A Single Amino Acid in the Cytoplasmic Domain of the β2 Integrin Lymphocyte Function-associated Antigen-1 Regulates Avidity-dependent Inside-out Signaling*

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Received for publication, October 2, 2000, and in revised form, December 20, 2000
Published, JBC Papers in Press, December 27, 2000, DOI 10.1074/jbc.M008967200

The leukocyte-specific β2 integrin lymphocyte function-associate antigen-1 (LFA-1) (αL/β2) mediates activation-dependent adhesion to intercellular adhesion molecule (ICAM)-1. In leukocytes, LFA-1 requires activation by intracellular messengers to bind ICAM-1. We observed malfunctioning of LFA-1 activation in leukemic T cells and K562-transfected cells. This defective inside-out integrin activation is only restricted to β2 integrins, since β1 integrins expressed in K562 readily respond to activation signals, such as phorbol 12-myristate 13-acetate. To unravel these differences in inside-out signaling between β1 and β2 integrins, we searched for amino acids in the β2 cytoplasmic domain that are critical in the activation of LFA-1. We provide evidence that substitution of a single amino acid (L732R) in the β2 cytoplasmic DLRE motif, creating the DRRE motif, is sufficient to completely restore PMA responsiveness of LFA-1 expressed in K562. In addition, an intact TTT motif in the C-terminal domain is necessary for the acquired PMA responsiveness. We observed that restoration of the PMA response altered neither LFA-1 affinity nor the phosphorylation status of LFA-1. In contrast, strong differences were observed in the capacity of LFA-1 to form clusters, which indicates that inside-out activation of LFA-1 strongly depends on cytoskeletal induced receptor reorganization that was induced by activation of the Ca2+-dependent protease calpain.

The lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18; αL/β2) is a member of the leukocyte integrin family. LFA-1 expression is leukocyte-specific and mediates adhesive interactions between cells. The β2 integrin LFA-1 consists of a common β2 subunit that is noncovalently associated with an αL subunit (1). By binding to the intercellular adhesion molecule (ICAM)-1, LFA-1 is important in mediating cellular interactions in the immune system such as cytotoxic T cells and natural killer cell-mediated cytotoxicity, helper T lymphocyte responses, and leukocyte adhesion (2–5).

LFA-1 has to be activated via outside-in or inside-out signals to efficiently bind ICAM-1. Outside-in signaling has been identified by LFA-1 activating antibodies (6) or immobilized ligands, resulting in cell spreading, rise in intracellular Ca2+ and pH, phosphorylation of proteins, and costimulatory signals (7, 8). Inside-out signals are initiated upon triggering of specific cell surface molecules, generating intracellular signals that induce a high affinity and/or avidity state of LFA-1 (9). Both conformational changes (affinity) in the presence of Mg2+ and altered surface distribution of LFA-1 into clusters (avidity) upon Ca2+ binding result in strong ligand binding (8, 10, 11).

Although the αL and β2 cytoplasmic domains of LFA-1 are relatively short and do not contain any intrinsic kinase activity, they are important for affinity and avidity regulation. Previous studies have shown that LFA-1 adhesiveness is controlled by the cytoplasmic domain of the β2 subunit, since truncation of the cytoplasmic β2 domain, but not the αL domain, eliminates LFA-1 binding to ICAM-1 (12). Complete deletion of the β2 cytoplasmic domain results in clustering and spontaneous activation of LFA-1. This constitutively active LFA-1 deletion mutant strongly binds to ICAM-1. The phorbol ester PMA that activates PKC cannot further increase the adhesion to ICAM-1 of this constitutive active LFA-1, in contrast to wild type LFA-1 (13). It has been proposed that the αL cytoplasmic domain of LFA-1 is involved in post-ligand binding events, since deletion of the cytoplasmic αL domain does not affect binding to ICAM-1 (12).

Also, cytoskeleton restraints play a crucial role in regulating LFA-1 avidity, since clustering of LFA-1 is induced on resting PBLs after treatment with cytochalasin D (14). Interestingly, this is not the case for β1 integrins, indicating that β2 and β1 integrins differ in their ability to cluster into specialized lipidic membrane microdomains, also termed rafts (15). Replacement of the β2 cytoplasmic domain for that of β1 (αL/β2/β1), creating a chimeric LFA-1 molecule containing a β1 cytoplasmic domain, provided us with additional evidence. The chimeric LFA-1 (αL/β2/β1) showed a clustered cell surface distribution when expressed in the erythroleukemic cell line K562. Furthermore, PMA activation of the chimeric LFA-1 molecule increased the adhesion to ICAM-1. This was in contrast to wild type LFA-1 that, when expressed in K562, is not clustered and is defective for PMA-induced activation (13).

Several regions within the β2 cytoplasmic domain are thought to be important in regulating LFA-1. Alanine substitutions of conserved threonines (TTT) in the β2 cytoplasmic domain reduce ICAM-1 binding, and a serine residue is phosphorylated upon PKC activation by PMA (16). There are different consensus sequences known in the β2 cytoplasmic do-
β2 Cytoplasmic Domain in LFA-1 Signaling

main that can associate with intercellular components, such as cytohesin-1 (17), Rack1 (18), and α-actinin (19).

The observation that, despite their homology, β2 and β1 integrins are differently regulated by inside-out signals, prompted us to identify residues within the β2 cytoplasmic domain that are involved in the PMA-induced LFA-1-mediated ligand binding. To this end, we substituted β2 amino acids for those of the β1 cytoplasmic domain that are critical for PMA-induced adhesion in K562 cells. We observed that a single β2/β1 amino acid substitution is sufficient to completely restore the PMA responsiveness by enhancing LFA-1 avidity but not the affinity. In addition, we observed that activation of LFA-1 by PMA is dependent on cytoskeletal rearrangements that seem to be mediated by the Ca2+–dependent protease calpain.

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 (20). The non-blocking mAb TS2/4 (IgG1) reactive with α6 (21), mAb 60.3 (IgG1) directed against β2 (22), and mAb KIM185 (IgG1) used to activate β2 integrins (6) were kindly provided by Drs. E. Martz, N. Hogg, J. Harlan, and M. Reiter (AgResearch, New Zealand). The monoclonal antibody SAM-1 (IgG2b) was directed against the α-chain of VLA-5 (23).

DNA Constructs—The 4.2-kilobases a chain of LFA-1 was cloned in the XbaI site of the pCDM8 vector, which directs expression of αL from the CMV AD169 immediate early promoter (pCDL1). The 3’-end of β2 was cloned as an EcoRI–BglII fragment in the pRc/CMV vector (containing a neomycin resistance gene; Invitrogen Corp., San Diego, CA). Within this sequence is a unique Apol site at position 1980. The terminal end was rebuilt from this site using 10 overlapping oligonucleotides and amplification by PCR to obtain the appropriate hybrids. For the β2/β1 chimeric protein, amino acid 752 of β1 cytoplasmic domain was joined to the amino acid 732 of β2. The deletion mutant of LFA-1 was generated by truncation of the β1 cytoplasmic domain from amino acid 724 (13). All point mutations in the β2 and β1 cytoplasmic domain were generated by the oligonucleotide-directed pAlter® mutagenesis system (Promega, Madison, WI) according to the protocol. The following oligonucleotides were used: L722R-β2, CTGGAGGCGCCGCGGCTAGTAC; Y735F-β2, CTCCCAGGTCATCAGGCTTTC; S756C-β2, CCTTCTTCAATGCGCAACCGACG; T775V-β2, TCTAAGAGCGCTCCAGGACGTCATGAAC; F766Y-β2, AACCCTAATGTGCTGAG; R732L-β2, ATAATCTGCTACCAAGGTGTTCG. For the deletion mutants, the following oligonucleotides were used: L732R-β2, CACCTGAGCGACCGCCGGGAGTAC; D732R-β2, AACCCCAAGTATGCTGAGAG; R732L-β2, CCTCCCTTCAATGCGCAACCGACG; T775V-β2, TCTAAGAGCGCTCCAGGACGTCATGAAC; F766Y-β2, AACCCTAATGTGCTGAG; R732L-β2, ATAATCTGCTACCAAGGTGTTCG. The deletion mutants of LFA-1 were generated by truncation of the β2 cytoplasmic domain from amino acid 724 (13). All point mutations in the β2 and β1 cytoplasmic domain were generated by the oligonucleotide-directed pAlter® mutagenesis system (Promega, Madison, WI) according to the protocol. The following oligonucleotides were used: L732R-β2, CTGGAGGCGCCGCGGCTAGTAC; Y735F-β2, CTCCCAGGTCATCAGGCTTTC; S756C-β2, CCTTCTTCAATGCGCAACCGACG; T775V-β2, TCTAAGAGCGCTCCAGGACGTCATGAAC; F766Y-β2, AACCCTAATGTGCTGAG; R732L-β2, ATAATCTGCTACCAAGGTGTTCG. For the deletion mutants, the following oligonucleotides were used: L732R-β2, CACCTGAGCGACCGCCGGGAGTAC; D732R-β2, AACCCCAAGTATGCTGAGAG; R732L-β2, CCTCCCTTCAATGCGCAACCGACG; T775V-β2, TCTAAGAGCGCTCCAGGACGTCATGAAC; F766Y-β2, AACCCTAATGTGCTGAG; R732L-β2, ATAATCTGCTACCAAGGTGTTCG. Both double mutations L732R, T775V-β2 and L732R, T775V-β2 were created using L732R-β2 as template and subsequent mutagenesis with the appropriate oligonucleotides for the S756C-β2 and T775V-β2 point mutation. All mutations were verified by nucleotide sequencing of the region encoding the cytoplasmic domain.

Cell Culture and Transfection—Stable LFA-1-expressing K562 transfectants were established by electroporation of 106 cells in 0.8 ml of phosphate-buffered saline at 280 V and 960 microfarads with the following oligonucleotides for the S756C-β2 and T775V-β2 mutants was determined by immunofluorescence. Cells (2 × 105) were incubated with FITC-conjugated anti-mouse IgG (Zymed Laboratories Inc., San Francisco, CA) for 30 min at 4 °C. The relative fluorescence intensity was measured by FACScan analysis (Becton Dickinson, Oxnard, CA).

Fluorescent Bead Adhesion Assay—for cell adhesion to ICAM-1, cells were reseeded in TSA (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2), 0.5% bovine serum albumin (w/v), Cip-A (200 U/ml), and 106 LFA-1 blocking mAb (20 μg/ml) for 10 min at room temperature in a 96-well V-shaped bottom plate. Carboxylate-modified TransFluoSpheres (485/ 645 nm, 1 μm; Molecular Probes, Inc., Eugene, OR) were coated with adhesion ligands (ICAM-1 Fc) as described earlier (24). The ligand-coated TransFluoSpheres (20 beads/cell) and different integrin stimuli (100 nM PMA (Calbiochem) or 10 μg/ml LFA-1-activating mAb KIM185 (25) for 20 min) were added, and the suspension was incubated for 30 min at 37 °C. Optionally, they werepretreated with 5 μg/ml cytochalasin D (Sigma) for 15 min at 37 °C or with 20–100 μg/ml calpeptin (Calbiochem) 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 of TSA. The LFA-1 transfectants that expressed similar levels of LFA-1, as determined by staining with TS2/4-FITC (minimum 30% of the cells with a mean fluorescence intensity of 70–80), were gated and analyzed for LFA-1-mediated adhesion measured by flow cytometry using the FACScan. Values are depicted as integrin-specific adhesion (i.e. cell adhesion percentage minus cell adhesion percentage in the presence of an integrin-blocking mAb).

Substitution of the β2 by the β1 Cytoplasmic Domain Restores PMA Responsiveness in K562 Cells Transfected with LFA-1—LFA-1 is a cell adhesion receptor that is exclusively expressed on leukocytes. Activation of LFA-1 is required for efficient binding to its ligand ICAM-1. The addition of the phorbol ester PMA has been shown to activate LFA-1 on leukocytes (10). When wild type LFA-1 is transfected into the erythroleukemic cell line K562 (K562-α1/β2) (Figs. 1 and 2), the β2-activating antibody KIM185 can activate LFA-1 and induce LFA-1-mediated adhesion to ICAM-1; however, an inside-out activator of LFA-1 such as PMA cannot activate LFA-1 (Fig. 3A) (13). This lack of PMA responsiveness of LFA-1 is not caused by a general defect of intracellular signal molecules, since other endogenous expressed integrins like VLA-5 can be activated by PMA to bind its ligand fibronectin. Our finding that expression of chimeric LFA-1 containing the β1 cytoplasmic domain (αL/β1/β2) can completely restore the PMA responsiveness of LFA-1 in K562 cells prompted us to search for single amino acids that differ between the β1 and β2 cytoplasmic domain.

Substitution of Leucine for the β2 Arginine in the DLRE Motif
of the \( \beta_2 \) Cytoplasmic Domain Restores PMA Responsiveness of LFA-1 in K562 Cells—To analyze in detail the regions in the \( \beta_2 \) cytoplasmic domain that are responsible for the PMA responsiveness of \( \beta_1/\beta_2 \) chimeric LFA-1 molecule transfected in K562, several point mutations were created in the \( \beta_2 \) cytoplasmic domain. Amino acids of the \( \beta_2 \) cytoplasmic domain were substituted for the residues present in the \( \beta_1 \) cytoplasmic domain (Fig. 1). K562 cells were transfected with \( \alpha \)-chain together with the \( \beta_2 \)-chain containing cytoplasmic domains of \( \beta_1 \) or \( \beta_2 \), as described under “Experimental Procedures.” The expression levels of K562 cells transfected with the LFA-1 chimeras and point mutants were determined by fluorescence-activated cell sorting analysis using anti-CD11a and anti-CD18 antibodies (Fig. 2). The mutations in the \( \beta_2 \) cytoplasmic domain did not affect the \( \alpha/\beta \) heterodimerization based on the expression of \( \alpha/\beta \) heterodimer dependent MHM23 epitope, and immunoprecipitation of LFA-1 from all mutants confirmed that mutant LFA-1 was expressed as \( \alpha/\beta \) heterodimers (data not shown).

The capacity of the LFA-1 mutants to adhere to ICAM-1 was determined using an ICAM-1-coated fluorescent bead adhesion assay (24) in the absence or presence of PMA or the LFA-1-activating antibody KIM185 (Fig. 3B). This adhesion assay allows analysis of only those cells that have similar expression levels of LFA-1. Only cells with a mean fluorescence of 70–80 were analyzed for ICAM-1-coated fluorescent bead binding. All LFA-1 mutants were able to adhere to ICAM-1 for at least 40% when activated by KIM185, indicating that the LFA-1 molecules are functionally expressed on the K562 cells. The level of
The δ2 cytoplasmic domain in LFA-1 signaling

LFA-1-mediated adhesion to ICAM-1 without any activation is low (<8%) except for the mutant δ1/L732R-β2 (18%). Mutation of potential tyrosine and serine phosphorylation sites within the β2-chain to β1 residues (δ1/Y735F-β2 and δ1/S756C-β2) do not restore the PMA responsiveness (Fig. 3B). The same holds true for the threonine at position 758, which has been reported to be involved in cell spreading, and the phenylalanine at position 766, which affects ligand binding (16). Of all mutants, only substitution of leucine for the β1 amino acid arginine (δ1/L732R-β2) in the DRRE motif of the β2 cytoplasmic domain results in a significant increase in adhesion to ICAM-1 upon PMA stimulation. Since the adhesion of unstimulated δ1/L732R-β2 cells is already high (18%), which might facilitate PMA activation, we investigated adhesion to decreasing ICAM-1 concentrations to a level in which the default adhesion of δ1/L732R-β2 was similar to that of wild type LFA-1 (Fig. 3B, inset). At low ICAM-1 concentration, PMA could still enhance the adhesion of mutant δ1/L732R-β2, indicating that the PMA responsiveness is truly induced by the point mutation and not by inherent stronger adhesion. In addition, similar as δ1/L732R-β2, mutant δ1/S756C-β2 has also a default adhesion of 10% but does not respond to PMA. To investigate whether this single mutation is crucial for PMA responsiveness, we mutated in the β2/β1 chimera the arginine present in the DRRE motif of the β1 cytoplasmic domain to a leucine (δ1/R732L-β1). However, in mutant δ1/R732L-β2, the PMA responsiveness was not abolished, suggesting that this arginine residue within the full β2 cytoplasmic domain is not essential for PMA responsiveness and thus that the PMA activation of β1 integrins is differently regulated than β2 integrins. The PMA-induced adhesion of the latter mutant seems somewhat lower than in mutant β2/β1 chimera. However, comparing the relative PMA inducibility between the PMA-responsive mutants demonstrates that there are no significant differences (Table I). Taken together, these results indicate that the created DRRE motif in the β2 cytoplasmic domain is essential for PMA-mediated activation of β2 integrin LFA-1.

Avidity but Not Affinity Changes Correlate with PMA Induction of β2 Cytoplasmic Mutants—To investigate whether the restored PMA responsiveness of the δ1/L732R-β2 mutant is due to a change in avidity and/or affinity, we performed confocal microscopy to detect avidity alterations and soluble ICAM-1 binding studies as affinity measurements. Analysis of the LFA-1 cell surface distribution by confocal microscopy shows that substitution of the β2 cytoplasmic domain for the β1 domain leads to clustering of LFA-1 on the cell surface (13), whereas wild type LFA-1 expressed in K562 cells shows homogeneous distribution of LFA-1 (Fig. 4A). All point mutants in the β2 cytoplasmic domain have a homogeneous LFA-1 distribution (Fig. 4, C and D, and data not shown), with the exception of the PMA-responsive LFA-1 mutant δ1/L732R-β2 that exhibits clusters of LFA-1 (Fig. 4B). Surprisingly, LFA-1 is clustered in all of the β1 cytoplasmic domain point mutants investigated, whereas the reversed mutation δ1/R732L-β2 has a homogeneous LFA-1 distribution (data not shown) despite the fact that the mutant is able to bind to ICAM-1 upon stimulation by PMA. These results suggest that the cluster status of the β2 integrin LFA-1 in δ1/β2 and δ1/L732R-β2 may facilitate the PMA responsiveness of LFA-1.

Whether also the avidity of LFA-1 for ICAM-1 is altered in the δ1/L732R-β2 mutant that responds to PMA, we determined the concentration of soluble ligand (ICAM-1Fc) that yielded half-maximal direct ligand binding activity (ED50). High affinity of LFA-1 for ICAM-1 results in a low concentration of ICAM-1Fc needed to obtain 50% of maximal binding. Strong binding of ICAM-1Fc was observed after stimulation of

![Figure 3](https://www.fic.org/)

**Figure 3.** Binding of LFA-1 mutants expressed in K562 cells to ICAM-1-coated fluorescent beads measured by flow cytometry. A, adhesion of K562-δ1/β2 and δ1/β2/β1 cells to ICAM-1 or fibronectin. Cells were incubated in medium, PMA (100 nm), the activating anti-β1 mAb TS2/16 (10 μg/ml), or the activating anti-β2 mAb KIM185 (10 μg/ml) together with ligand-coated TransFluoSpheres for 30 min at 37°C as described under “Experimental Procedures.” Depicted is the percentage ± S.D. of either VLA-5- or LFA-1-specific adhesion to fibronectin or ICAM-1, respectively. Specific adhesion is the percentage of cells binding minus the percentage of cells binding in the presence of soluble ICAM-1Fc concentrations with or without activation by PMA. Data are representative of three experiments. Inset, adhesion of mutant δ1/L732R-β2 to various soluble ICAM-1Fc concentrations with or without activation by PMA. One of two independent experiments is shown, S.D. ± 10%.

| Table I | Relative PMA induction |
|---------|-------------------------|
| K-αL/β2 | αL/β2/β1 | αL/L732R-β2 | αL/R732L-β1 |
| Exp. 1° | 1.8 ± 1.5 | 67.3 ± 66.0 | 59.1 ± 50.1 |
| Exp. 2 | 2.2 ± 1.4 | 36.5 ± 42.5 | 45.8 ± 55.2 |
| Exp. 3 | 4.3 ± 2.2 | 69.2 ± 51.2 | 53.0 ± 53.2 |
| Exp. 4 | 10.8 ± 5.7 | 57.4 ± 44.8 | 81.3 ± 63.5 |
| Exp. 5 | 2.2 ± 1.2 | 57.5 ± 37.3 | 33.5 ± 35.5 |
| Exp. 6 | 11.9 ± 6.2 | 20.3 ± 25.0 | 35.7 ± 50.7 |
| Exp. 7 | 21.2 ± 4.7 | 49.2 ± 36.8 | 45.5 ± 45.7 |
| Mean ± S.D. | 9.3 ± 7.0 | 52.1 ± 16.8 | 42.4 ± 12.23 | 49.4 ± 18.94 |

° Exp., experiment.  
* Percentage PMA induction calculated from absolute adhesion values: ([PMA – medium]/[KIM185 – medium]) × 100%.  
* Student’s t test: K-αL/β2, p value < 0.0001. No significant differences between αL/β2/β1, αL/L732R-β2, and αL/R732L-β1.
LFA-1 with the activating mAb KIM185 (Table II). Binding of ICAM-1Fc to the mutants was LFA-1-dependent, since LFA-1-blocking antibodies completely inhibited adhesion (data not shown). When the concentration of ICAM-1Fc that yielded half-maximal binding was calculated, we observed an ED50 of ~2 μg/ml for soluble ICAM-1Fc binding to LFA-1 of K562-L/F-β2, α1/β2/β1, α1/L732R-β2, α1/β756C-β2, and α1/T758V-β2. The double mutant α1/T735F-β2 has a slightly, but not significantly (p = 0.136), lower affinity (ED50 = 4.9 ± 0.14 μg/ml) compared with wild type LFA-1 (ED50 = 3.0 ± 1.53 μg/ml). Activation of α1/L732R-β2 with PMA (ED50 = 6.0 ± 1.63 μg/ml) does not significantly (p = 0.248) increases the affinity compared with unstimulated cells (ED50 = 4.5 ± 2.25 μg/ml) as shown in Fig. 3B (inset). Together, these findings indicate that not affinity but avidity changes are responsible for the PMA responsiveness of mutant α1/L732R-β2 to bind ICAM-1.

Importance of the β2 Cytoplasmic Domain for PMA Responsiveness of LFA-1 in K562 Cells—To determine whether only L732R in the β2 domain was enough to generate PMA-induced adhesion, we deleted the β2 cytoplasmic domain directly after the wild type leucine at position 732 in the DLRE motif, at the similar position in the mutant α1/L732R-β2, or after the aspartic acid at position 731 (Figs. 1A and 2). Deleting the cytoplasmic domain immediately after position 731 or 732 completely abolished the PMA-induced adhesion to ICAM-1 (Fig. 5A), whereas the LFA-1-activating antibody KIM185 induced ICAM-1 binding equally well (50%). These results demonstrate that next to the β2 residue L732R in the β2 cytoplasmic domain also other residues within the C-terminal part of the β2 cytoplasmic domain are necessary for the acquired PMA responsiveness of mutant L732R-β2.

Threonine 758 Is Important for PMA Responsiveness of L732R-β2—To investigate which amino acids C-terminal of position 732 in the β2 cytoplasmic domain are necessary together with L732R for PMA-induced LFA-1 activation, double mutants were created that contained both L732R and serine and threonine mutations located C-terminal of L732R (Fig. 1). Serine and threonine residues have been shown to be important in LFA-1 phosphorylation and function (16, 25). These two double mutants, designated α1/L732R,S756C-β2 and α1/L732R,T758V-β2, were stained for functional expression of LFA-1 (data not shown), and the LFA-1-mediated adhesion to ICAM-1 was studied using the ICAM-1-coated fluorescent bead adhesion assay (Fig. 5B). To our surprise, the double mutant α1/L732R,T758V-β2 disrupted the L732R-induced PMA response from 35 to 5%. Mutation of the potential serine phosphorylation site (S756C) did not alter the PMA responsiveness of double mutant α1/L732R,S756C-β2 (37%). These data suggest that the acquired PMA responsiveness of mutant α1/L732R-β2 depends on the threonine residue at position 758 but not the serine residue at position 756.

The β2 cytoplasmic domain contains several phosphorylation-sensitive serine and threonine residues that are phosphorylated upon phorbol ester stimulation (25). Since the α1/L732R-β2 mutant could restore the PMA response, whereas the double mutant α1/L732R,T758V-β2 blocked this responsiveness, we investigated the importance of serine or threonine phosphorylation of the β2-cytoplasmic domain due to PMA activation. Both mutants and wild type LFA-1 in K562 cells are serine- and threonine-phosphorylated on the α2- and β2-cytoplasmic domain with or without PMA stimulation (data not shown), suggesting that the lack of PMA responsiveness of mutant α1/L732R,T758V-β2 is not caused by an impaired phosphorylation on serine or threonine residues in the LFA-1 molecule.

To further investigate whether affinity and/or avidity changes regulate the PMA response of the double mutants, we determined the affinity of ICAM-1 by measuring the soluble ICAM-1Fc concentration needed to yield half-maximal binding activity (Table II). Although the double mutants α1/L732R,S756C-β2 and α1/L732R,T758V-β2 differed in PMA responsiveness, no significant changes could be observed for the ED50 (1.9 ± 0.70 μg/ml ICAM-1 and 3.3 ± 1.79 μg/ml ICAM-1, respectively). However, analysis of the cell surface distribution of LFA-1 revealed that LFA-1 was homogeneously distributed on the cell surface, similar to α1/S756C-β2 (C), α1/T758V-β2 (D), and α1/L732R,T758V-β2 (E). LFA-1 is localized in large clusters on α1/L732R-β2 (B) and α1/L732R,S756C-β2 (F) as indicated by arrows. The instrument settings of the CLSM were the same for the four different panels as follows: lens, × 60; gain, 1300; pinhole, 1.5 μm; and magnification, × 2.0. One out of three experiments is shown.
changes in LFA-1 due to the activation of a Ca\(^{2+}\)-dependent protease calpain that disrupts the cytoskeletal association with LFA-1 (26). Activation of calpain can be blocked by reagent calpeptin. To investigate whether our LFA-1-transfected K562 cells regulate their cell surface distribution by the activation of calpain, we inhibited adhesion to ICAM-1 with calpeptin at concentrations previously demonstrated to block calpain activity (26). Surprisingly, calpeptin completely abrogated the PMA responsiveness of mutant \(\alpha_L/L732R-\beta_2\), whereas a lower concentration of calpeptin (20 \(\mu\)g/ml) could not fully block the PMA-induced adhesion (Fig. 6). These results indicate that PMA acts via calpain to promote activation of LFA-1 through partial dissociation from the cytoskeleton facilitating clustering of LFA-1 molecules.

**DISCUSSION**

Using a cell transfection system in which inside-out signaling of the \(\beta_2\) integrin LFA-1 could be modified by substitution of the \(\beta_2\) for the \(\beta_1\) cytoplasmic domain, we searched for single amino acids in the \(\beta_2\) cytoplasmic domain that regulate inside-out signaling. We identified one amino acid at position 732, in which a leucine is substituted for an arginine that could restore the PMA responsiveness of LFA-1 completely. PMA inside-out signaling also depends on a threonine located more C-terminally at position 758. Of all LFA-1 mutants that respond to PMA, it is surprising that a substitution of a leucine for an arginine could fully restore the PMA responsiveness of LFA-1.

### Table II

| K562 transfectants | Concentration soluble ICAM-1Fc (\(\mu\)g/ml) | ED\(_{50}\)^a | Mean ± S.D. |
|--------------------|-----------------------------------|--------|----------|
| K-\(\alpha_L/\beta_2\)   | 32.0^c | 16.1 | 2.8 | 1.7 | 3.03 | 1.53 |
| \(\alpha_L/L732R-\beta_2\) | 28.0 | 17.1 | 8.0 | 2.1 | 1.0 | 0.9 | 2.53 | 1.07 |
| \(\alpha_L/L732R/S756C-\beta_2\) | 32.9 | 30.9 | 17.0 | 3.7 | 3.4 | 4.4 | 2.50 | 1.21 |
| \(\alpha_L/L732R/T758V-\beta_2\) | 27.2 | 22.0 | 3.6 | 1.0 | 0.6 | 0.8 | 1.1 | 4.90 | 0.14 |
| \(\alpha_L/L732R/S756C-\beta_2\) | 25.8 | 26.9 | 16.0 | 10.7 | 4.2 | 3.0 | 3.2 | 2.16 | 1.06 |
| \(\alpha_L/L732R/T758V-\beta_2\) | 24.9 | 22.2 | 12.9 | 4.8 | 2.5 | 1.9 | 2.2 | 2.10 | 0.36 |
| \(\alpha_L/L732R/T758V-\beta_2\) | 64.2 | 68.3 | 40.4 | 24.2 | 5.7 | 3.5 | 1.1 | 1.87 | 0.70 |
| \(\alpha_L/L732R/T758V-\beta_2\) | 76.6 | 69.4 | 29.9 | 10.4 | 2.7 | 1.1 | 1.1 | 3.32 | 1.79 |

^a Half-maximum binding.  
^b Mean ED\(_{50}\) ± S.D. of at least three independent experiments.  
^c Percentage adhesion to soluble ICAM-1Fc.
PMA, avidity alterations and not affinity changes or β2 phospho-
rylation seemed important for proper function. We propose a
model for this PMA responsiveness regulated by position 732
and 758 and avidity changes regulated by activation of a Ca2+
dependent protease calpain that releases LFA-1 from the cyto-
skeleton, thereby allowing the formation of a signaling com-
plex leading to active LFA-1.

Integrin-dependent adhesion is strongly induced upon in-
side-out signaling when PKC is activated through the addition
of PMA or via T cell receptor triggering. It remains still obscure
how “inside-out” signaling by PMA results in LFA-1 activation.

The newly identified single amino acid mutation (L732R) re-
sponsible for PMA activation of LFA-1 is situated in the
β2 DLRE motif, which is conserved throughout the other integrins
but distinct in one amino acid (DRRE) in β1 and β2 integrins.

PMA can activate β1 integrins, but not β2 and β3 in K562 cells,
suggesting that besides the DRRE motif also lymphocyte-spe-
cific elements are involved (13). In addition, creating the DLRE
motif in the β1 cytoplasmic domain (α1/β2 R732L-β2) did not abol-
ish the PMA responsiveness, indicating that the PMA activa-
tion of β1 integrins is differently regulated compared with β2
integrins. The DLRE motif has been proposed to bind the
GFFKR motif in the α-chain, and both of these cytoplasmic
domains serve to constrain LFA-1 into a default low affinity
state (27). Mutations in the TTT region (positions 758–760)
to alanines residues of the β2 cytoplasmic domain have been
shown to reduce the default adhesion to ICAM-1 and the phor-
dol ester-mediated LFA-1 phosphorylation when expressed in
COS cells or B lymphoblastoid cells, but they do not abrogate
the binding to ICAM-1 in response to PMA (16). In contrast, we
observed with the double mutant α1/β2 L732R, T758V-β2 that sub-
stitution of the threonine into the β2 residue valine in K562 cells
completely decreased the PMA-induced adhesion restored
by the L732R mutation and altered the LFA-1 surface distribu-
tion. The phosphorylation level of these threonines after
stimulation with PMA is strongly increased upon pretreatment
with okadaic acid, which inhibits serine and threonine phospho-
phatases (25). Threonine-phosphorylated CD18 molecules have
been shown to associate with the cytoskeleton (28) and play an
important role in the formation of stress fibers and specialized
microdomains, such as rafts (15, 29, 30). However, in our sys-
tem we could not identify any role of threonine phosphorylation
and the PMA responsiveness of LFA-1, although the threonines
themselves are a prerequisite for the PMA response together
with the mutation L732R. In line with the threonine phos-
phorylation, we observed also no differences in serine phospho-
rylation, even in double mutant α1/β2 L732R, S756C-β2 that was still
able to adhere to ICAM-1 upon stimulation with PMA. This
further questions the relevance of this serine residue in phos-
phorylation and ICAM-1 binding as shown in other studies
(16). The tyrosine-based NP9Y motif in the β1 cytoplasmic
domain has been implicated in regulating integrin function
(31). However, substitution of the phenylalanine for the β1
tyrosine (F766Y-β2) in this motif did not restore PMA sensitiv-
ity. Thus, phosphorylation of the β2 cytoplasmic domain is not
a prerequisite for the acquired PMA responsiveness.

Cytokinesis, a member of the guanine nucleotide exchange
factors for ADP-ribosylation factor G-proteins, specifically in-
teracts with the β2 cytoplasmic domain directly after the trans-
membrane region (positions 723–725), thereby controlling T
cell receptor or phorbol ester-induced activation of LFA-1 (17).
Cytokinesis expression is involved in maintaining LFA-1 in a
high avidity state. Since cytokinesis is expressed in K562 cells
and associates with LFA-1 (32), it is likely that the high avidity
state of our DRRE mutant is a direct result of cytokinesis bind-
ing. Double staining of LFA-1 and cytokinesis in the β2 point
mutants did not demonstrate differences in colocalization of
LFA and cytokinesis (data not shown). Upon PMA activation,
many proteins are phosphorylated and activated via PKC such
as the β2-linked proteins Rack1, MacMARCKS, and L-plastin.

Phosphorylated Rack1 binds PKC, allowing subsequent re-
cruitment of Rack1 to the KALI region in the β2 cytoplasmic
domain, which is the same binding region for cytokinesis (17, 18).

The WD repeats 5–7 of Rack1 interact with β integrins, leaving
the other repeats free for binding to PKC and possible cytohe-
sin. Thus, Rack1 merely functions as a scaffold protein to
recruit PKC and other β2 regulators to the site of action. Mac-
MARCKS is a PKC substrate phosphorylated upon PMA acti-
vation, which leads to an increase in the lateral diffusion of β2
integrins and enhanced LFA-1-dependent cell clustering (33).
We could not demonstrate by confocal microscopy any differ-
ces in cytosolic localization of MacMARCKS after activation
with PMA (data not shown). Hence, it remains unclear whether
MacMARCKS directly binds the β2 cytoplasmic domain or
Rack1. The leukocyte-specific actin-bundling protein L-plastin
proved to be important in enhanced integrin avidity through
PKC and PI-3 kinase (34). Upon PMA activation, calcium is
released from intracellular stores and binds to the EF-hand
type calcium-binding domain of L-plastin, thereby inhibiting
actin bundling activity. Thus, MacMARCKS as well as L-plas-
tin play a crucial role in the association of the integrin with
the cytoskeleton and subsequently integrin activation. Besides
PKC, phosphatidylinositol 3-kinase and the small GTPase Rap1 modulate LFA-1 avidity in leukocytes (35, 36). Both Rap1
and the phosphatidylinositol 3-kinase but not PKC mediated
activation up-regulated the NKI-L16 epitope, indicating in-
creased LFA-1 avidity (35).

The actin cytoskeleton plays a critical role in integrin acti-
vation and signaling by acting as a platform to bring different
components close together, leading to a signaling complex. The
β cytoplasmic domain has been demonstrated to be associated
with the cytoskeletal component α-actinin (19), vinculin (37),
filamin (38), or talin (39). Treatment of cells with cytochalasin
D, which disrupts the cytoskeleton network, results in activa-
tion of LFA-1 that coincides with clustering of LFA-1, indicat-
ing that the cytoskeleton restraints keep integrins inactive
(14). However, cytochalasin D had no effect on activation or
clustering of wild type LFA-1 expressed in K562 cells, indicat-
ing a different mechanism of cytoskeletal organization. In con-
trast, the PMA-responsive mutant α1/β2 L732R-β2 could be sponta-
nously activated by cytochalasin D. The release of LFA-1
from the cytoskeleton in lymphocytes is also thought to be
regulated by the cysteine protease calpain that is activated by
local Ca2+ fluxes (26). Indeed, we have evidence that the PMA-
induced activation of LFA-1 is mediated by calpain, since inhib-
ition with calpeptin abrogated the PMA responsiveness of
mutant α1/β2 L732R-β2. Calpeptin also has been reported to in-
duce stress fiber formation in fibroblasts due to its inhibitory
action on protein-tyrosine phosphatases upstream of the small
GTPase Rho (40). It is rather unlikely that this additional
effect of calpeptin on protein-tyrosine phosphatases also occurs
during the integrin-mediated adhesion of the nonadherent
K562 cells that do not induce stress fiber formation. Proteins
identified as potential calpain targets include talin, filamin,
and α-actinin. Talin forms the bridge between the β2 integrin
and the actin filaments. Upon activation, LFA-1 is released
from the cytoskeleton as a result of proteolysis of talin, proba-
bly by calpain, leading to freely mobile integrin as postulated
by Sampath et al. (46). Next, α-actinin binds the β2 cytoplasmic
domain between residues 736 and 746 directly C-terminal of
the DLRE motif, thereby stabilizing the cytoskeleton-integrin
interaction necessary for strong adhesion. We speculate that
this $\beta_2$-specific event is impaired in K562, whereas the DRRE mutant partly restores the lateral mobility of LFA-1, resulting in increased LFA-1 avidity. The $\alpha$-actinin binding motif is also important for endoplasmic reticulum retention, assembly, and transport to the cell surface of LFA-1 (37). Peptides from the $\beta_1$ cytoplasmic domain reveal that pp125FAK and paxillin bind the cytoplasmic domain with the binding sites (underlined) for cytohesin, Rack1, and $\alpha$-actinin. The threonine residues at positions 758–760 together with the phenylalanine (position 766) are required for ligand binding. The mutated lysine creating the DRRE motif and the essential threonine at position 758 restores the PMA responsiveness of LFA-1 expressed in K562 cells.

The acquired PMA signaling of our LFA-1 mutants in K562 cells coincides with a change in the clustered LFA-1 cell surface distribution. Much attention has been recently given to specialized lipidic membrane microdomains, also termed "rafts." They function as platforms for signaling molecules and are involved in the regulation of LFA-1 function and adhesion through avidity changes (15, 30). Whether the PMA-responsive LFA-1 mutant is differently organized in rafts compared with wild type LFA-1 remains so far unsolved. Our results suggest that increased avidity facilitates PMA-induced adhesion to ICAM-1 rather than affinity changes. Clustering of LFA-1 molecules probably leads to a higher concentration of signaling components involved in LFA-1 signaling such as cytohesin, Rack1, paxillin, or MacMARCKS as shown in a model in Fig. 7. Furthermore, activation of LFA-1 by PMA is dependent on calpain, which cleaves cytoskeletal components. This is probably a crucial event, because one can imagine that dislodgement from the cytoskeleton facilitates binding of signaling molecules, leading to a reorganization of the cytoskeleton and activation of LFA-1.

We have shown that this process can be overruled by adding cytochalasin D. However, PMA seems ineffective in cells with a homogeneous distribution of LFA-1. In the case of the mutants with a clustered LFA-1 distribution, the threshold for triggering the PMA signaling cascade is lower, since the concentrations of signaling molecules directly or indirectly connected to the cytoplasmic domain of LFA-1 are higher. Therefore, we cannot exclude the possibility that PMA indeed has an effect on LFA-1 in K562 cells, albeit small and not detectable with our assays. By using the soluble ICAM-1 binding assay, we can detect relatively small changes in LFA-1 affinity. However, all of our mutants showed an equal ability to bind soluble ICAM-1, indicating no affinity differences. Previous reports suggest that affinity changes play an important role in regulating integrin-mediated adhesion, although we showed earlier that this is not true for $\beta_2$ integrins (43, 44). Activation of LFA-1 by EGTA or Mg$^{2+}$ leads to enhanced expression of the M24 epitope, indicating that Mg$^{2+}$ binding induces conformational changes in LFA-1, leading to enhanced ICAM-1 binding (45). Since we observed no changes in affinity and M24 expression of the mutants, we conclude that the acquired PMA responsiveness has no effect on the extracellular conformational changes of LFA-1.

In summary, we present in this study evidence that substitution of a single amino acid in the $\beta_2$ DLRE motif together with an intact C-terminal TTT sequence is sufficient to restore PMA-induced LFA-1 adhesion to ICAM-1. The gained PMA
signaling is probably due to the presence of a dense LFA-1 intracellular signaling complex, since these mutants have a clustered surface distribution of LFA-1. The activation of LFA-1 is dependent on rearrangements of the cytoskeleton through a mechanism involving a Ca$^{2+}$-dependent protease calpain. This work clearly demonstrates that the function of LFA-1 is strictly regulated and involves a leukocyte-specific signaling element.

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