild type	1	1.58 \pm 0.190*
Δ 1107	0.94 \pm 0.085	1.53 \pm 0.046*
Δ 1095	0.98 \pm 0.056	1.11 \pm 0.118
K/A	0.95 \pm 0.061	1.01 \pm 0.237
KK/AA	1.02 \pm 0.040	1.02 \pm 0.010
A5	0.99 \pm 0.049	0.95 \pm 0.038
β2 mutants		
Δ 744	0.90 \pm 0.146	1.63 \pm 0.030*
Δ 731	0.91 \pm 0.159	1.45 \pm 0.055*
Y/A	1.09 \pm 0.149	1.44 \pm 0.110*
F/A	1.16 \pm 0.244	1.78 \pm 0.175*
TTT/AAA	0.97 \pm 0.146	1.70 \pm 0.176*

Soluble ICAM-1-Fc bindings of BAF/LFA-1 transfectants with or without Rap1V12 were determined by flow cytometry as described in MATERIALS AND METHODS. Abbreviations of LFA-1 mutants are used as in Figures 2 and 3. Mean fluorescence of soluble ICAM-1 binding of each mutant was normalized to the wild type LFA-1 expression. Data shown are the average ratios to wild type \pm SD determined from three independent experiments. *Statistical significance was determined using Student's *t* test ($p < 0.01$).

1997b) to reconstitute the wild-type, K1097A/K1099A, and A5 mutants of α L and then examined the mutation effects on TCR-stimulated adhesion. Transfection of J β 2.7 cells with wild-type or mutant α L recovered expressions of both α L and β 2 (Figure 5A). J β 2.7 expressing the wild type, but not parent cells adhered to ICAM-1 with TCR cross-linking, which was inhibited with anti-LFA-1 or anti-ICAM-1 antibody (Figure 5B). In contrast, J β 2.7 expressing α L with K1097A/K1099A or A5 poorly adhered to ICAM-1 with TCR cross-linking, whereas CBR-LFA1/2 stimulated adhesion equivalent to J β 2.7 expressing the wild-type α L, indicating the α L mutants in Jurkat cells are not defective for cell adhesion (Figure 5B). Collectively, these results demonstrate that the Rap1-responsive site in the α L cytoplasmic region plays a major role in adhesion of Jurkat cells stimulated with TCR.

We have recently found that SDF-1 activates Rap1 rapidly and increases ICAM-1 binding peaking at 10 min after stimulation in BAF cells (our unpublished results). We took advantage of this response and compared SDF-1-stimulated adhesion to ICAM-1 of BAF cells expressing wild-type and α L-K1097A/K1099A mutant (Figure 6). Shear-resistant attachment to ICAM-1 was increased ~2–4-fold in BAF expressing wild-type in the presence of SDF-1 (Figure 6). In contrast, BAF cells expressing α L-K1097A/K1099A stimulated with SDF-1 were poorly attached on ICAM-1 under flow with little significant increase at an early time point. BAF cells with α LA5 also showed a defective response similar to those expressing K1097A/K1099A (our unpublished results). Therefore, mutations of the Rap1V12-responsive site in the α L cytoplasmic region greatly compromised shear-resistant adhesion induced by SDF-1.

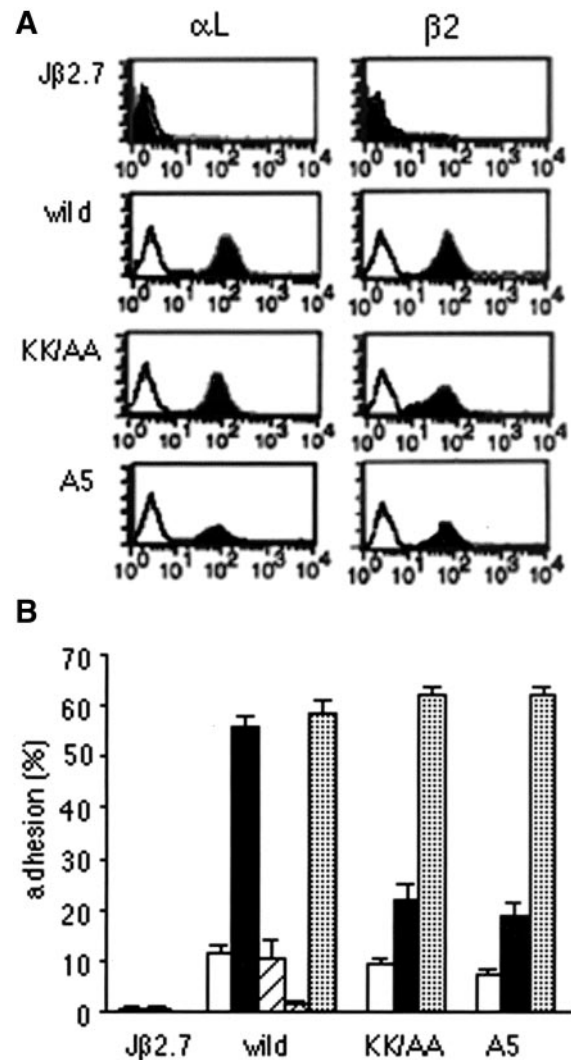


Figure 5. Effects of α L mutations on adhesion to ICAM-1 induced by TCR cross-linking. (A) Expressions of α L and β 2 in parental J β 2.7 cells and J β 2.7 transfectants introduced with wild-type α L (wild-type), α L K1097A/K1099A (KK/AA), or α LA5. LFA-1 expression was examined by staining with TS2/4 (α L) and TS1/18 (β 2), followed by FITC-labeled anti-mouse IgG F(ab')₂ fragments. (B) Adhesion to ICAM-1 of J β 2.7 transfectants stimulated by TCR cross-linking. Adhesion of parental J β 2.7 cells and J β 2.7 transfectants as in A was unstimulated (open) or triggered by anti-CD3 antibody (OKT3; closed). Adhesion of J β 2.7 transfectants with wild-type α L triggered by anti-CD3 antibody was also examined in the presence of anti-LFA-1 antibody (TS1/22; hatched), anti-ICAM-1 antibody (RR1/1; double hatched), or CBR-LFA1/2 (dotted). Means and SEs of triplicate determinations are shown.

Mutation at Y735 in the β 2 Cytoplasmic Region Impaired Internalization of LFA-1

The β 2 cytoplasmic region has a unique tyrosine residue at 735 in a short sequence (YRRF) that is similar to tyrosine-based endocytosis motif (YXX Φ , Φ is a bulky hydrophobic amino acid). We examined the effect of this mutation on internalization of LFA-1 (Figure 7). Surface expressions of

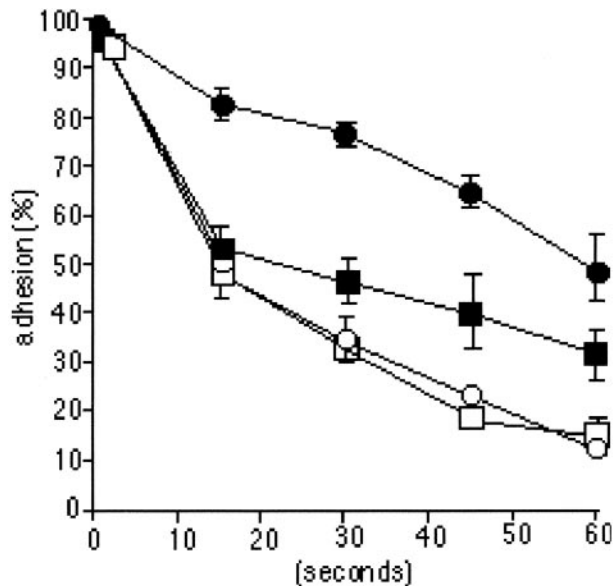


Figure 6. Effect of α L-K1097/K1099A mutation on SDF-1-induced adhesion to ICAM-1. Adhesion assays under shear flow were performed as described in MATERIALS AND METHODS. Cells of wild-type LFA-1 (\circ , \bullet) or KK/AA mutant (\square , \blacksquare) were incubated for 10 min in the ICAM-1-coated flow chamber with (closed) or without (open) 20 nM SDF-1, followed by washing with shear stress at 2.0 dyn/ml for 1 min. Shear-resistant attached cells were recorded at the time indicated and plotted as a percentage of input cells in the microscopic field. Injected cell numbers were 5×10^5 and ~ 120 –170 cells were accumulated in the microscopic field before washing. There were no significant difference of accumulated cell numbers in each experiment. Shear-resistant attachment in the presence of anti-LFA-1 or anti-ICAM-1 was $<10\%$ after washing for 30 s. Few cells were attached with BSA coating alone. Means and SEs of triplicate experiments are shown.

wild-type LFA-1 was spontaneously reduced to $\sim 40\%$ of the initial expressions after 15-min incubation at 37°C and returned to $\sim 80\%$ over the next 30 min (Figure 7A). The surface LFA-1 reduction was accompanied with the appearance of cytoplasmic punctate spots stained with anti-LFA-1 antibody (Figure 7B). In contrast, the Y735A mutant was found to be defective in internalization with reduction of surface expressions <10 – 20% and no significant accumulation of LFA-1 in the cytoplasm. An alanine substitution for F754 in the NPLF sequence did not affect the internalization and recycling (our unpublished results). Introduction of Rap1V12 did not affect the level and kinetics of LFA-1 internalization of wild-type and mutant Y735A (Figure 7A). Internalization and recycling of LFA-1 also occurred in T lymphoblasts in a similar extent and time course. These results indicate that wild-type LFA-1 is spontaneously internalized and recycled in lymphoid cells and Y735 in the $\beta 2$ cytoplasmic region plays a critical role in the internalization process.

Impaired Deadhesion of Rap1V12-induced Migration in Y735A-expressing Cells

As shown above, Y735A mutation inhibited internalization of LFA-1, but did not affect adhesion to ICAM-1 induced by

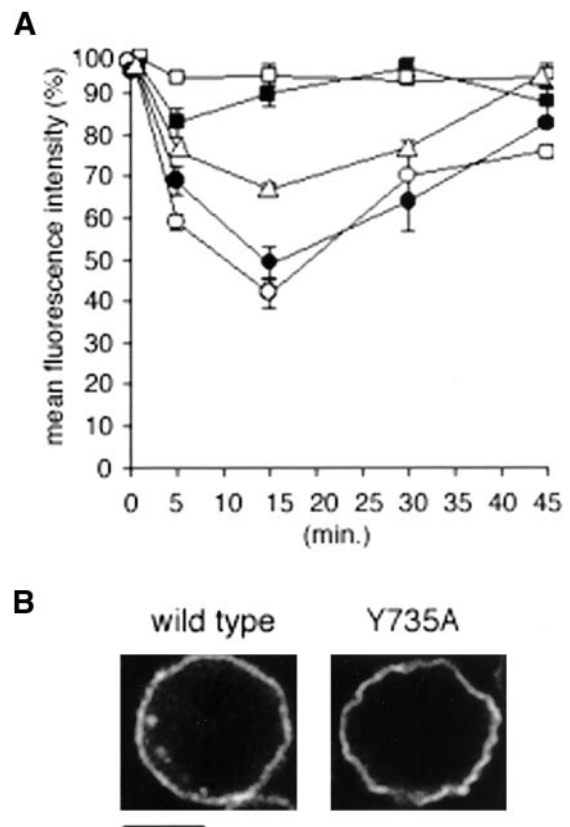


Figure 7. Y735A mutation inhibits the internalization of LFA-1. (A) Internalization of LFA-1. BAF transfectants of wild-type LFA-1 (\circ , \bullet) and α L/ β 2Y735A (\square , \blacksquare) with (closed) or without (open) Rap1V12 introduction, and T lymphoblasts (triangle) were examined for kinetics of modulation of surface LFA-1 levels at 37°C as described in MATERIALS AND METHODS. The averages of the mean fluorescence intensities of LFA-1 expressions detected by a flow cytometer from three (BAF cells) or two (T lymphoblasts) representative experiments are shown with SEs. (B) Distribution of LFA-1. Wild-type LFA-1 or α L/ β 2Y735A in BAF cells were labeled with Alexa488-conjugated T52/4 on ice. Cells were then incubated in suspension at 37°C for 15 min, a peak time point of downmodulation of surface LFA-1, and fixed for observation using a confocal laser microscope. Note that wild-type LFA-1, but little α L/ β 2 Y735A if any, was detected as multiple cytoplasmic punctate spots. Bar, 10 μm .

Rap1V12 (Figure 3). However, we found that the morphology of Y735A-expressing cells attached on ICAM-1 was quite different from that of wild-type cells. As shown in Figure 8, cells expressing Y735A showed a long-stretched cell shape, which was often $>100 \mu\text{m}$ in length. Time-lapse observation on attached cells revealed that Rap1V12-expressing cells with wild-type LFA-1 were highly motile. In contrast, Rap1V12 expressing cells with Y735A were often forced to be stretched because of impaired detachment at the rear end when the front end moved forward, leading to sudden rear end detachment, or pulling back toward the rear. To confirm firm attachment by LFA-1 on the rear in Y735A cells, we visualized attached sites with mAb24, which recognizes a conformational epitope that appears on

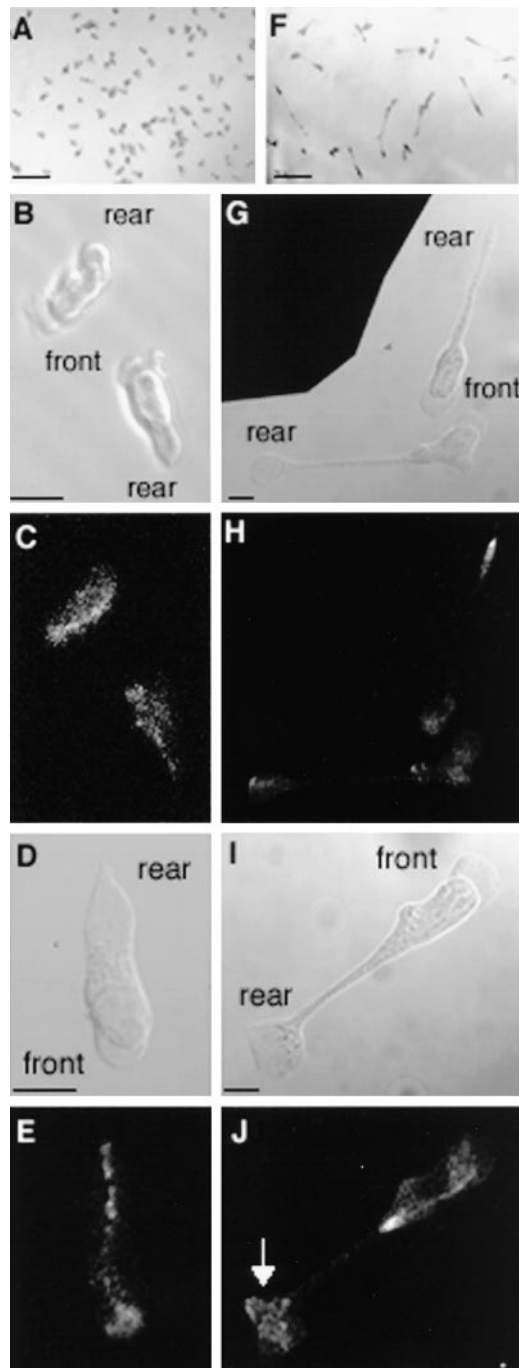


Figure 8. Defective cell detachment expressing α L/ β 2Y735A. Rap1V12 expressing BAF cells of wild-type LFA-1 (A–E) and α L/ β 2Y735A (F–J) were incubated on ICAM-1-coated glass-chambers at 37°C for 30 min. After washing off unattached cells, cells were fixed and stained with mAb24 (C, E, H, and J), which recognizes ligand-engaged LFA-1. Morphology of attached cells at lower (A and F) or higher (B, D, G, and I) magnifications are shown. Bars, 100 μ m (A and F) and 10 μ m (B, D, G, and I). In G and H, ROI was set to the field where cells were attached. Note that elongated cell shape of BAF cells expressing Y735A with broad attachment areas of the cell rear through LFA-1 and ICAM-1 (arrow), as indicated by mAb24 staining. Cell front and rear are also indicated. Time-lapse videos for cell migration of BAF cells on ICAM-1 are available on-line.

activated or ligand-engaged LFA-1 (Cabanas and Hogg, 1993; Lu *et al.*, 2001). A small increase of the mAb24 expression by Rap1V12 expressions was detected in suspension with FACS analysis, but was not clearly seen under microscope (our unpublished results). However, upon attachment of wild-type expressing cells to ICAM-1, the mAb24 epitope was upregulated and broadly distributed in the front and cell body and waned toward the rear (Figure 8, C and E), suggesting that firm attachment at the front is attenuated toward the rear. In contrast, cells expressing Y735A showed intense staining of the mAb24 epitope in broadly spread rear areas in addition to the cell body and the front (Figure 8, H and J), suggesting that ICAM-1-engaged LFA-1 are remained on the rear. The mAb24 epitope intensity of the rear (the stretched rear part) in Y735A was increased approximately twofold when compared with that of the constricted rear in wild type. We also found that detachment processes are impaired in Y735A-expressing cells without Rap1V12 when stimulated with SDF-1. In this case, BAF cells expressing wild-type LFA-1 showed active random migration on ICAM-1. However, BAF cells expressing Y735A were stuck at the rear with active movement of the front. Cells were not stretched out, probably because of rather weak adhesion when stimulated with SDF-1. Taken together, these results strongly suggest that internalization of LFA-1 mediated by the tyrosine-based endocytosis motif in the β 2 subunit plays a pivotal role in detachment during cell migration.

DISCUSSION

In this study, we identified previously unrecognized lysine residues in the α L subunit that are critical in adhesion by constitutively active Rap1. The K1097A/K1099A mutation in the α L subunit suppressed the conformation (NKI-L16) and ligand-binding affinity of LFA-1, which also resulted in defective LFA-1 binding to ICAM-1 stimulated by TCR cross-linking and SDF-1. Although the β 2 cytoplasmic region was not required for Rap1V12-induced adhesion, disruption in the β 2 tyrosine-based endocytosis motif inhibited LFA-1 internalization, and surprisingly impaired deadhesion processes in cell migration stimulated by Rap1V12 or SDF-1. Our study demonstrates the primary role of the α L cytoplasmic region in the response to Rap1 and the inside-out signaling by SDF-1 and TCR and the β 2 tyrosine-based endocytosis motif in the subsequent deadhesion event.

The lysine residue at 1097 is conserved in α L, α M, α X, and α D in the β 2 integrin family. α L has an additional lysine at 1099 that is unique to α L. An alanine substitution for K1097 inhibited more than half, but not completely. Because both lysines had to be mutated in order to inhibit adhesion induced by Rap1V12 almost completely, the K1099 probably plays a compensatory role in Rap1V12 responsiveness. The cytoplasmic regions of the integrin α subunits have the conserved GFFKR motif, but otherwise were diverse amino acid sequences (Sastri and Horwitz, 1993). However, there are single or multiple of lysine or homologous arginine residues at various positions after the GFFKR motif in the α subunit of other integrins. α 4 has lysine (human) or arginine (mouse) at the position identical to α L K1097. Indeed, it was reported that Rap1V12 also stimulated VLA-4 (Reedquist *et al.*, 2000). It will be interesting to examine whether these lysine or arginine residues contribute to Rap1V12-induced adhesion.

The requirement of the lysine residues by Rap1V12-induced adhesion is physiologically relevant, because the K1097A/K1099A mutation greatly compromised adhesion stimulated by TCR and SDF-1. These results are consistent with our previous study that showed that Rap1 was the major inside-out signal triggered by TCR (Katagiri *et al.*, 2002). Furthermore, our recent study has shown that Rap1 activation by SDF-1 and SLC stimulates adhesion through LFA-1 (Shimonaka, *et al.*, 2003). Therefore, it is likely that Rap1 activation by physiologically relevant stimuli also modulates the LFA-1 adhesive activity through this site.

We previously reported that Rap1 increased the NK1-L16 epitope of LFA-1 as well as ligand-binding affinity to ICAM-1 (Katagiri *et al.*, 2000). We showed in this study that the K1097A/K1099A mutation suppressed these changes of LFA-1, suggesting that Rap1 modulates conformations and ligand-binding activity of LFA-1 through this site. NK1-L16 recognizes dimerized LFA-1 (Binnerts and van Kooyk, 1999). Because ICAM-1 is expressed on the cell surface mostly as a dimer (Miller *et al.*, 1995; Reilly *et al.*, 1995), dimerized LFA-1 should facilitate binding to ICAM-1. It should be pointed out that upregulation of the L16 epitope does not necessarily mean the active conformation of LFA-1 (van Kooyk *et al.*, 1991), LFA-1 clustering was reported to be correlated with increased adhesion by LFA-1 in primary lymphocytes derived from Rap1V12-transgenic mice (Sebzda *et al.*, 2002). Our results showed that ligand-binding activity to soluble ICAM-1 was also increased significantly by Rap1V12 and inhibited by the specific mutation of the α L cytoplasmic region, suggesting that K1097/K1099 is also involved in LFA-1 affinity modulation by Rap1V12.

Rap1-induced ligand binding affinity detected by soluble ICAM-1-Fc is rather modest (up to twofold), which was often below detection levels when Rap1V12 expression was limited. It was previously demonstrated that adhesion-stimulatory divalent cations such as Mg^{2+} increased soluble ICAM-1-Fc binding threefold (Stewart *et al.*, 1998). The activating antibody, CBR-LFA1/2 induced soluble ICAM-1 bindings and adhesion to immobilized ICAM-1, which were comparable to those of Rap1V12-expressing cells (Figures 2 and 5). Therefore, this small increase of ligand-binding affinity is probably enough to trigger strong adhesion. The affinity of the α L I domain can range from 200 nM to 2 mM by stabilizing the open and closed conformations of the I domain (Shimaoka *et al.*, 2001). Although we could not measure accurately ligand-binding affinity of LFA-1 induced by Rap1V12 with conventional methods, Rap1-induced affinity change is likely in the order of the micromolar range, considering from the soluble ICAM-1 concentration used here ($\sim 4.5 \mu$ M). This small increase of ligand-binding affinity may reflect that Rap1 induces limited numbers of the high-affinity state or possible intermediate affinity states (Shimaoka *et al.*, 2002). It could be envisioned that the initial weak ligand binding could result in a shift of equilibrium toward the high-affinity state by stabilizing with immobilized ligands. Alternatively, it could lead to further conformational changes of LFA-1 that result in a fully open, high-affinity conformation, as is proposed for LFA-1 (Cabanas and Hogg, 1993) and α IIb β 3 (Takagi *et al.*, 2002). As mentioned above, Rap1 also enhances LFA-1 clustering on the cell surface. This may further help stabilize the initial weak ligand bindings.

Unlike the β 2 cytoplasmic region, the α L cytoplasmic region has not been investigated intensively with only a few proteins reported that interact with the α L cytoplasmic region. The GFFKR motif is well conserved in the integrin α subunit and its disruption made α L constitutively active (Lu and Springer, 1997). It is proposed that the arginine residue in the motif interacts with acidic amino acids in the β 1 cytoplasmic region through a salt bridge and acts as "hinge" (Hughes *et al.*, 1996). Calreticulin was reported to bind the GFFKR motif (Rojiani *et al.*, 1991), but its physiological significance in leukocytes is yet unclear (Coppolino *et al.*, 1997). Paxilin is reported to bind the α 4 cytoplasmic region through the α 4 unique sequence and facilitate cell migration (Liu *et al.*, 1999; Liu and Ginsberg, 2000). Identification of critical amino acids in Rap1-induced adhesion facilitates the search for the interactor and helps understand how Rap1 modulates clustering and ligand-binding affinity of LFA-1.

PMA, a strong activator of PKC, is also known to activate Rap1 (Reedquist and Bos, 1998). Our study showed that K1097A/K1099A mutation of α L abrogated Rap1V12-induced adhesion and partially affected PMA-induced adhesion. The reduction was less obvious at higher concentrations of ICAM-1 (our unpublished results), showing that Rap1 dependency of PMA-stimulated adhesion tends to be more apparent at lower concentrations of ICAM-1. This result is consistent with the notion that PMA-induced adhesion is mediated in part by Rap1, and PMA-induced, Rap1-independent adhesion is presumably through PKC. The result that SpaI reduced PMA-stimulated adhesion to the level of that in K1097A/K1099A supports this notion. Our result is in line with the recent report (Liu *et al.*, 2002), showing that Rap1 dependency on PMA-stimulated FN adhesion in Jurkat cells. The relative contribution of Rap1 and PKC may vary in the different cell context and integrin types and ligand concentrations. Although Rap1 contributed to PMA-induced adhesion at least in part, PMA did not increase detectable soluble ICAM-1 binding either in wild type or K1097/K1099 (0.95 ± 0.06 - and 0.99 ± 0.09 -fold increase, respectively). This result is consistent with the earlier reports that PMA stimulated diffusion and clustering of LFA-1, but not ligand binding affinity (Kucik *et al.*, 1996; Stewart and Hogg, 1996; Stewart *et al.*, 1998). Failure to detect affinity change upon PMA stimulation could be due to insufficient levels of Rap1 activation, resulting in affinity change below detectable levels or unknown inhibitory effects of PMA on affinity modulation. In either cases, this result also confirmed that the soluble ICAM-1-Fc used for affinity measurement could not detect clustered LFA-1 with low-affinity states.

Our attempt to identify the specific amino acid residues that are required in PMA-induced, Rap1-independent adhesion was unsuccessful because an alanine substitution of all amino acids from 1097 to 1101 failed to abrogate adhesion. There are no other conserved amino acids between 1095 and 1107. This result is in line with the previous study that demonstrated that adhesive activities of α 2 and α 4 were lost by deletion just after the GFFKR motif and recovered by adding a short, nonspecific stretch of amino acid residues (Kassner *et al.*, 1994). Our result supports the idea that PMA does not require specific amino acid residues in the α L cytoplasmic region.

The β 2 cytoplasmic region was not needed for adhesion stimulated by Rap1V12. Instead, truncation of the β 2 cyto-

plasmic region had a tendency to increase basal levels of adhesion. The complete deletion of the $\beta 2$ cytoplasmic region also resulted in increased basal adhesion in K562 cells (van Kooyk *et al.*, 1999). However, we did not detect an increase in ligand-binding affinity of $\beta 2$ mutants (Table 1). The cytoplasmic region of $\beta 2$ can associate with talin, filamin, vinculin, and α -actinin (Sampath *et al.*, 1998). The linkage of integrin with cytoskeleton promotes adhesion by integrin clustering, formation of focal contacts, and cell shape changes (Hynes, 1992). However, it has been increasingly realized that cytoskeleton association also functions as a restraint of integrin mobility on the cell surface that suppresses basal adhesion levels (Kucik *et al.*, 1996; Zhou *et al.*, 2001), and a release from the cytoskeletal restraint can serve as an important regulatory point as the inside-out signals (Stewart *et al.*, 1998; van Kooyk and Figdor, 2000). Thus, the increase in basal adhesion by truncations or point mutations of the $\beta 2$ cytoplasmic region may reflect a release of cytoskeletal restraint on LFA-1. Our result that the $\beta 2$ cytoplasmic region was not needed for Rap1 adhesion suggests that the Rap1 regulatory point is distinct from modulation of cytoskeletal linkage through the $\beta 2$ cytoplasmic region.

The more important function of the $\beta 2$ cytoplasmic region demonstrated in our study is that the tyrosine-based endocytosis motif mediates LFA-1 internalization and plays a pivotal role in detachment. LFA-1 internalization and recycling occurs spontaneously and was blocked by an alanine substitution of Y735. This tyrosine-based endocytosis motif (YXX ϕ) is conserved in the $\beta 2$ and $\beta 7$ integrins that are expressed specifically in leukocytes. LFA-1 internalization through this site was previously unrecognized in the study using CHO cells (Fabbri *et al.*, 1999), probably because the two NPXF motifs act as a redundant endocytosis signal in CHO cells. In integrin-mediated migration, it is important to recruit integrins to the front to form new attachments. In CHO cells, the Y735A mutation impaired LFA-1 recycling to the ruffling membrane and decreased haptotactic migration (Fabbri *et al.*, 1999). We could not examine whether the Y735A mutation is also involved in recycling because its internalization was blocked by this mutation. Internalized LFA-1 was detected as punctate spots that were not colocalized with internalized TRITC-transferrin (our unpublished results), in contrast to the report that showed internalized $\beta 1$ integrin was colocalized with transferrin/the transferrin receptor in breast carcinoma cells (Ng *et al.*, 1999). These results suggest that the internalization and recycling pathway of LFA-1 is distinct from that of the $\beta 1$ integrin and the transferrin receptor.

Leukocyte migration is much faster than that of fibroblasts, and Rap1V12-expressing BAF cells also moves fast (10–15 $\mu\text{m}/\text{min}$). In constitutively adherent cells, such as fibroblasts, mechanical dissociation of dragged rear parts from the rest of the cell body is often observed (Regen and Horwitz, 1992). In contrast, a migrating leukocyte has a distinct cellular structure at the cell rear called uropod, which is thought to facilitate detachment of migrating leukocytes (Sanchez-Madrid and del Pozo, 1999). In addition to the morphological advantage, our result suggests that LFA-1 internalization of mediated by the YXX ϕ motif promotes rapid detachment of the leukocyte rear end. Considering the fact that Rap1V12 stimulates cell adhesion and migration at the same time, we favor the idea that detachment of the rear

end in migrating leukocytes is regulated through internalization, rather than the overall switch-on and-off of integrin activity. It is possible that ICAM-1-bound LFA-1 is inactivated before internalization and this inactivation process is also impaired by the Y735A mutation. However, a rapid, transient increase of LFA-1 adhesive activity of the Y735A was induced upon SDF-1 stimulation, peaking at 10 min, and rapidly downregulated after 20 min, which was very similar to that of the wild-type-expressing cells (our unpublished results). Thus, it is unlikely that the Y735A mutation impairs LFA-1 inactivation. Recent advancement of microscopic technology to visualize a single molecule will be helpful to clarify LFA-1 activation and conformational changes during migration.

Our study clearly shows that attachment and detachment are regulated through the distinct αL and $\beta 2$ cytoplasmic regions in cells stimulated by Rap1V12 and SDF-1. Identification of Rap1 effector molecules and interactors to the αL cytoplasmic region is crucial in elucidating how integrin adhesive activities are modulated in response to chemokines and the TCR. Our study provides an important clue for the better understanding of this process.

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