Cytoplasmic Tails of \( \beta_1 \), \( \beta_2 \), and \( \beta_7 \) Integrins Differentially Regulate LFA-1 Function in K562 Cells

Marijke Lub,* Sandra J. van Vliet,* Sigrid P.M.A. Oomen,* Reagan A. Pieters,* Martyn Robinson,† Carl G. Figdor,* and Yvette van Kooyk**

*Department of Tumor Immunology, University Hospital Nijmegen St. Radboud, Nijmegen, the Netherlands; and †Department of Exploratory Research, Celltech, United Kingdom

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The \( \beta_2 \) integrin lymphocyte function-associated antigen 1 (LFA-1) mediates activation-dependent adhesion of lymphocytes. To investigate whether lymphocyte-specific elements are essential for LFA-1 function, we expressed LFA-1 in the erythroleukemic cell line K562, which expresses only the integrin very late antigen 5. We observed that LFA-1-expressing K562 cannot bind to intercellular adhesion molecule 1-coated surfaces when stimulated by phorbol 12-myristate 13-acetate (PMA), whereas the LFA-1-activating antibody KIM185 markedly enhanced adhesion. Because the endogenously expressed \( \beta_1 \) integrin very late antigen 5 is readily activated by PMA, we investigated the role of the cytoplasmic domain of distinct \( \beta \) subunits in regulating LFA-1 function. Transfection of chimeric LFA-1 receptors in K562 cells reveals that replacement of the \( \beta_2 \) cytoplasmic tail with the \( \beta_1 \) but not the \( \beta_7 \) cytoplasmic tail completely restores PMA responsiveness of LFA-1, whereas a \( \beta_2 \) cytoplasmic deletion mutant of LFA-1 is constitutively active. Both deletion of the \( \beta_2 \) cytoplasmic tail or replacement by the \( \beta_1 \) cytoplasmic tail alters the localization of LFA-1 into clusters, thereby regulating LFA-1 activation and LFA-1-mediated adhesion to intercellular adhesion molecule 1. These data demonstrate that distinct signaling routes activate \( \beta_1 \) and \( \beta_2 \) integrins through the \( \beta \)-chain and hint at the involvement of lymphocyte-specific signal transduction elements in \( \beta_2 \) and \( \beta_7 \) integrin activation that are absent in the nonlymphocytic cell line K562.

INTRODUCTION

The \( \beta_1 \) integrin lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18 or \( \alpha_\text{L}\beta_2 \)) is a lymphocyte-specific adhesion receptor that coordinates different adhesive and signaling interactions within the immune system (Kurzinger et al., 1981; Martz, 1987; Arnaout, 1990; Springer, 1990). LFA-1 mediates cell-cell adhesion upon binding to any one of its ligands, which are intercellular adhesion molecule (ICAM) 1 (Marlin and Springer, 1987), ICAM-2 (Staunton et al., 1989), and ICAM-3 (de Fougerolles and Springer, 1992; Fawcett et al., 1992; Vazeux et al., 1992; de Fougerolles et al., 1993). Integrins are heterodimeric transmembrane molecules composed of an \( \alpha \)-chain that is noncovalently linked to a \( \beta \)-chain. Various integrin subfamilies can be distinguished by differences in their \( \beta \)-chains (Hynes, 1987).

Besides \( \beta_3 \) integrins, lymphocytes also express \( \beta_1 \) and \( \beta_2 \) integrins. Similar to the \( \beta_2 \) integrins, \( \alpha_\text{L}\beta_2 \) is only expressed on lymphocytes and mediates adhesion to vascular cell adhesion molecule 1, mucosal addressing cell adhesion molecule, \( \alpha_\text{C} \) or the extracellular matrix component fibronectin (Ruegg et al., 1992; Allevogt et al., 1995; Berlin et al., 1995). In contrast, the \( \beta_1 \) integrins \( \alpha_\text{L}\beta_1 \) and \( \alpha_\text{M}\beta_1 \) are not lymphocyte-specific adhesion receptors, since they are found on a variety of other cell types (Hemler, 1988, 1990; Hemler et al., 1989).
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1990. \( \alpha_\beta \) mediates adhesion to VCAM-1, \( \alpha_\text{p} \) and the extracellular matrix component fibronectin, whereas \( \alpha_\beta \) predominantly binds fibronectin (Hemler et al., 1987; Takada et al., 1987; Hemler et al., 1988; Altevogt et al., 1995).

LFA-1/ICAM-1 adhesion requires activation of LFA-1 through intracellular signals (Martz, 1987; Dustin and Springer, 1989; van Kooyk et al., 1989; Hyynes, 1992). This activation process, termed “inside-out” signaling, is common to all integrins including the \( \beta_1 \) and \( \beta_2 \) integrins (Keizer et al., 1988; Dustin and Springer, 1989; van Kooyk et al., 1989; Altieri, 1991; Dransfield et al., 1992b; Andrew et al., 1993; Landis et al., 1993). Activation of LFA-1 is thought to result in a conformational change in the \( \alpha/\beta \) heterodimer, leading to an enhanced binding affinity for LFA-1 for its ligand ICAM-1 (Lollo et al., 1993). Both affinity (active conformation) and avidity (clustering) changes are required to obtain strong binding of LFA-1 to ICAM-1 (Dustin, 1990). Affinity/avidity changes in LFA-1 depend on an intact cytoskeleton, physiological temperature, and binding of divalent cations, in particular \( \text{Mg}^{2+} \) (Rothlein and Springer, 1986; Dransfield and Hogg, 1989; Figdor et al., 1990; Dransfield et al., 1992a).

Binding of \( \text{Ca}^{2+} \) to LFA-1 supports clustering (high-affinity state) of LFA-1 on the cell surface, resulting in enhanced LFA-1-mediated adhesion (Figdor et al., 1990; van Kooyk et al., 1994).

Alternatively, binding of certain activating anti-integrin antibodies, or the divalent cation \( \text{Mn}^{2+} \), to the extracellular part of the integrin, can also induce an active conformation (high-affinity state) of the \( (\beta_1, \beta_2, \beta_2) \) integrin, resulting in increased adhesion to the ligand (Keizer et al., 1988; Robinson et al., 1992; Andrew et al., 1993; Landis et al., 1993). It is thought that these activating anti-integrin monoclonal antibodies (mAbs) mimic ligand binding and stimulate postligand binding signaling (“outside-in” signaling). Outside-in signaling generates different intracellular signals, including phosphorylation of distinct tyrosine kinases and other proteins (Hyynes, 1992; Kanner et al., 1993; Arroyo et al., 1994).

Although the cytoplasmic tail of the \( \alpha \)- and \( \beta \)-chains of \( \beta_1, \beta_2, \) and \( \beta_2 \) integrins are relatively short (45, 45, and 51 amino acids for the \( \beta_1, \beta_2, \) and \( \beta_2 \) cytoplasmic tail, respectively) and do not contain any intrinsic kinase activity, the cytoplasmic tails seem to be involved in transmitting inside-out signals as well as outside-in signals to and from the integrin molecule. It has been demonstrated that the adhesiveness of LFA-1 is controlled by the cytoplasmic domain of the \( \alpha_\text{p} \) subunit, because truncation of the cytoplasmic \( \beta_2 \) tail, but not the \( \alpha_\text{p} \) tail, eliminates LFA-1 binding to ICAM-1 (Hibbs et al., 1991b). In particular, mutations of a triplet of threonines (positions 758–760) and the phenylalanine residue at position 766, in the \( \beta_2 \) cytoplasmic tail profoundly reduced the adhesiveness of LFA-1 (Hibbs et al., 1991a; Peter and O’Toole, 1995). 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 (Peter and O’Toole, 1995). Because deletion of the cytoplasmic domain of the \( \alpha_\text{p} \) subunit does not affect binding to ICAM-1, it is hypothesized that the cytoplasmic tail of \( \alpha_\text{p} \) is predominantly involved in “postligand role in regulating binding” events of this integrin (Hibbs et al., 1991b).

Similarly, truncation of the cytoplasmic domain of the \( \beta_1 \) integrin subunit impairs adhesion to both fibronectin and laminin and has been shown to be important for cell spreading and localization to focal contacts (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Reszka et al., 1992). Moreover, partial removal of the \( \beta_7 \) cytoplasmic domain displayed no ligand binding activity of \( \alpha_\beta_7 \) (Crowe et al., 1994).

In this study, we investigated whether a lymphocytic environment is required to mediate adhesion through the \( \beta_2 \) integrin LFA-1 and whether this depends on the cytoplasmic tail of the \( \beta \)-chain. Therefore, we used the erythroleukemic K562 cells to express either wild-type LFA-1, LFA-1 in which the entire cytoplasmic tail of the \( \beta_2 \) was deleted (\( \Delta \beta_2 \)), or LFA-1 in which the cytoplasmic domain of the \( \beta_2 \) was exchanged for the cytoplasmic domains of \( \beta_1 \) or \( \beta_7 \). We demonstrate that the cytoplasmic tail of the \( \beta_2 \)-chain of integrins plays a pivotal role in regulating ligand binding affinity (active conformation) and avidity (clustering) and suggest that K562 and lymphocytes use different signaling elements to activate integrins.

**MATERIALS AND METHODS**

**mAbs**

mAbs SFV-L7 (IgG1), NK1-L15 (IgG2a), and NK1-L16 (IgG2a) reactive with the \( \alpha \)-chain of LFA-1 were raised as described previously (Keizer et al., 1985, 1988). The nonblocking mAb TS2/4 (IgG1) reactive with \( \alpha_2 \) was provided by Dr. E Martz (Sanchez Madrid et al., 1982), mAb 60.3 (IgG1), directed against \( \beta_2 \) was obtained from Dr. M. Harlan (Beatty et al., 1983). The anti-\( \beta_2 \) mAb KIM18S (IgG1) was used to activate \( \beta_2 \) integrins (Andrew et al., 1993) and the anti-\( \beta_2 \) mAb TS2/16 to activate \( \beta_2 \) integrins (Hemler et al., 1984; van de Wiel-van Kemenade et al., 1992). The anti-\( \alpha_\text{p} \) mAb SAM-1 (IgG1) was used to block very late antigen 5-dependent adhesion (Keizer et al., 1987).

**DNA Constructs**

The 4.2-kb \( \alpha \)-chain of LFA-1 was cloned in the XbaI site of the pCDM8 vector that directs expression of from the cytomegalovirus (CMV) AD169 immediate early promoter (pCDL1). The 3' end of \( \beta_2 \) was cloned as an EcoRI-BglII fragment in the pRC/CMV vector (containing a neomycin resistance gene; Invitrogen, San Diego, CA). Within this sequence is a unique Apal site at position 1980. The C-terminal end was rebuilt from this site with 10 overlapping oligonucleotides and amplification by the polymerase chain reaction to obtain the appropriate hybrids. For the \( \beta_2/\beta_7 \) chimeric protein,
plasmic tail from amino acid 723 (cytoplasmic domain) was joined to amino acid 723 of β2 and for the β2/β2 chimeric protein, amino acid 747 of β2 (cytoplasmic domain) was joined to amino acid 723 of β2. The deletion mutant of LFA-1 was made by truncation of the β2 cytoplasmic tail from amino acid 724 (see Figure 2).

Cell Culture and Transfection

Stable LFA-1-expressing K562 transfectants were established by electroporation of 10^7 cells in 0.8 ml of phosphate-buffered saline at 280 V and 960 pulses with the electroporation of 10^7 cells in 0.8 ml of phosphate-buffered saline at 4°C. Stably transfected CD11a/CD18 K562 cells were labeled with Na125I (Amersham International). For immunoprecipitation, 10^7 cells were solubilized for 1 h at 4°C in immunoprecipitation buffer (IPB), which contained 1% Nonidet P-40 (Sigma, St. Louis, MO), 50 mM triethanolamine (pH 7.8, Sigma), 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2, and as protease inhibitors (Sigma) 1 mM phenylmethylsulfonyl fluoride, 0.02 mg/ml ovomucoid trypsin inhibitor, 0.02 mg/ml leupeptin, and 1 mM N-o-tosyl-l-lysine chloromethyl ketone were added. Nuclear debris was removed from the lysates by centrifugation at 13,000 x g for 15 min at 4°C. Lysates were precleared by successive incubation with mouse IgG covalently coupled to protein A-Sepharose CL-4B beads (Pharmacia, Piscataway, NJ). Precleared cell lysates were immunoprecipitated with specific mAb, directed against either LFA-1 or VLA-5, coupled to protein A-Sepharose CL-4B for 1 h at 4°C. The immunoprecipitates were removed from the lysates by centrifugation at 13,000 x g. Subsequently, immunoprecipitates were washed extensively in IPB and analyzed under reducing conditions with 5% β-mercaptoethanol in SDS sample buffer. SDS-PAGE was carried out on vertical slab gels (5-15%) according to a modification of the Laemmli procedure (Laemmli, 1970). Kodak XAR film was used in combination with intensifier screens (Cronex Lightning Plus; DuPont, Newton, CT) for autoradiography of 125I-labeled materials.

Immunofluorescence Analysis

Expression of LFA-1 on the transfectants was determined by immunofluorescence. Cells (2 x 10^5 cells) were incubated (30 min, 4°C) in phosphate-buffered saline, containing 0.5% (wt/vol) bovine serum albumin (BSA, Boehringer Mannheim, Mannheim, Germany) and 0.01% sodium azide (10 mM, Merck, Hohenbrunn, Germany), with appropriate dilutions of either an anti-integrin mAb or an isotype-matched control antibody, followed by incubation with FITC-labeled goat anti-mouse IgG mAb (Zymed Laboratories, San Francisco, CA) or anti-human IgF mAb (Jackson Immunoresearch Laboratories, Westgrove, PA) for 1 h at 37°C and blocked with 1% BSA in TSM (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2) for 30 min at 37°C. Radiolabeled cells were washed and preincubated for 15 min at room temperature with different stimuli (50 nM phorbol 12-myristate 13-acetate (PMA, Calbiochem, La Jolla, CA), 5 μg/ml KIM185, or 5 μg/ml TSZ/161 and/or blocking mAbs (10 μg/ml)). Cells were allowed to adhere for 45 to 60 min at 37°C. Unbound cells were removed by washing with TSM supplemented with 0.5% (wt/vol) BSA. The adherent cells were lysed with 100 μl of 2% Triton X-100 and radioactivity was quantified in a gamma counter. Results are expressed as the mean percentage of cells binding to triplicate wells. Values are depicted as integrin-specific adhesion: percentage of cells binding — percentage of cells binding in the presence of an integrin-blocking mAb (KIM185 or SAM-1).

Radiolabeling and Immunoprecipitation

Transfectants were surface labeled with Na125I (Amersham International). For immunoprecipitation, 10^7 cells were solubilized for 1 h at 4°C in immunoprecipitation buffer (IPB), which contained 1% Nonidet P-40 (Sigma, St. Louis, MO), 50 mM triethanolamine (pH 7.8, Sigma), 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2, and as protease inhibitors (Sigma) 1 mM phenylmethylsulfonyl fluoride, 0.02 mg/ml ovomucoid trypsin inhibitor, 0.02 mg/ml leupeptin, and 1 mM N-o-tosyl-l-lysine chloromethyl ketone were added. Nuclear debris was removed from the lysates by centrifugation at 13,000 x g for 15 min at 4°C. Lysates were precleared by successive incubation with mouse IgG covalently coupled to protein A-Sepharose CL-4B beads (Pharmacia, Piscataway, NJ). Precleared cell lysates were immunoprecipitated with specific mAb, directed against either LFA-1 or VLA-5, coupled to protein A-Sepharose CL-4B for 1 h at 4°C. The immunoprecipitates were removed from the lysates by centrifugation at 13,000 x g. Subsequently, immunoprecipitates were washed extensively in IPB and analyzed under reducing conditions with 5% β-mercaptoethanol in SDS sample buffer. SDS-PAGE was carried out on vertical slab gels (5-15%) according to a modification of the Laemmli procedure (Laemmli, 1970). Kodak XAR film was used in combination with intensifier screens (Cronex Lightning Plus; DuPont, Newton, CT) for autoradiography of 125I-labeled materials.

Confocal Microscopy

Cells were fixed with 0.5% paraformaldehyde. Fixed cells were stained with TSZ/4 mAb (10 μg/ml) for 30 min at 37°C followed by incubation with FITC-labeled goat F(ab')2 anti-mouse IgG mAb (Zymed Laboratories) 30 min at room temperature. 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, Hercules, CA). The CLSM settings were: lens, 60X; gain, 1300; pinhole, 1.5 μm; and magnification, 1.5 X. The same instrument settings of the CLSM were used throughout the distinct experiments.

RESULTS

Expression and Function of Wild-Type LFA-1 in the Erythroleukemic K562 Cells

LFA-1 (αι/β2) is an adhesion receptor that is exclusively expressed on lymphocytes. To investigate the importance of a lymphocytic environment for the adhesion function of LFA-1, we transfected both wild-type αι- and β2-chain cDNA in erythroleukemic K562 cells that do not express the lymphocyte-specific β2 and β1 integrins endogenously. The transfectants express high levels of the heterodimer LFA-1 on their cell surface as detected by staining of the cells with mAb directed against either αι (SPV-L7) or β2 (60.3; see Figure 3A).

The capacity of LFA-1 on K562 cells to bind its ligand ICAM-1 was determined by activation of LFA-1 with PMA or the activating anti-β2 mAb (KIM185) and was compared with the adhesion capacity of VLA-5, the only endogenous expressed β1 integrin. Figure 1A demonstrates that the LFA-1-trans-
Fibronectin

Control PMA TS2/16

transmembrane part of cytoplasmic domain of the acid sequences implicated in integrin function and activation. Between brackets is depicted the amino acid position of Erythroleukemic K562 Cells

Figure 1. Capacity of K562 αβ transfectants to bind to ICAM-1 or fibronectin. K562-αβ cells were preincubated in medium (control), PMA (50 nM), the activating anti-β2 mAb KIM185, or the activating anti-β2 mAb TS2/16 (5 μg/ml). Depicted is the mean percentage of either LFA-1-specific adhesion to ICAM-1 or VLA-5-specific adhesion to fibronectin. Integrin-specific adhesion: percentage of cells binding – percentage of cells binding in the presence of an integrin blocking mAb (NKI-L15 or SAM-1). Data are representative of four experiments.

Amino acid sequence of the different LFA-1 chimeras. Asterisks mark the amino acid sequence homology of the cytoplasmic domain of the three distinct β-chains. Both βα and βγ integrins are predominantly expressed by lymphocytes, whereas βα integrins are widely distributed.

The chimeric βα-chains: βα/β1, βα/βγ and βα/Δ724 were transfected along with the wild-type α-chain (αδ) in K562. All transfectants expressed equally high levels of LFA-1 (Figure 3A). Our unpublished observations demonstrated that LFA-1 was expressed as a heterodimer on the cell surface, since all transfectants expressed equally high levels of the MHM23 epitope that has been reported to detect an α/β-association-dependent epitope on LFA-1 (Hildreth and August, 1985). These data demonstrate that deletion of the βγ cytoplasmic tail or replacement of the βγ cytoplasmic tail for the βα or βγ cytoplasmic tail does not alter the overall conformation of the LFA-1/αδ heterodimer. All transfectants show similar levels of the endogenously expressed βα integrin, VLA-5 (Figure 3A).

Expression of βγ-Chimeric LFA-1 Molecules in Erythroleukemic K562 Cells

To determine whether the observed differences between PMA responsiveness of βα and βγ integrins are due to differences in the cytoplasmic tail, we generated LFA-1 molecules in which the cytoplasmic tail of the βγ-chain was truncated close to the transmembrane region at amino acid position 724 (K562-αδ/Δ724) or replaced with that of the βα-chain or the βγ-chain (K562-αδ/βα and K562-αδ/βγ, respectively). Figure 2 shows the amino acid sequence of the different LFA-1 chimeras. Asterisks mark the amino acid sequence homology of the cytoplasmic domain of the three distinct β-chains. Both βα and βγ integrins are predominantly expressed by lymphocytes, whereas βα integrins are widely distributed.

The chimeric βα-chains: βα/β1, βα/βγ and βα/Δ724 were transfected along with the wild-type α-chain (αδ) in K562. All transfectants expressed equally high levels of LFA-1 (Figure 3A). Our unpublished observations demonstrated that LFA-1 was expressed as a heterodimer on the cell surface, since all transfectants expressed equally high levels of the MHM23 epitope that has been reported to detect an α/β-association-dependent epitope on LFA-1 (Hildreth and August, 1985). These data demonstrate that deletion of the βγ cytoplasmic tail or replacement of the βγ cytoplasmic tail for the βα or βγ cytoplasmic tail does not alter the overall conformation of the LFA-1/αδ heterodimer. All transfectants show similar levels of the endogenously expressed βα integrin, VLA-5 (Figure 3A).

To verify that the chimeric LFA-1 molecules αδβα/β1 and αδβα/βγ did not associate with endogenously expressed βα-chain in K562, LFA-1 and VLA-5 were immunoprecipitated from all transfectants. βα wild-type, chimeric βα-chains, and αδβα/Δ724 associated with αδ and not with αδ (Figure 3B, lanes C, D, F, G, I, J, L, and M, observed as the thick bands of 185 and 95 kDa). Similarly, immunoprecipitation with an anti-αδ mAb showed that VLA-5 does not associate with αδ or βγ (Figure 3B, lanes A, E, H, and K, observed as one thick band of 130–135 kDa, because, under reduced conditions, the αδ and βγ-chains have approximately the same molecular weight).

Cytoplasmic βα Domain Restores PMA Responsiveness of LFA-1 in K562 Cells

Next, we investigated the capacity of the cytoplasmic tail of the βα or βγ integrin to restore activation of LFA-1 by PMA. Wild-type LFA-1 (Figure 4) and the chimeric LFA-1 transfectants αδβα/β1 and αδβα/βγ showed increased binding when activated by the LFA-1-activating antibody KIM185. The cytoplasmic tail of βγ in the αδβα/βγ transfectant did not restore PMA-
induced binding to ICAM-1. In contrast, the cytoplasmic tail of $\beta_1$ in the chimeric $\alpha_1\beta_2/\beta_1$ transfectant restored PMA responsiveness, which is not dependent on expression levels of chimeric $\alpha_1\beta_2/\beta_1$, since our unpublished results have shown that transfectants expressing low levels of $\beta_2/\beta_1$ also respond to PMA. In addition, increasing the concentration of coated ICAM-1Fc did not alter the PMA nonresponsiveness of wild-type LFA-1 in K562 (Figure 5A). Whereas PMA stimulated binding of chimeric $\alpha_1\beta_2/\beta_1$ to ICAM-1Fc to an extent similar to the KIM185-stimulated adhesion of these cells, both independent of the concentration of coated ICAM-1Fc (Figure 5B). Similarly, also titration using higher doses of PMA did not alter the PMA nonresponsiveness of wild-type LFA-1 in K562 (Figure 5C). In contrast to both the chimeric and wild-type LFA-1 transfectants, the $\alpha_1\beta_2/\Delta724$ transfectant binds ICAM-1 equally well in the absence or presence of the activating mAb KIM185 or PMA (Figure 4). Adhesion was LFA-1 mediated since antibodies directed against LFA-1 (Figure 4) or ICAM-1 inhibited the cell binding completely and mock transfectants always showed less than 2% adhesion to ICAM-1 as demonstrated by our unpublished results. These data indicate that the $\beta_1$, but not the $\beta_2$, cytoplasmic domain is capable of restoring PMA responsiveness of LFA-1 in K562 and that the cytoplasmic tail of $\beta_2$ is important in regulating LFA-1 activation. The observation that PMA could still enhance $\beta_2$ integrin (VLA-5)-mediated adhesion to fibronectin of all transfectants (Figures 1 and 4) again suggests that distinct intracellular routes are involved in $\beta_2$ and $\beta_7$ compared with $\beta_1$ integrin activation.

**Clustering of LFA-1 on the Cell Surface Is Regulated by the $\beta$-Chain**

We determined whether truncation of the $\beta_2$ cytoplasmic domain or replacement of the $\beta_2$ cytoplasmic domain by the corresponding $\beta_1$ or $\beta_7$ cytoplasmic domains affected the distribution of $\alpha_1\beta_2$ integrins at the cell surface. Altered distribution of integrins may affect the avidity state of the receptors, facilitating ligand binding (Figgior et al., 1990; van Kooyk et al., 1994; Lub et al., 1995). Therefore, wild-type ($\alpha_1\beta_2$), the deletion mutant ($\alpha_1\beta_2/\Delta724$), and the $\beta_2$-chimeric LFA-1 transfectants ($\alpha_1\beta_2/\beta_1$ and $\alpha_1\beta_2/\beta_7$) were stained with the anti-LFA-1 antibody NKI-L16, which detects Ca$^{2+}$-dependent clustering of LFA-1 on the cell surface (Keizer et al., 1988; van Kooyk et al., 1994). Figure 6 demonstrates that wild-type LFA-1 transfectants express low levels of the L16 epitope compared...
with expression of a regular anti-LFA-1 (NKI-L16; SPV-L7 peak channel ratio is approximately 0.3). Similarly, \( \alpha_1 \beta_2/\beta_1 \) transfectants show low L16 expression indicating that the cytoplasmic domain of \( \beta_2 \) does not affect LFA-1 distribution (NKI-L16:SPV-L7 peak channel ratio is approximately 0.3). In marked contrast, \( \alpha_1 \beta_2/\beta_1 \) and \( \alpha_1 \beta_2/\gamma_2 \) transfectants show high expression of the L16 epitope, comparable to expression of the SPV-L7 epitope, indicating that all LFA-1 molecules express the L16 epitope (NKI-L16:SPV-L7 peak channel ratio is roughly 1).

To determine whether deletion or replacement of the \( \beta_2 \) cytoplasmic domain for the \( \beta_1 \) cytoplasmic domain directly affects the distribution of LFA-1 on the cell surface, CLSM studies were performed with all LFA-1 transfectants. Figure 7 demonstrates that LFA-1 is homogenously distributed on the cell surface of both wild-type (Figure 7A, only two of seven cells show some clustering of LFA-1) and chimeric \( \alpha_1 \beta_2/\beta_1 \) transfectants (Figure 7C), whereas on the cell surface of all chimeric \( \alpha_1 \beta_2/\beta_7 \) (Figure 7B) and \( \alpha_1 \beta_2/\gamma_2 \) transfectants (Figure 7D), LFA-1 is distributed in clusters. Clustering was not attributed to a higher expression level of LFA-1 (Figure 3A). These data indicate that either deletion or replacement of the \( \beta_2 \) cytoplasmic domain for the \( \beta_1 \) cytoplasmic domain dramatically affects the distribution of LFA-1, as well as results in an increment in the L16 epitope expression. Moreover, our unpublished results demonstrated that VLA-5 is similarly distributed into small clusters on all K562 transfectants. It should be noted that despite the strong clustering of \( \alpha_1 \beta_2/\beta_1 \) receptors on the cell surface stable binding to ICAM-1 still depends on activation of the receptor with PMA, in contrast to the \( \alpha_1 \beta_2/\gamma_2 \) transfectants that express constitutively active LFA-1 molecules.

DISCUSSION

Our results demonstrate that 1) PMA cannot activate LFA-1 when expressed in erythroleukemic K562 cells, despite the fact that PMA readily activates endogenously expressed \( \beta_1 \) integrins. 2) Expression of \( \beta_2 \) chimeric receptors in K562 reveals that the \( \beta_1 \) cytoplasmic tail but not the \( \beta_2 \) cytoplasmic tail restores PMA responsiveness of LFA-1. 3) Lymphocyte-specific signal transduction elements may be involved in \( \beta_2 \) and \( \beta_7 \) integrin activation that are absent in K562 cells. 4) Inside-out signaling (by PMA) is mediated by the \( \beta \)-chain of integrins. 5) Replacement of the \( \beta_2 \) cytoplasmic tail with that of \( \beta_7 \) alters the surface distribution of LFA-1 into clusters and facilitates ICAM-1 binding. 6) Deletion of the entire \( \beta_2 \) cytoplasmic domain localizes LFA-1 molecules into clusters and results in constitutively active receptors.

The PMA nonresponsiveness of LFA-1 in K562 cells is not restricted to this integrin only but holds also for the other two \( \beta_2 \) integrins Mac-1 and p150.95 (Ortlepp et al., 1995) and is not dependent on the concentration of the ligand presented or on the concentration of the stimulus (Figure 5). Our finding that the cytoplasmic tail of the \( \beta_7 \) integrin cannot revert the PMA nonresponsiveness of LFA-1 in these cells predicts that transfection of \( \beta_7 \) integrins into K562 (these cells do
not endogenously express $\beta_2$ integrins, such as $\alpha_4\beta_2$, will not allow the cells to respond to PMA. This suggests that K562 cells lack intracellular signaling elements required to regulate adhesion through the lymphocyte-specific $\beta_2$ and $\beta_1$ integrins. The finding that the cytoplasmic tail of the $\beta_1$ integrin completely restores PMA responsiveness of LFA-1 in K562 cells cannot be attributed to a higher expression level of the $\beta_2$/$\beta_1$ chimeric receptors, since our unpublished results demonstrate that transfectants expressing low levels of $\beta_2$/$\beta_1$ also respond to PMA. Our finding that the $\beta_1$ integrin VLA-5, which is endogenously expressed by the K562 cells, responds to PMA by allowing the cells to bind its ligand fibronectin demonstrates that the intracellular signaling elements activated by PMA are different for $\beta_1$ integrins compared with $\beta_2$ and $\beta_3$ integrins.

Our results demonstrate that the cytoplasmic domain of the $\beta$-chain of integrins plays an important role in the inside-out signaling initiated by PMA. Whether PMA directly or indirectly activates $\beta_2$ integrins via its $\beta$-chain requires further study. Previ-

[Figure 5. PMA nonresponsiveness of LFA-1 expressed in K562 is independent on the concentration ICAM-1 coated (A and B) or the amount of stimulus PMA to induce adhesion (C and D). K562-$\alpha_2\beta_2$ (A) and K562-$\alpha_2\beta_3$/ICAM-1 (B) were preincubated in medium (W), PMA (50 nM, A), or with the activating anti-$\beta_2$ mAb KIM185 (5 $\mu$g/ml, B) and allowed to adhere to different concentrations of ICAM-1Fc (500-25 ng/ml) for 45 to 60 min at 37°C in the absence or presence of the LFA-1-blocking mAb (NKI-L15). Data are representative of three experiments.]

[Figure 6. Expression of the LFA-1-clustering-dependent L16 epitope. K562-$\alpha_2\beta_2$, K562-$\alpha_2\beta_3$/ICAM-1, and K562-$\alpha_2\beta_3$/KIM185 were stained with isotype-matched control antibodies, the mAb NKI-L16, which reports LFA-1 clustering, or with a regular mAb directed against the $\alpha$ subunit (SPV-L7) of LFA-1. Data are representative of five experiments.]
These distinct findings can be attributed to the sites of the J37 cytoplasmic domain (A773) of the DRRE sequence is conserved between the different amino acid sequences. The different cytoplasmic tails share high homology in their membrane-proximal region, especially the DRRE sequence is conserved between the different β cytoplasmic tails (Figure 3, D759RRE762 of β1). Comparison of the positions at which the distinct β cytoplasmic tail were truncated suggests that deletion of the conserved aspartic acid residue corresponding to position 731 in the β2 tail results in a constitutively active molecule, indicating that this residue is most important in regulating integrin activation. In contrast, it seems that in deletion mutants in which this conserved aspartic acid residue is not removed, the integrin activity can still be regulated. Moreover, amino acid sequences more C-terminal of the β cytoplasmic tail are more likely important in ligand binding capacity rather than in regulation of integrin activation (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Hibbs et al., 1991a,b; Crowe et al., 1994; Hughes et al., 1995). The hypothesis that the conserved aspartic acid residue (Figure 3, D759 of β1) is pivotal in the regulation of integrin activation, is also supported by our finding that the LFA-1 deletion mutant K562-αβ2/Δ724, which lacks the entire cytoplasmic β2 tail including the conserved DRRE sequence, has shown to be constitutively active.

The observation that β2/β1 chimeric LFA-1 receptors localize in clusters on the cell membrane but the β2/β2 chimeric LFA-1 and wild-type LFA-1 do not demonstrates that the β2 cytoplasmic domain plays an important role in mobilizing LFA-1 into clusters. Others have demonstrated that the NPNY motif within the cytoplasmic β2 tail plays an essential role in mobilizing integrins into focal contacts (Reszka et al., 1992; Mauro and Dixon, 1994; O’Toole et al., 1995). Interestingly, this motif is absent in the β2 and β2 cytoplasmic domain (Figure 2), which may explain the absence of a clustered LFA-1 distribution on the wild-type αβ2 and the chimeric αβ2/β2 transfectants. This finding again demonstrates that clustering of LFA-1 in itself is essential but not sufficient to stimulate stable LFA-1/ICAM-1 adhesion. Both high-avidity (clustering) and high-affinity (active conformation induced by PMA) states of LFA-1 cooperated for strong adhesion.

It has been demonstrated that integrins can associate with cytoskeletal components (α-actinin and talin), particularly through the β-chain, and thereby regulate the cell surface distribution of the integrin (Burn et al., 1988; Pavalko and LaRocque, 1993). Deletion of the cytoplasmic tail may disconnect the integrin from the cytoskeleton and allow lateral movement of the integrin at the cell membrane, explaining the clustered distribution of LFA-1 on the αβ2/Δ724 transfectants. Furthermore, it may well be that clustering of integrins on the cell surface but also the extracellular conformation is altered, as evidenced by enhanced L16 epitope expression when the β2 cyto-
plasmic domain was deleted or replaced for the β1 cytoplasmic domain. This may be attributed to distinct interactions with cytoplasmic proteins that affect the extracellular conformations of the integrin molecule.

We have demonstrated that the cytoplasmic domain of the β-chain of integrins is responsible for the cell surface distribution of the integrin, regulating the activation of the integrin and that it plays an essential role, either directly or indirectly, in PMA-induced signaling. Furthermore, PMA can activate β3 integrins on K562, whereas it failed to activate the lymphocyte-specific β2 and β3 integrins in K562, suggesting that PMA activates β2 and β3 integrins through lymphocyte-specific elements that are absent in the nonlymphocytic cell line K562.

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