The Actin Cytoskeleton Regulates LFA-1 Ligand Binding through Avidity Rather than Affinity Changes*

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To elucidate the role of the cytoskeleton regulating avidity or affinity changes in the leukocyte adhesion receptor lymphocyte function-associated antigen-1 (LFA-1) (αβ2), we generated mutant cytoplasmic LFA-1 receptors and expressed these into the erythroleukemic cell line K562. We determined whether intercellular adhesion molecule-1 (ICAM-1)-mediated adhesion of LFA-1, lacking parts of its cytoplasmic tails, is regulated through receptor diffusion/clustering and/or by altered ligand binding affinity. All cytoplasmic deletion mutants that lack the complete β2 cytoplasmic tail and/or the conserved KVGFFKR sequence in the αL cytoplasmic tail were constitutively active and expressed high levels of the activation epitopes NKI-L16 and M24. Surprisingly, whereas these mutants showed a clustered cell surface distribution of LFA-1, the ligand-binding affinity as measured by titration of soluble ligand ICAM-1 remained unaltered. The notion that redistribution of LFA-1 does not alter ligand-binding affinity is further supported by the finding that disruption of the actin cytoskeleton by cytochalasin D did not alter the binding affinity nor adhesion to ICAM-1 of these mutants. Most cytoplasmic deletion mutants that spontaneously bound ICAM-1 were not capable to spread on ICAM-1, demonstrating that on these mutants LFA-1 is not coupled to the actin cytoskeleton. From these data we conclude that LFA-1-mediated cell adhesion to ICAM-1 is predominantly regulated by receptor clustering and that affinity alterations do not necessarily coincide with strong ICAM-1 binding.

The β2 integrin lymphocyte function-associated antigen-1 (LFA-1) (1) (CD11a/CD18 or αβ2) is a leukocyte-specific adhesion receptor that coordinates different adhesive and signaling interactions within the immune system (1–4). LFA-1 mediates cell-cell adhesion upon binding to its cellular ligand intercellular adhesion molecule-1 (ICAM-1) (5). At distinct sites in the body, leukocytes behave as adherent cells, whereas at other sites they have to circulate as nonadherent cells. This dynamic control of adhesion is regulated by binding strength, and the kinetics of interactions between adhesive ligands and β2 integrins. In addition, events such as lateral diffusion of integrins (6–11) and interactions with and reorganization of the cytoskeleton enforce adhesion (12, 13). Integrins are heterodimeric transmembrane molecules composed of an α chain that is noncovalently linked to a β chain. The cytoplasmic tails of LFA-1, as well as other integrins, are essential for control of adhesion. Mutation or deletion of specific cytoplasmic sequences causes integrins to become constitutively active and have also revealed amino acids located in the β2 cytoplasmic tail that interact with the cytoskeleton (14–16). On resting, lymphocytes LFA-1 is inactive. It is thought that the attachment of LFA-1 to the actin cytoskeleton keeps the integrin in an inactive state. Binding to its ligand ICAM-1 is only observed through intracellular signals (3, 17–19) by TCR/CD3 cross-linking that causes LFA-1 activation (17, 18). A temporary dislodgment from the actin cytoskeleton may facilitate lateral diffusion of β2 integrins into clusters (9, 10, 20, 21), as can be observed after disruption of the cytoskeleton by cytochalasin D or activation of calpains that facilitate adhesion of β2 integrins (22).

Activation of LFA-1 likely results in a conformational change in the α/β heterodimer, as evidenced by the expression of neoepitopes or activation epitopes (L16 and M24) (6, 23). This has led to the speculation that affinity changes in LFA-1 are associated with conformational alterations, leading to an enhanced binding to its ligand ICAM-1 (24). Both affinity (active conformation) and avidity (clustering) changes have been considered to be important for strong LFA-1-mediated cell binding (25). These affinity/avidity-induced conformational changes in LFA-1 depend on an intact cytoskeleton, physiological temperature, and on binding of divalent cations, Mg2+ in particular (7, 23, 26, 27). Binding of Ca2+ to LFA-1 supports clustering (high avidity state) of LFA-1 on the cell surface resulting in enhanced LFA-1-mediated adhesion (7, 8). Antibodies such as NKI-L16, which recognizes a Ca2+-dependent epitope on the α chain of LFA-1 have been used to detect clustered LFA-1 on the cell surface, whereas the antibody M24 recognizes a Mg2+-dependent epitope on LFA-1 that coincides the high affinity state of LFA-1 or ligand bound state (23, 28, 29). Although the cytoplasmic tails of the α and β chain LFA-1 are relatively short (58 and 45 amino acids, respectively) and do not contain any intrinsic kinase activity, the cytoplasmic tails seem to be involved in affinity or avidity regulation and cytoskeleton association. It has been demonstrated that the adhesiveness of LFA-1 is controlled by the cytoplasmic domain of the β2 subunit, because truncation of the cytoplasmic β2 tail, but not the αL tail, eliminates LFA-1 binding to ICAM-1 (30). Because deletion of the cytoplasmic domain of the αL subunit does not affect binding to ICAM-1, it is hypothesized that the cytoplasmic tail of αL is
predominantly involved in “post-ligand binding” events by integrins (30).

In this work, we have examined the role of the αL and β2 cytoplasmic domains of LFA-1 on their capacity to regulate ligand binding affinity and avidity, and on the interaction with the actin cytoskeleton network. We observed that the cytoplasmic tail of both the β2 chain and the GFFKR sequence in the αL cytoplasmic tail play a pivotal role in regulating ligand binding through induction of avidity changes rather than by affinity changes.

**EXPERIMENTAL PROCEDURES**

**Monoclonal Antibodies**—The monoclonal antibodies (mAbs) SPV-L7 (IgG1), NKI-L15 (IgG2a), and NKI-L16 (IgG2a) reactive with the α chain of LFA-1 were raised as described previously (31, 32). NKKI-L16 recognizes a Ca2+-dependent epitope on the α chain of LFA-1 (32). The nonbinding mAb TS2/4 (IgG1) reactive with αL (33), M24 (IgG1) that recognizes a Mg2+-dependent epitope on αL (23), mAb 60.3 (IgG1) directed against β2 (34), and mAb KIM185 (IgG1) used to activate β2 integrins (35), were kindly provided by Drs. E. Mertz, N. Hogg, J. Harlan, and M. Robinson, respectively.

**DNA Constructs**—The chimeric α chain constructs αLαS and αLαx were generated by PCR using oligonucleotides for the αL-containing at the 5’ end a StuI site (GATGACGGAGCGTCGCTCC, respectively) and at the 3’ end an AatII site (TTATAGCCGTCAATTTGGAGGACAT). Oligonucleotides for the αL and αx contained at the 5’ end an AatII site (ATTATTGACATCTAATACTTAC, respectively). Digestion of the PCR products with AatII followed by ligation of the two products was furthermore confirmed by cloning StuI and EcoXI digestion in the pBluescript vector containing the αL cDNA thereby creating an AatII site in the chimeric product. The ΔKGGFKKR mutant was created by PCR with primers containing at the 5’ end a StuI site and at the 3’ end an AatII site for αL (see above) and an αx PCR product using primers containing at the 5’ end a RsaI site (ATATGTTAACCACTGGAGAAGATGG) and at the 3’ end an EcoXI (TCTTCAAAGGCATTGGCTTCTGTTCTGCAC). Two PCR products were ligated and digested with RsaI in a chain removing only the KVVFKKR sequence from the αx cytoplasmic tail. The αL cytoplasmic deletion mutants Δ1088xα and Δ1095xα were generated by introduction of a termination codon into the αx cDNA with P-ALter. The following antisense oligonucleotides were used for mutagen and amplification (Δ1088xα: AGGCGTGCTTGTAACTGTTGTTTCCTC; Δ1095xα: TCTTCAAGGCTGCTAACTGTTGGCTTCC, respectively). Digestion of the PCR products with AatII followed by ligation of the two products was furthermore confirmed by cloning StuI and EcoXI digestion in the pBluescript vector containing the αL cDNA thereby creating an AatII site in the chimeric product.

**Delta 80 Transfectants**—LFA-1 transfectants were generated by electroporation of 107 cells in 0.8 ml of PBS at 250 V and 960 μF with either the wild-type αL (in pRc/CMV) and wild-type β2 subunit (in pCDMB), or truncated αL with β2 wild-type, truncated β2 and wild-type αL or double truncation mutants (36). K562-LFA-1 transfectants were cultured in RPMI 1640 medium (Life Technologies Ltd., Paisley, Scotland), supplemented with 10% fetal calf serum (Biowhittaker, Verviers, Belgium), 1% antibiotics/antimycotics (Life Technologies, Inc.). After 48 h, the neomycin analogue, gentamicin (2 mg/ml; Life Technologies Ltd.) was added to the culture medium. The different transfectants were sorted three or more times to obtain a homogeneous population of cells expressing high levels of LFA-1. Positive cells were stained with FITC-conjugated TS2/4 mAb and isolated using a Coulter Epics Elite cell sorter (Coulter, Hialeah, FL).

**Immunofluorescence Analysis**—Expression of LFA-1 on the transfectants was determined by immunofluorescence. Cells (2 × 106) were incubated (30 min, 4°C) in PBS containing 0.5% w/v bovine serum albumin (Roche Molecular Biologicals, Mannheim, Germany) and 0.01% sodium azide (10 μM, Merck, Hohenbrunn, Germany), with appropriate dilutions of either an anti-integrin mAb or an isotype-matched control antibody. For L16 staining, cells were washed twice with cation-free PBS and staining was performed in the presence or absence of 1 mM CaCl2. For M24 staining, LFA-1 was activated by 1 mM MnCl2 for 30 min at 37°C in TSM, cells were washed and followed incubation with monoclonal antibody M24 for 30 min, 4°C. Subsequently, cells were incubated with FITC-labeled goat (Fab’2) anti-mouse IgG mAb (Zymed Laboratories, Inc., San Francisco, CA) for 30 min at 4°C. The relative fluorescence intensity was measured by FACSscan analysis (Becton Dickinson).

**Ligand Coating of Fluorescent Microspheres**—Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes) were coated with adhesion ligands as follows. Streptavidin was covalently coupled to the TransFluorSpheres as described by the manufacturer. 20 μl of streptavidin (5 mg/ml in 50 mM MES buffer) was added to 50 μl TransFluorSpheres. 30 μl of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (1.33 mg/ml) was added and the mixture was incubated at room temperature for 2 h. The reaction was stopped by the addition of glycine to a final concentration of 0.2 M. The coated beads were washed three times with PBS (50 mEq NaCl, pH 7.4) and resuspended in 150 μl of PBA (PBS, 0.5% bovine serum albumin (w/v), 0.002% NaAz). This suspension remained stable for 2 months if stored at 4°C. The streptavidin-coated beads (15 μl) were incubated with biotinylated goat-anti-human Fc (Fab’2) fragments (6 μg/ml) in 0.5 ml PBA for 2 h at 37°C. The beads were washed once with PBA and incubated with human IgG1 Fc-fused ligands (2500 ng/ml) in 0.5 ml overnight at 4°C. The IgG1 Fc-fused ligands used in this study were ICAM-1Fc. ICAM-1Fc consists of the extracellular part of both proteins fused to a human IgG1 Fc fragment. ICAM-1Fc was produced in Chinese hamster ovary K1 cells cotransfected with the ICAM-1-gIgG1Fc (37) and pE14 vector similarly as was described for CD4 T lymphocyte glycoprotein (38). The ICAM-1Fc concentration in the supernatant was monitored by an IgG1 enzyme-linked immunosorbent assay and the supernatant was used without further purification. The ligand-coated beads were washed with PBA, resuspended in 100 μl PBA, and stored at 4°C.

**Fluorescent Beads Adhesion Assay**—For cell adhesion to ICAM-1, cells were resuspended in TSM (20 μl Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2, 0.5% bovine serum albumin (w/v), 5 × 106 cells/ml). 50,000 cells were preincubated with/without LFA-1-blocking mAb (20 μg/ml) for 10 min at room temperature in a 96-well V-shaped bottom plate. The ligand-coated TransFluoroSpheres (20 beads/cell) and different integrin stimuli (100 nM PMA (Calbiochem), LFA-1-activating mAbs, KIM185 (10 μg/ml), respectively), were added, and the suspension was incubated for 30 min at 37°C. The cells were washed with TSA and incubated for 10 min at room temperature with FITC-conjugated anti-TS2/4-antibody. The cells were washed with TSA and resuspended in 100 μl TSA. The LFA-1 transfectants that expressed distinct levels of LFA-1, as determined by staining for TS2/4-FITC, were gated (mean fluorescence intensity of 40–60), to analyze only those cells that have similar expression levels. Thus, this assay allows discrimination of different transfectants that express distinct levels of LFA-1. LFA-1-mediated adhesion was measured by flow cytometry using the FACSScan. Values are depicted as integrin specific adhesion, i.e. cell adhesion percentage minus cell adhesion percentage in the presence of a LFA-1 blocking mAb (NKI-L15), which was always less than 3%.

**Soluble ICAM-1Fc Binding**—Transfectants were resuspended in TSM (20 μl Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2, 0.5% bovine serum albumin (w/v), 5 × 106 cells/ml). 50,000 cells were preincubated with/without LFA-1-blocking mAb (20 μg/ml) for 10 min at room temperature in a 96-well V-shaped bottom plate. Different concentrations of purified soluble ICAM-1Fc was added together with medium or the LFA-1-activating mAbs, KIM185 (10 μg/ml), and the suspension was incubated for 30 min at 37°C. The cells were washed with TSA and incubated for 30 min at room temperature with FITC-conjugated goat-anti-human Fc-specific antibody (Jackson Immunoresearch Labs, West Grove, PA). The cells were washed with TSA and resuspended in 100 μl TSA. The percentage of positive cells was measured by flow cytometry using the FACSscan. Values are depicted as percentage of positive cells, i.e. cell adhesion percentage minus cell adhesion percentage in the presence of an integrin blocking LFA-1 mAb (NKI-L15), which was always less than 2% Alternatively, the concentration of soluble ICAM-1Fc that gives half-maximal adhesion (ED50) is depicted.

**Confocal Microscopy**—Cells were fixed with 0.5% paraformaldehyde. Fixed cells were stained with TS2/4 mAb (10 μg/ml) for 30 min at 4°C, followed by incubation with FITC-labeled goat (Fab’2) anti-mouse IgG mAb (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at room temperature. The cells were attached to poly-l-lysine-coated glass slides, after which cell surface distribution of integrins was determined by confocal laser scanning microscopy (CLSM) at 488 nm with a krypton/argon laser (Bio-Rad 1000). The CLSM settings were: lens, 60×; gain, 1300; pinhole, 1.5 μm; and magnification, 2.0×. The same instrument settings of the CLSM were used throughout the different experiments.
studies revealed that these chimeric cytoplasmic LFA-1 mutants did not show any altered cell surface distribution of LFA-1 compared with wild-type LFA-1 (data not shown). From these data we conclude that the C-terminal part of LFA-1, immediately after the KVGFFKR region, is not involved in the active connection of LFA-1 to the actin cytoskeleton network, regulating avidity or affinity changes.

Domains in the α and β Cytoplasmic Tails Important in Regulating LFA-1 Function—To analyze in more detail, regions in the cytoplasmic α and β tail of LFA-1 that are involved in the avidity and/or affinity regulation of LFA-1/ICAM-1 binding, different αK and βK cytoplasmic tail deletion mutants were generated (Fig. 2). Cytoplasmic deletion mutants of the LFA-1 α chain were created by deleting the cytoplasmic tail before the highly conserved KVGFFKR region (Δ1088αK) and just after the KVGFFKR region (Δ1095αK), or by deleting only the KVGFFKR region (ΔKVGFFKRαK). Mutant ΔβK was generated by truncation of the β2 cytoplasmic tail from the amino acid 724 tail of β2, deleting the conserved DLRE motif (36). Thus, we created LFA-1 transfectants that lack complete or parts of the αK or βK cytoplasmic tail alone or that lack both αK and βK cytoplasmic tails (Δ1088αK/ΔβK and Δ1095αK/ΔβK). LFA-1 surface expression on these K562 transfectants was evaluated by staining for anti-β2 and anti-αK antibodies using flow cytometry (Fig. 3). All mutants expressed similar levels of LFA-1 except those LFA-1 mutants that lack both the αK and βK cytoplasmic tail (Δ1088αK/ΔβK and Δ1095αK/ΔβK) or that lacks only the KVGFFKR region (ΔKVGFFKRαK) expressed low amounts of LFA-1. Mutations in the cytoplasmic tail of αK or βK did not affect αβ association of LFA-1 based on expression of the MHM23 epitope, which has been reported to detect an αβ association-dependent epitope on LFA-1 (39), and immunoprecipitation of LFA-1 from all mutants confirmed that mutant LFA-1 was expressed as αβ heterodimers (data not shown).

Affinity and Avidity Regulation by the LFA-1 αK and βK Cytoplasmic Tails—The capacity of the LFA-1 tail deletion mutants to bind ICAM-1 was determined by the ICAM-1 fluorescent beads adhesion assay we developed (see “Experimental Procedures”), which allows analysis of only those cells that have similar expression levels of LFA-1, by staining LFA-1 with the FITC-conjugated nonblocking mAb TS2/4 and are under similar gate settings (fluorescence intensity 40–60). Thus, this new adhesion assay is suitable to investigate and compare various cell lines that express distinct levels of adhesion receptors and excludes variation in adhesion due to variation in expression levels of LFA-1. In contrast to wild-type LFA-1 and the chimeric LFA-1 transfectants, deletion of the βK cytoplasmic tail (ΔβK) or αK cytoplasmic tail (Δ1088αK) and deletion of only the KVGFFKR region in the αK cytoplasmic tail (ΔKVGFFKRαK) resulted in high spontaneous adhesion that was as high as adhesion after stimulation with the activating LFA-1 antibody KIM185 (Fig. 4B). Surprisingly, the αK deletion mutant that contained the GFFKR region (Δ1095αK) did not show any spontaneous adhesion and was similar to wild-type LFA-1. The two mutants that contained deletion of both the αK cytoplasmic tail and the βK cytoplasmic tails (Δ1088αK/ΔβK and Δ1095αK/ΔβK) also expressed a constitutive active receptor that bound to ICAM-1 spontaneously. These observations are in line with findings of others showing that integrins exert high spontaneous ligand binding when both cytoplasmic tails are deleted (14, 16). Furthermore these data demonstrate that the somewhat lower expression levels of LFA-1 on the double cytoplasmic deletion mutants and ΔKVGFFKRαK does

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**RESULTS**

Active Regulation of LFA-1 Ligand Binding by Disconnecting the Actin Cytoskeleton Network—LFA-1 expressed on resting peripheral blood lymphocytes is inactive and poorly binds ICAM-1, even when the cells are stimulated by PMA (Fig. 1). This might enable leukocytes by a mechanism to migrate through the blood vessel wall in a nonadhesive state. Several findings have demonstrated that a number of cytoskeletal components (α actin, talin) are attached to the cytoplasmic tails of LFA-1. LFA-1 connected to the cytoskeleton remains inactive whereas temporary dislodgment of the cytoskeleton by treatment of cells with cytochalasin D, reduces the cytoskeleton restraints and results in enhanced LFA-1-ICAM-1-mediated binding (Fig. 1) (10, 22). We have demonstrated that this temporary dislodgment of the cytoskeleton alters the otherwise homogeneous cell surface distribution of LFA-1 into the formation of clusters (10). Here we analyzed in detail whether the connection of the cytoskeleton to the cytoplasmic tails of LFA-1 regulates cell adhesion by altering the cell surface distribution of LFA-1 (avidity) and/or the affinity for ICAM-1. We therefore generated different αK and βK cytoplasmic tail deletion mutants and chimeric cytoplasmic mutants that were analyzed for these conditions (see “Experimental Procedures”) (Fig. 2).

Chimeric LFA-1 Molecules and the Actin Cytoskeleton—To discriminate between the role of the intact conserved KVGFFKR sequence present in the α cytoplasmic tail in avidity/avidity regulation and that of other amino acids C-terminal of this sequence, we generated chimeric LFA-1 receptors in which the αK cytoplasmic tail was swapped for that of αK or αK, K562 transfectants expressing high levels of αK chimeric LFA-1 receptors (Fig. 3) were generated as described under “Experimental Procedures.” Analysis of expression levels of the L16 and the M24 epitopes, which have been described to be reporters for clustered or activated LFA-1, respectively, are expressed on low levels on both wild-type LFA-1-activated chimeric LFA-1 receptors, as compared with expression of regulatory LFA-1 epitopes (i.e. SPV-L7) (Table I). As we earlier reported wild-type LFA-1 expressed in K562 cells does not bind ICAM-1 unless activated by the activating anti-β2 mAb (KIM185), whereas PMA is incapable of activating LFA-1 expressed in these cells (36). Also disconnection from the actin cytoskeleton network did not alter the LFA-1-mediated adhesion after treatment with cytochalasin D (Table II). Confocal laser microscopy...
not affect the adhesive state of the receptor, because they remain extremely active and bind extremely well to ICAM-1. We demonstrated previously that an altered distribution of integrins might affect the avidity state of the receptors, thus facilitating ligand binding (7–9). We therefore investigated whether adhesive properties of the α or β cytoplasmic tail transfectants correlate with the cell surface distribution of LFA-1. Analysis by confocal microscopy revealed that the cytoplasmic deletion mutants that strongly bound to ICAM-1 (ΔKVGFFKRα, Δβ2, Δ1088αL/Δβ2, and Δ1095αL/Δβ2) all show clusters of LFA-1 on the cell surface. Mutant Δ1088αL-LFA-1 contained tiny clusters, compared with K562 expressing wild-type LFA-1 or Δ1095αL, who both show a homogeneous distribution of LFA-1 and did not adhere spontaneously to ICAM-1 (Fig. 5). Again the lower expression levels of LFA-1 present on the cytoplasmic deletion mutants could rule out its effect on surface distribution.

To investigate whether the affinity of LFA-1 for ICAM-1 is altered upon deletion or mutation of the cytoplasmic tails also, we determined the concentration of soluble ligand (ICAM-1Fc) that yielded half-maximal direct ligand binding activity (ED_{50}) (11, 40). The lower the concentration of ICAM-1Fc needed to bind to 50% of the positive cells, the higher the affinity of LFA-1 for ICAM-1. Similar to the beads adhesion assay, strong binding of sICAM-1 was observed for the cytoplasmic deletion mutants that spontaneously adhered to ICAM-1 (ΔKVGFFKRα, Δβ2, Δ1088αL/Δβ2, and Δ1095αL/Δβ2) (Fig. 6). Binding of sICAM-1 was completely LFA-1 dependent, because anti-LFA-1 antibodies completely blocked the binding (data not shown). When we calculated the concentration of sICAM-1 that yielded half-maximal binding we observed that sICAM-1 bind to LFA-1 for all the mutants ranged from ED_{50} of less than 1 μg/ml ICAM-1Fc (ΔKVGFFKRα) to an ED_{50} of 2 μg/ml for all the mutants (Table III). These findings indicate that, although the cytoplasmic tail deletion mutants ΔKVGFFKRα, Δ1088αL/Δβ2, and Δ1095αL/Δβ2 all show high spontaneous binding to ICAM-1, the affinity of LFA-1 for ICAM-1 on these transfectants is not higher than that of wild-type LFA-1.

Expression of the LFA-1 Activation Epitopes (L16 and M24) on the LFA-1 Cytoplasmic Deletion Mutants—Next we studied the expression of the L16 epitope and the M24 epitope that both have been described as reporters for clustered or activated forms of LFA-1, respectively. Although the L16 epitope is a Ca^{2+}-dependent epitope on the α chain of LFA-1 and correlates with the clustering status of LFA-1 on the cell surface (8, 31), the 24 epitope is a Mg^{2+}-dependent epitope expressed on the α chain of LFA-1 that has been used as an “activation reporter” of LFA-1, which can be induced by Mn^{2+} (23, 27). As depicted in Table I, wild-type LFA-1 transfected in K562 cells express only low levels of L16 (mean ratio 0.3 compared with expression of a regular LFA-1 epitope, SPV-L7) in line with previous findings (36). Similarly, low levels of M24 epitope are expressed on cells expressing wild-type LFA-1, but M24 can be induced by Mn^{2+} (mean ratio 0.3). In contrast, all cytoplasmic tail deletion mutants that express a constitutive active form of LFA-1 (ΔKVGFFKRα, Δβ2, Δ1088αL/Δβ2 and Δ1095αL/Δβ2), express high levels of the L16 epitope, as well as the M24 epitope, without prior activation with Mn^{2+} (both mean ratio 0.9–1.0). The only exception is mutant Δ1088αL, which also spontaneously binds ICAM-1 but does not express high levels of the L16 and M24 epitope and has little clustered LFA-1. In conclusion these data clearly demonstrate that when LFA-1 is found in large clusters on the cell surface, L16 and M24 epitopes are expressed. Furthermore, our findings demonstrate that clustering of LFA-1 is not associated with an enhanced affinity for ICAM-1 (Fig. 6).

Disruption of Cytoskeleton Restraints Enhances LFA-1-mediated Ligand Binding and Is Associated with Avidity but Not Affinity Alterations—We studied the role of the actin cytoskeleton by treating all LFA-1 cytoplasmic tail mutants with cytochalasin D and subsequently determined binding to ICAM-1-coated beads. As expected, the high spontaneous binding to ICAM-1 of mutants Δ1088αL and Δβ2 and the double α1β2 cytoplasmic deletion mutants (Δ1088αL/Δβ2 and Δ1095αL/Δβ2) were not affected by disruption of the actin cytoskeleton network (Table II). Probably deletion of the cytoplasmic tail of the β2 or α1 chain reduces the interaction with the cytoskeleton causing aggregates of LFA-1 receptors (avidity) that favors ligand binding. Surprisingly, Δ1095αL, containing the wild-type β2 cytoplasmic tail together with the α1 cytoplasmic tail deleted immediately after the GFFKR sequence, showed enhanced spontaneous binding to ICAM-1 (from 2 to 32%, Table 

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**Fig. 2. Schematic diagram of LFA-1 α and β subunit deletion mutants.** The wild-type α and β subunits are composed of a large extracellular part, a transmembrane (black box), and a cytoplasmic domain (right side). The conserved cytoplasmic KVGFFKR sequence corresponding to amino acids 1088–1094 is adjacent to the transmembrane domain. Mutant ΔKVGFFKRαL contained an internal deletion of the KVGFFKR sequence. Mutants Δ1088αL and Δ1095αL were generated by truncation of the cytoplasmic tails immediately after the KVGFFKR sequence, respectively. The chimeric α2αL and α1αL, the α2 and α1 cytoplasmic tails, respectively, are joined to the α3 cytoplasmic tail just after the KVGFFKR sequence of α3. The β2 cytoplasmic deletion mutants were created by truncation immediately after the transmembrane at amino acid position 724 (38).
form aggregates, thereby enhancing the avidity of LFA-1-ICAM-1 interactions.

Post-ligand Binding Events Are Regulated by Both the $\alpha$ and $\beta_2$ Cytoplasmic Tail—We also investigated the post-adhesion cell spreading on ICAM-1 of the LFA-1 cytoplasmic deletion mutants (Table IV) to study in more detail the role of the cytoskeleton in LFA-1-ICAM-1 adhesion. When activated by KIM185, wild-type LFA-1 transfected in K562 cells binds to ICAM-1, and cells are able to spread on ICAM-1, because a flattening of the cell is observed after 60 min of binding. Disruption of the actin cytoskeleton network after treatment of cells with cytochalasin D dramatically reduces the spreading, whereas no effect is seen on ICAM-1 binding. (Table II). Also the chimeric LFA-1 molecules $\alpha_2\alpha_2\alpha_2$ and $\alpha_2\alpha_3\alpha_3$ spread on ICAM-1 after KIM185 treatment similar to wild-type. Deletion of the $\beta_2$ cytoplasmic tail resulted in high binding to ICAM-1, however, binding was not accompanied by any spreading of cells also not after KIM185 activation. Similarly, the double cytoplasmic tail deletion mutants did not spread on ICAM-1, indicating that spreading requires connections with the actin cytoskeleton. In contrast, truncation of the $\alpha_3$ cytoplasmic tail before the KVGFFKR or just after this domain, results in strong binding of ICAM-1 and normal spreading, whereas the mutant in which only the KVGFFKR domain was deleted binds ICAM-1 very well, but does not spread. Together these data demonstrate that both the $\alpha_3$ and the $\beta_2$ cytoplasmic tails contain sequences that contribute to the reorganization of the cytoskeleton.

**DISCUSSION**

From our results we conclude the following. 1) Deletion of the $\beta_2$ and/or $\alpha_L$ cytoplasmic tail including the KVGFFKR sequence leads to strong ICAM-1 binding by reducing cytoskeleton restraints, enabling the formation of LFA-1 clusters without the necessity of affinity alterations. 2) Together with earlier reports, this indicates that attachment of the actin cytoskeleton network to LFA-1 keeps the integrin in a default-inactive state by inhibiting the lateral movement of the receptors on the cell membrane. 3) Both the $\alpha_3$ and $\beta_2$ cytoplasmic tails are connected to the actin cytoskeleton network, because (a) cytochalasin D did not inhibit the spontaneous adhesion of the $\alpha_3$ and $\beta_2$ cytoplasmic deletion mutants, and (b) spreading of LFA-1 transfecants was impaired when the $\beta_2$ or the KVGFFKR region in the $\alpha_3$ tail were deleted. 4) LFA-1 deletion mutants that are constitutively active express both the L16 “clustering” epitope and the M24 activation reporter epitope, demonstrating that all clustered LFA-1 is active and binds ICAM-1. 4) Cytochalasin D enhances adhesion of $\Delta 1095\alpha_3$ to ICAM-1 by disrupting cytoskeleton restraints that are attached to the short remaining $\alpha_3$ tail and enhances the avidity (clustering) but not the affinity of LFA-1.

On normal resting lymphocytes, LFA-1 is in an inactive state that poorly binds ICAM-1. Several recent publications have demonstrated that temporary dislodgment of LFA-1 from the cytoskeleton network facilitates ICAM-1 binding, following TCR/CD3 or PMA activation (inside-out activation of LFA-1) by increasing lateral diffusion of LFA-1 (9, 10, 20–22). This temporary disconnection from the cytoskeleton constraints by cytochalasin D may facilitate redistribution of LFA-1 receptors on the cell membrane altering LFA-1-ICAM-1 avidity interaction. In this study, we aimed to investigate the role of the $\alpha$ and $\beta$ cytoplasmic tails of LFA-1 in regulating ICAM-1 binding through avidity changes or affinity changes.

Most integrins contain the conserved DLRE motif in the $\beta$ cytoplasmic tail. Deletion of this sequence has already been shown to lead to an active receptor for various integrins, enabling it to bind spontaneously ligand (14, 16, 41). We confirmed...
Expression of LFA-1 activation epitopes on the α and β cytoplasmic deletion mutants

NKI-L16 expression was determined in the absence or presence of 1 mM CaCl$_2$. The mean expression levels (relative fluorescence intensity) and the ratio (Ca$^{2+}$/Mn$^{2+}$) are depicted. Table I. M24 expression was determined in the absence or presence of 1 mM MnCl$_2$. The mean expression levels and ratio (Mn$^{2+}$/Ca$^{2+}$) are depicted. Expressions of isotype control antibody and LFA-1 staining by an anti-CD11a mAb SPV-L7 are presented as the mean expression levels.

| Cells               | Control | SPV-L7 | NKI-L16 | M24  | Binding to ICAM-1 medium |
|---------------------|---------|--------|---------|------|--------------------------|
|                     | PBS$^a$ | Ca$^{2+}$/Mn$^{2+}$ Ratio$^b$ | PBS$^a$ | Ca$^{2+}$/Mn$^{2+}$ Ratio | PBS$^a$ | Ca$^{2+}$/Mn$^{2+}$ Ratio | PBS$^a$ | Ca$^{2+}$/Mn$^{2+}$ Ratio | PBS$^a$ | Ca$^{2+}$/Mn$^{2+}$ Ratio |
| K-LFA-1             | 4       | 5      | 1.0     | 153  | 155                      | 24     | 129                     | 0.2     | 6                | 0.1    | 7                        |
| K-α1,α2             | 3       | 3      | 1.0     | 72   | 72                       | 28     | 73                      | 0.4     | 30               | 0.4    | 9                        |
| K-α1,α4             | 3       | 3      | 1.0     | 69   | 68                       | 25     | 65                      | 0.4     | 22               | 0.4    | 9                        |
| K-ΔKVGFKKR$_{α1}$   | 4       | 4      | 1.0     | 33   | 31                       | 11     | 12                      | 0.9     | 14               | 0.6    | 74                       |
| K-Δ1088sL$_{α1}$    | 3       | 6      | 1.0     | 152  | 150                      | 19     | 70                      | 0.3     | 5                | 0.1    | 64                       |
| K-Δ1095sL$_{α1}$    | 3       | 4      | 1.0     | 178  | 176                      | 20     | 86                      | 0.2     | 12               | 0.1    | 4                        |
| K-Δ1088sL$_{α1}/β_2$| 4       | 3      | 1.0     | 34   | 33                       | 14     | 15                      | 0.9     | 19               | 0.3    | 54                       |
| K-Δ1095sL$_{α1}/β_2$| 4       | 3      | 1.0     | 36   | 34                       | 23     | 25                      | 0.9     | 23               | 0.4    | 55                       |
| K-Δβ_2              | 4       | 5      | 1.0     | 45   | 44                       | 18     | 21                      | 0.9     | 23               | 0.7    | 60                       |

$^a$ Relative fluorescence intensity.

$^b$ Ratio relative fluorescence intensity PBS/relative fluorescence intensity in the presence of divalent cations.

$^c$ Percentage of cells binding to ICAM-1-coated beads.

Effect of cytochalasin D (CD) on the spontaneous ICAM-1 adhesion of LFA-1 cytoplasmic deletion mutants

Various K-LFA-1 transfectants were pretreated with 10 μg/ml cytochalasin D or Me$_2$SO as control for 15 min at 37 °C and subsequently allowed to adhere to ICAM-1Fc-coated fluorescent beads as described in the Materials and Methods section. Percentage of cells that spontaneously bind ICAM-1-coated fluorescent beads is depicted in the presence or absence of cytochalasin D (% adhesion ± CD), and factor by which adhesion is enhanced (adhesion index) is depicted (% adhesion with CD/% adhesion without CD). Whereas cytochalasin D has no effect on binding of wild-type LFA-1 to ICAM-1 nor on the spontaneous binding of the cytoplasmic deletion mutants, it enhances adhesion of mutant Δ1088sL/$β_2$ 16-fold.

| Cells               | % Adhesion | % Adhesion + CD | Adhesion index$^d$ |
|---------------------|------------|----------------|-------------------|
| K-LFA-1             | 2          | 5              | 3                 |
| K-α1,α2             | 5          | 5              | 1                 |
| K-α1,α4             | 6          | 6              | 1                 |
| K-ΔKVGFKKR$_{α1}$   | 75         | 77             | 1                 |
| K-Δ1088sL$_{α1}$    | 65         | 72             | 1                 |
| K-Δ1095sL$_{α1}$    | 2          | 32             | 16                |
| K-Δβ_2              | 59         | 48             | 1                 |
| K-Δ1088sL$_{α1}/β_2$| 54         | 58             | 1                 |
| K-Δ1095sL$_{α1}/β_2$| 55         | 46             | 1                 |

$^d$ Adhesion index is the factor by which the adhesion is enhanced (% adhesion with CD/% adhesion without CD).

In Adhesion to ICAM-1-coated beads, medium, PMA, and the double Δβ_2 deletion mutants; C, adhesion of β_2 deletion mutants. K562-α1,β_2 cells were preincubated in medium (control), PMA (50 nM, III), or the activating anti-β_2 mAb KIM185 (5 μg/ml, □), respectively, for 15 min at 37 °C in the absence or presence of the LFA-1 blocking mAb (NKI-L15). Depicted is the mean percentage of LFA-1-specific adhesion to ICAM-1 of the gated cells that expressed equal amounts of LFA-1 (mean fluorescent intensity 40–60) as determined by staining with the FITC-conjugated nonblocking anti-LFA-1 antibody (TS2/4). Integrin-specific adhesion: percentage of cells binding – percentage of cells binding in the presence of an integrin-blocking mAb (NKI-L15). Data are representative of four experiments.

To investigate whether the spontaneous activation of LFA-1, due to truncation of the cytoplasmic tail, was the result of affinity or avidity alterations, we determined the minimal concentration of soluble ICAM-1 to bind the various LFA-1-transfectants (affinity), as well as the surface distribution of LFA-1 by confocal microscopy (avidity). We observed that all LFA-1 mutants that spontaneously bound ICAM-1 (Δ1088sL$_{α1}$, ΔKVGFKKR$_{α1}$, Δβ_2, and the double Δβ deletion mutants) showed a clustered cell surface distribution of LFA-1, correlating well with our earlier findings in which we addressed the importance of clustering of LFA-1 to facilitate ICAM-1 binding.
Fig. 5. Surface distribution of LFA-1 as determined by CLSM. Cells were fixed (0.5% paraformaldehyde) and subsequently stained with the anti-LFA-1 mAb TS2/4 and goat anti-mouse-(Fab')2-FITC second antibodies. Wild-type LFA-1 is found homogeneous on the cell surface similar as Δ1095α2β2, although it is localized in little clusters on Δ1088α2β2 and large clusters on ΔKVGFFKRα2β2 and the double αβ deletions (Δ1088α1Δβ2 and Δ1095α1Δβ2). The instrumental settings of the CLSM were the same for the four different panels: lens, 60×; gain, 1200; pinhole, 1.5 μm; and magnification × 2.0. One of three experiments is shown.

Fig. 6. Analysis of soluble ICAM-1Fc binding to K562 cytoplasmic deletion mutants by direct ligand binding. Recombinant ICAM-1Fc fusion protein was incubated for 30 min at 37 °C with the transfectants in the presence of medium. The concentration of ligand varied 100–0.1 μg/ml. Binding was detected by staining with FITC-conjugated goat anti-human Fc, and analyzed on FACSscan. The percentage of positive cells represent the percentage of cells binding soluble ICAM-1Fc. The specific adhesion could be blocked by blocking anti-LFA-1 mAbs (not shown). Depicted is the concentration of sICAM-1Fc that gives half maximal binding to the LFA-1 transfectants; data are representative of three experiments.

Because clustering of LFA-1 on these cytoplasmic tail mutants might be due to a reduced capacity to interact with the cytoskeleton, we investigated whether disruption of the actin cytoskeleton by cytochalasin D affected adhesion. As expected, no reduction of spontaneous adhesion to ICAM-1 was observed, indicating that the cytoskeleton is not attached to LFA-1 when the β or α cytoplasmic tails are truncated. Therefore, no post-receptor-binding events that depend on the attachment of cytoskeleton are observed (cell spreading on ICAM-1). Probably deletion of the cytoplasmic tail disconnects the integrin from the cytoskeleton and allows lateral movement of the integrin at the cell membrane, explaining the clustered distribution of LFA-1 on all the Δβ2 mutants or the ΔKVGFFKR mutants. By contrast, when the entire α1 cytoplasmic tail was deleted (Δ1088α1), LFA-1 was less clustered and was still able to spread on ICAM-1 similar to wild-type LFA-1.

It has been demonstrated that integrins can associate with cytoskeletal components (α-actin, talin), particularly through the β chain and thereby may regulate the cell surface distribution of the integrin (49, 50). In particular, mutations of a tripeptid of threonines (position 758–760) and the phenylalanine residue at position 766 in the β2 cytoplasmic tail profoundly reduced the adhesiveness of LFA-1 (13, 41). It has been suggested that the altered adhesiveness due to mutation of the threonine triplet is caused by an altered cytoskeletal association/organization and not to an affinity change in LFA-1 (13).

Clustering of integrins on the cell surface can also co-localize important kinases essential for proper signal transduction (51). Not only the intracellular conformation or association with regulatory proteins is affected by clustering of integrins on the cell surface, but the extracellular conformation is altered also, as evidenced by enhanced L16 and M24 epitope expression when the β2 or α1 cytoplasmic domain was deleted (ΔKVGFFKR). This may be attributable to distinct interactions with cytoplasmic proteins affecting the extracellular conformations of the integrin molecule.

Because K562-LFA-1 transfectants express ICAM-1, we investigated whether initial cell contact with ligand during culture may result in the dynamic clustering of integrins thereby augmenting the avidity for ligand. To rule out that the cluster-

![Image](https://example.com/image.png)

TABLE III

| K562 transfectants | Affinity of LFA-1 for sICAM-1 (ED50) μg | Medium | Medium + CD |
|--------------------|----------------------------------------|--------|-------------|
| K-LFA-1            | 2                                      | 2      |             |
| K-ΔKVGFFKRα1       | 1                                      | 1      |             |
| K-Δ1088α1          | 2                                      | 3      |             |
| K-Δ1095α1          | 2                                      | 2      |             |
| K-Δβ2              | 2                                      | 2      |             |
| K-Δ1088α1/Δβ2      | 2                                      | 2      |             |

(7–9). This notion is supported by the demonstration that expression of the clustering sensitive epitope on LFA-1 (L16) on all these mutants is increased except Δ1088α1/β2, which has less clustered LFA-1 than ΔKVGFFKR, Δβ2 and the double αβ deletion mutants.

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FIG. 7. Disruption of the interaction of LFA-1 with the actin cytoskeleton network by cytochalasin D induces clustering of LFA-1 transfectant Δ1095αL/β2. LFA-1 is homogeneously distributed on Δ1095αL/β2, whereas distribution shifts toward a clustered distribution upon cytochalasin D treatment (10 μg/ml). Cells were stained for 30 min with anti-LFA-1 mAb TS2/4 (10 μg/ml) and FITC-labeled goat (Fab')2 anti-mouse IgG. Fluorescence distribution was determined by confocal laser scanning microscopy at 488 nm. The same instrument settings of the CLSM were used throughout the experiment.

TABLE IV

| Cells                  | Spreading | Adhesion |
|------------------------|-----------|----------|
| K-LFA-1                | 20        | 4        |
| K-α1,α2                | 20        | 10       |
| K-α1,α2                | 20        | 10       |
| K-ΔKVGGFKRαL           | 0         | 65       |
| K-Δ1088αL              | 80        | 60       |
| K-Δ1095αL              | 20        | 4        |
| K-Δβ2                  | 0         | 60       |
| K-Δ1088αL/Δβ2          | 0         | 60       |
| K-Δ1095αL/Δβ2          | 0         | 65       |

Spreading of the various LFA-1 cytoplasmic tail deletion mutants on ICAM-1-coated wells

The various LFA-1-K562 transfectants were activated with the activating anti-LFA-1 mAb KIM185 to adhere to ICAM-1-Fe-coated wells (200 μg/ml). Cells were allowed to adhere for 1 h at 37 °C, after which spreading was scored (percentage of cells spreading) visually on a microscope using a 10× objective. Spontaneous adhesion was measured by incubation of the LFA-1 mutants, without further activation, with the fluorescent ICAM-1-coated beads as described in Fig. 4.

observation that dimers of ICAM-1 have been shown to bind LFA-1 with much greater affinity than monomer ICAM-1 (53). This is in accordance with the finding that ICAM-1 is mostly expressed as dimer on the cell surface (54, 55). The recent crystal structure of a dimeric form of ICAM-1 containing only the outer two Ig-like domains, provides additional evidence that dimerization of ICAM-1, and consequently LFA-1, plays an important role in receptor-ligand interactions and downstream signaling (56).

Cytochalasin D treatment enhanced spontaneous LFA-1-mediated adhesion of the LFA-1 mutant Δ1095αL, truncated immediately after the KVGGFKR. Enhanced adhesion after cytochalasin D treatment of Δ1095αL was associated with clustering of LFA-1 but did not affect the ICAM-1 binding affinity. These findings correlate well with similar results that have been shown for α4 tail deletions (11). The cytoplasmic domain of the α chain may cover a negative site in the β tail, the unshielded and unregulated interactions of β tails with cytoskeletal proteins may lead to increased constitutive cytoskeletal anchoring, and thus diminished diffusion and clustering at adhesive sites.

Although previous reports for various integrins (β1 and β3) suggest that affinity alterations play an important role in regulating integrin-mediated adhesion (57, 58), we were not able to measure any affinity alterations for the β2 integrin LFA-1. It remains largely unknown whether affinity changes are involved in the regulation of cell adhesion. Thus far, LFA-1 affinity studies, by competition between sICAM-1 and function blocking antibody for binding to LFA-1 on T cells, have determined only low affinity of LFA-1 on resting T cells (100 nM), whereas activation of T cells increases the affinity up to 400 nM (24). Studies on the affinity of purified LFA-1, which is constitutively active, for binding sICAM-1 was calculated somewhat higher (130 nM) (53, 59). Other evidence for possible affinity changes of LFA-1, comes from the finding that activation of LFA-1 by EGTA and Mg2+ leads to enhanced expression of the Mg2+-dependent 24 epitope on CD11a, implying that Mg2+ binding involves induction of conformational changes in LFA-1 that coincides with ICAM-1 binding (28, 29, 40).

We observed that the cytoplasmic deletion mutants ex-

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3 Y. van Kooyk, S. J. van Vliet, and C. G. Figdor, manuscript in preparation.

4 Y. van Kooyk, S. J. van Vliet, and C. G. Figdor, unpublished results.
pressed a constitutively active LFA-1 receptor, all exhibited LFA-1 in large clusters on the cell surface, and expressed high levels of L16 and M24 epitopes. Both mutant $\Delta \alpha_1 \beta_2$, as well as $\Delta \alpha_1 \beta_2$ after cytochalasin D treatment, were spontaneously active, but did not express the L16 and M24 epitope, which correlated with less clustered LFA-1 distribution on these mutants than the double deletion mutants. From this study we may conclude that the small changes in LFA-1 clustering, as detected by confocal microscopy, can have huge consequences on the adhesion capacity, which cannot be detected by L16 and M24 expression.

All LFA-1 cytolsmic tail deletion mutants that showed enhanced M24 expression were also L16 positive and showed large clusters of LFA-1 on the cell surface. This did not coincide with an enhanced affinity of LFA-1 for ICAM-1, as determined by the half-maximal concentration of soluble ICAM-1 to bind LFA-1. To date, only activation of LFA-1 after Mg$^{2+}$/EGTA treatment has been shown to lead to an affinity change (60). In contrast, activation of LFA-1 through intracellular signaling routes, or through inducing a conformational change upon binding of activating anti-LFA-1 antibodies, such as KIM185 (data not shown) enhance LFA-1-mediated adhesion through avidity alterations in LFA-1 (22). In this study, we demonstrate that deletion of the cytolsmic tails of LFA-1 detaching LFA-1 from the cytoskeleton facilitates motility of LFA-1 stimulating LFA-1 clustering and adhesion without apparent affinity alterations.

In summary, we have demonstrated that both the $\beta_2$ cytolsmic tail and the $\alpha_1$ cytolsmic tail (KVGFKKKR region) are involved in the attachment of LFA-1 to the cytoskeleton that by default keep LFA-1 in an “off” situation. Activation of LFA-1 leads to a temporary dislodgment of LFA-1 from these actin cytoskeleton restraints and increases the lateral mobility of LFA-1, which enables LFA-1 to form clusters (avidity) leading to strong ICAM-1 binding, whereas the affinity for ICAM-1 remains unaltered. These results may emphasize that clustering of $\beta_2$ integrins may be of general importance for regulating adhesion, especially in the absence of affinity changes for ligand binding (61).

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