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

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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 $\beta_2$ integrin lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18 or $\alpha_\lambda \beta_2$) 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 $\beta_2$ integrins, lymphocytes also express $\beta_1$ and $\beta_7$ integrins. Similar to the $\beta_2$ integrins, $\alpha_\lambda \beta_2$ is only expressed on lymphocytes and mediates adhesion to vascular cell adhesion molecule 1, mucosal addressing cell adhesion molecule, $\alpha_\lambda$, or the extracellular matrix component fibronectin (Ruegg et al., 1992; Altevogt et al., 1995; Berlin et al., 1995). In contrast, the $\beta_1$ integrins $\alpha_\lambda \beta_1$ and $\alpha_\lambda \beta_7$ are not lymphocyte-specific adhesion receptors, since they are found on a variety of other cell types (Hemler, 1988, 1990; Hemler et al., 1993).
LFA-1/ICAM-1 adhesion requires activation of LFA-1 through intracellular signals (Martz, 1987; Dustin and Springer, 1989; van Kooyk et al., 1989; Hynes, 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 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 divergent cations, in particular Mg$^{2+}$ (Rothlein and Springer, 1986; Dransfield and Hogg, 1989; Figdor et al., 1990; Dransfield et al., 1992a). Binding of Ca$^{2+}$ to LFA-1 supports clustering (high-avidity 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 Mn$^{2+}$, to the extracellular part of the integrin, can also induce an active conformation (high-affinity state) of the ($\beta_1$, $\beta_2$, $\beta_3$) 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 (Hynes, 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 $\beta_3$ integrins are relatively short (46, 45, and 51 amino acids for the $\beta_1$, $\beta_2$, and $\beta_3$ 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 $\alpha_5$ subunit, because truncation of the cytoplasmic $\beta_3$ tail, but not the $\alpha_5$ 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 $\beta_3$ 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/orientation and not to an affinity change in LFA-1 (Peter and O'Toole, 1995). Because deletion of the cytoplasmic domain of the $\alpha_5$ subunit does not affect binding to ICAM-1, it is hypothesized that the cytoplasmic tail of $\alpha_5$ is predominantly involved in "postligand role in regulating avidity state) of LFA-1 on the cell surface, resulting in increased LFA-1-mediated adhesion (Figdor et al., 1990; van Kooyk et al., 1994).

In this study, we investigated whether a lympho-cytic 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 ($\Delta T24$), 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. J.M. Harlan (Beatty et al., 1983). The anti-$\beta_2$, mAb KIM185 (IgG1) was used to activate $\beta_2$ integrins (Andrew et al., 1993) and the anti-$\beta_1$ mAb TS2/16 to activate $\beta_1$ integrins (Hemler et al., 1984; van de Wiel-van Kemenade et al., 1992). The anti-$\alpha_5$ 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 the gene from the cytomegalovirus (CMV) ADI69 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 ApaI site at position 1980. The C-terminal end was replaced from this site with 10 overlapping oligonucleotides and amplification by the polymerase chain reaction to obtain the appropriate hybrids. For the $\beta_1$/$\beta_2$, chimeric protein,
amino acid 752 of β2 (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 μF with the αL (in pCDM8) and the wild-type β2 subunit (in pRC/CMV), the αL and β2 cytoplasmic truncated subunit (Δ724), or the αL and the chimeric β2/β2- or β2/β2-chain of LFA-1. K562-LFA-1 transfectants were cultured in RPMI 1640 medium (Life Technologies, Paisley, Scotland), supplemented with 10% fetal calf serum (BioWhittaker, Verviers, Belgium), and 1% antibiotics/antimycotics (Life Technologies). After 48 h the neomycin analogue, Geneticin (2 mg/ml, Life Technologies) was added to the culture medium. The different transfectants were sorted three times to obtain a homogenous population of cells expressing high levels of LFA-1. Positive cells were stained directly with fluorescein isothiocyanate (FITC)-labeled TS2/4 mAb (Zymed Laboratories, San Francisco, CA).

Immunofluorescence Analysis

Expression of LFA-1 on the transfectants was determined by immuno-fluorescence. Cells (2 × 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 (Fab′)2 anti-mouse IgG mAb (Zymed Laboratories, San Francisco, CA) for 30 min at 4°C. The relative fluorescence intensity was measured by FACSscan analysis (Becton Dickinson, Oxnard, CA).

Adhesion Assay

Binding of LFA-1-positive cells to ICAM-1 was performed with ICAM-1 fusion proteins consisting of the five Ig-like domains of ICAM-1 fused to a human IgG1 Fc fragment (ICAM-1Fc). ICAM-1Fc was generated by transfecting mouse L cells with the vector pICAM-1-IgG1 by calcium phosphate precipitation (calcium phosphate transfection system, Life Technologies) using a standard protocol (Fawcett et al., 1992). Culture supernatant was purified by protein A chromatography and eluted with 3.5 M MgCl2 and 10% glycerol.

Ninety-six-well flat-bottomed plates (MaxiSorp, Nunc, Roskilde, Denmark) coated with 50 μg/ml goat anti-human Fc-specific Fab′2 (4 μg/ml, 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 were coated with 0.2 μg/ml ICAM-1Fc protein overnight at 4°C. Stably transfected CD11a/CD18 K562 cells were labeled with Na2[35S]CrO4 (Amersham International, Buckinghamshire, England) for 45 min at 37°C. Radiolabeled cells were washed and preincubated for 15 min at room temperature with different stimuli (300 mM phorbol 12-myristate 13-acetate (PMA), Calbiochem, La Jolla, CA), 5 μg/ml KIM185, or 5 μg/ml TS2/16 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 (NKI-L15 or SAM-1).

β2, Cytoplasmic Tail in LFA-1 Function

Radioiodination 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 7.5, 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-ethylmaleimide. Nuclear debris was removed from the lysates by centrifugation at 13,000 × g for 15 min at 4°C. Lysates were preclarified by successive incubation with mouse IgG covalently coupled to protein A-Sepharose CL-4B beads (Pharmacia, Piscataway, NJ). Preclarified cell lysates were immunoprecipitated with specific mAb, directed against either LFA-1 or VLA-5, coupled to protein A-Sepharose CL-4B for 1 to 2 h at 4°C. The immunoprecipitates were removed from the lysates by centrifugation at 13,000 × 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 washed with T/S/24 mAb (10 μg/ml) for 30 min at 37°C followed by incubation with FITC-labeled goat (Fab′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, 60×; gain, 1300; pinhole, 1.5 μm; and magnification, 1.5×. 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 (αLβ2) 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 αL- and β2-chain cDNA in erythroleukemic K562 cells that do not express the lymphocyte-specific β2- and β2 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 αL (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 after 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 endogenously expressed β1 integrin. Figure 1A demonstrates that the LFA-1-trans-
fected K562 cells express functional LFA-1 molecules because KIM185 readily induces LFA-1-mediated ICAM-1 binding. Surprisingly, PMA was not able to induce LFA-1-mediated adhesion to ICAM-1. This is not due to a general nonresponsiveness of K562 cells to PMA because it significantly enhances VLA-5-mediated binding to fibronectin. Similar to KIM185, the activating anti-β, mAb TS2/16 effectively stimulated VLA-5-mediated adhesion (Figure 1B).

The observation that PMA could enhance β1 integrin (VLA-5)-mediated adhesion but not β2 integrin (LFA-1) mediated adhesion suggests that distinct signaling elements are involved in the PMA-induced β2 and β1 integrin activation.

Expression of β2-Chimeric LFA-1 Molecules in Erythroleukemic K562 Cells

To determine whether the observed differences between PMA responsiveness of β1 and β2 integrins are due to differences in the cytoplasmic tail, we generated LFA-1 molecules in which the cytoplasmic tail of the β2-chain was truncated close to the transmembrane region at amino acid position 724 (K562-α/β, Δ724) or replaced with that of the β1-chain or the β7-chain (K562-α/β, β1 and K562-α/β, β7, 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 β2 and β7 integrins are predominantly expressed by lymphocytes, whereas β1 integrins are widely distributed.

The chimeric β2-Chains: β2/β1, β2/β7 and β2/Δ724 were transfected along with the wild-type α-chain (αL) 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 MHH23 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 β2 cytoplasmic tail or replacement of the β2 cytoplasmic tail for the β1 or β7 cytoplasmic tail does not alter the overall conformation of the LFA-1 α/β heterodimer. All transfectants show similar levels of the endogenously expressed β1 integrin, VLA-5 (Figure 3A).

To verify that the chimeric LFA-1 molecules α/β2/β1 and α/β2/β7 did not associate with endogenous α/β-chain in K562, LFA-1 and VLA-5 were immunoprecipitated from all transfectants. β1 wild-type, chimeric β2-chains, and α/β2/Δ724 associated with αL and not with α5 (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-αL mAb showed that VLA-5 does not associate with α5 or β2 (Figure 3B, lanes A, E, H, and K, observed as one thick band of 130-135 kDa). Because of the high expression levels of the VLA-5, observed as the thick bands of 185 and 95 kDa, all transfectants were used to express the chimeric αL/β2/β7 integrin.

Cytoplasmic β2 Domain Restores PMA Responsiveness of LFA-1 in K562 Cells

Next, we investigated the capacity of the cytoplasmic tail of the β1 or β2 integrin to restore activation of LFA-1 by PMA. Wild-type LFA-1 (Figure 4) and the chimeric LFA-1 transfectants α/β2/β1 and α/β2/β7 showed increased binding when activated by the LFA-1-activating antibody KIM185. The cytoplasmic tail of β7 in the α/β2/β7 integrin transfected did not restore PMA-
induced binding to ICAM-1. In contrast, the cytoplasmic tail of β1 in the chimeric α1β2/β1 transfectant restored PMA responsiveness, which is not dependent on expression levels of chimeric α1β2/β1, since our unpublished results have shown that transfectants expressing low levels of β2/β1 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 α1β2/β1 to ICAM-1 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 α1β2/Δ724 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 β1, but not the β2, cytoplasmic domain is capable of restoring PMA responsiveness of LFA-1 in K562 and that the cytoplasmic tail of β2 is important in regulating LFA-1 activation. The observation that PMA could still enhance β2 integrin (VLA-5)-mediated adhesion to fibronectin of all transfectants (Figures 1 and 4) again suggests that distinct intracellular routes are involved in β2 and β7 compared with β1 integrin activation.

Clustering of LFA-1 on the Cell Surface Is Regulated by the β-Chain

We determined whether truncation of the β2 cytoplasmic domain or replacement of the β2 cytoplasmic domain by the corresponding β1 or β7 cytoplasmic domains affected the distribution of α1β2 integrins at the cell surface. Altered distribution of integrins may affect the avidity state of the receptors, facilitating ligand binding (Figdor et al., 1990; van Kooyk et al., 1994; Lub et al., 1995). Therefore, wild-type (α1β2), the deletion mutant (α1β2/Δ724), and the β7-chimeric LFA-1 transfectants (α1β2/β1 and α1β7/β1) were stained with the anti-LFA-1 antibody NKi-L16, which detects Ca++-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 the...
Figure 4. Cytoplasmic β₂ domain restores PMA responsiveness of LFA-1 in K562 cells. K562-αLβ₂/β₁ and K562-αLβ₂/β₂ transfectants and K562-αLβ₂/Δ724 were preincubated in medium (control), PMA (50 nM), or with the activating anti-β₂ mAb KIM185 (5 µg/ml) or activating anti-β₁ mAb TS2/16 (5 µg/ml) and allowed to adhere for 45–60 min at 37°C. Adhesion was performed in the absence or presence of the LFA-1 blocking mAb (NKI-L15) or VLA-5 blocking mAb (Sam-1) to ICAM-1 or fibronectin, respectively. Depicted is the mean percentage of LFA-1-specific binding to ICAM-1 and VLA-5-specific adhesion to fibronectin of three independent wells. Integrin-specific adhesion: Percentage of cells binding — percentage of cells binding in the presence of an integrin blocking mAb. Data are representative of three experiments.

with expression of a regular anti-LFA-1 (NKI-L16: SPV-L7 peak channel ratio is approximately 0.3). Similarly, αLβ₂/β₁ transfectants show low L16 expression indicating that the cytoplasmic domain of β₂ does not affect LFA-1 distribution (NKI-L16:SPV-L7 peak channel ratio is approximately 0.3). In marked contrast, αLβ₂/β₁ and αLβ₂/Δ724 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 β₂ cytoplasmic domain for the β₁ 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 homogeneously distributed on the cell surface of both wild-type (Figure 7A, only two of seven cells show some clustering of LFA-1) and chimeric αLβ₂/β₁ transfectants (Figure 7C), whereas on the cell surface of all chimeric αLβ₂/β₁ (Figure 7B) and αLβ₂/Δ724 transfected cells (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 β₂ cytoplasmic domain for the β₁ 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 αLβ₂/β₁ receptors on the cell surface stable binding to ICAM-1 still depends on activation of the receptor with PMA, in contrast to the αLβ₂/Δ724 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 β₁ integrins. 2) Expression of β₂ chimeric receptors in K562 reveals that the β₁ cytoplasmic tail but not the β₂ cytoplasmic tail restores PMA responsiveness of LFA-1. 3) Lymphocyte-specific signal transduction elements may be involved in β₂ and β₁ integrin activation that are absent in K562 cells. 4) Inside-out signaling (by PMA) is mediated by the β-chain of integrins. 5) Replacement of the β₂ cytoplasmic tail with that of β₁ alters the surface distribution of LFA-1 into clusters and facilitates ICAM-1 binding. 6) Deletion of the entire β₂ 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 β₂ integrin 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 β₂ integrin cannot revert the PMA nonresponsiveness of LFA-1 in these cells predicts that transfection of β₂ integrins into K562 (these cells do
not endogenously express $\beta_2$ integrins), such as $\alpha_4\beta_7$, 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 $\beta_7$ 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 $\beta_2/\beta_7$ chimeric receptors, since our unpublished results demonstrate that transfectants expressing low levels of $\beta_2/\beta_7$ 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 $\beta_7$ 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. Previoúsly, it has been shown that PMA leads to phosphorylation of the serine residue at position 756 in the $\beta_2$ cytoplasmic domain (Hibbs et al., 1991a). However, mutation of this serine residue does not impair ICAM-1 binding, demonstrating that phosphorylation at this position is not crucial for adhesion to ICAM-1 (Hibbs et al., 1991a). Alignment of the $\beta_1$ and $\beta_2$ cytoplasmic domains to the $\beta_2$ cytoplasmic domain revealed that the serine residue is conserved in both $\beta_1$ and $\beta_7$ (Figure 2), indicating that this serine residue cannot explain the differences observed in the chimeric receptors expressed in K562. Mutations of a triplet of threonines (amino acids 758–760) and a phenylalanine residue at position 766 in the $\beta_2$ cytoplasmic domain have been completely abrogated ICAM-1 adhesion. Interestingly, a similar triplet of threonines and phenylalanine residue are present in the $\beta_1$ cytoplasmic domain and are absent in the $\beta_7$ cytoplasmic domain. Therefore, it is tempting to speculate that these residues may be important in the lymphocyte-specific signal transduction pathway (Hibbs et al., 1991a). To determine the precise residues in the $\beta_1$ cytoplasmic domain involved in the PMA induced signaling, mutations studies should be performed (work in progress).

Our LFA-1 deletion mutant K562-$\alpha_4\beta_2/\Delta 724$, which lacks the complete $\beta_2$ cytoplasmic tail (45 amino acids long), was found to be constitutively active in K562.
cells. Similar observations were made when the complete cytoplasmic tail of the \( \beta_2 \) was deleted (Δ747, deletion of 51 amino acids) in \( \alpha_4 \beta_2 \) (Crowe et al., 1994) or when the complete cytoplasmic tail of the \( \beta_2 \) was deleted (Δ744, deletion of 45 amino acids, the \( \beta_2 \) cytoplasmic tail is 47 amino acids long) in \( \alpha_{2b} \beta_2 \) (Hughes et al., 1995). In contrast, when the first 28 amino acids of the N-terminal region of the cytoplasmic tail of \( \beta_2 \) were deleted, no effect on ICAM-1 binding activity was observed (Hibbs et al., 1991a). Similarly, mutants containing deletion greater than 5–15 amino acids at the C-terminal end of the \( \beta_1 \) cytoplasmic domain neither promoted adhesion nor localization of \( \beta_1 \) integrins in focal contacts (Solowska et al., 1989; Hayashi et al., 1990; Marcatonio et al., 1990). In contrast, deletion of 39 amino acids C-terminal of the \( \beta_1 \) cytoplasmic tail (Δ759, \( \beta_1 \) cytoplasmic tail is 46 amino acids long) impairs the binding capacity to both laminin and fibronectin. Likewise, partial removal of the C-terminal part of the \( \beta_2 \) cytoplasmic domain (Δ773) of the \( \alpha_\beta \) integrin displayed no ligand binding activity to VCAM-1 (Hayashi et al., 1990; Crowe et al., 1994). These distinct findings can be attributed to the sites where the cytoplasmic tails of the \( \beta \)-chains were deleted. The different \( \beta \) cytoplasmic tails share high homology in their membrane-proximal region, especially the DRRE sequence is conserved between the different \( \beta \) cytoplasmic tails (Figure 3, D\textsuperscript{759}RRE\textsuperscript{762} of \( \beta_2 \)). Comparison of the positions at which the distinct \( \beta \) cytoplasmic tail were truncated suggests that deletion of the conserved aspartic acid residue corresponding to position 731 in the \( \beta_2 \) 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 \( \beta \) cytoplasmic tail are more important in regulating integrin activation (Solowska et al., 1989; Hayashi et al., 1990; Marcatonio 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, D\textsuperscript{759} of \( \beta_2 \)) is pivotal in the regulation of integrin activation, is also supported by our finding that the LFA-1 deletion mutant K562-αLβ2/Δ724, which lacks the entire cytoplasmic \( \beta_2 \) tail including the conserved DRRE sequence, has shown to be constitutively active.

The observation that \( \beta_2/\beta_1 \) chimeric LFA-1 receptors localize in clusters on the cell membrane but the \( \beta_2/\beta_2 \) chimeric LFA-1 and wild-type LFA-1 do not demonstrates that the \( \beta_2 \) cytoplasmic domain plays an important role in mobilizing LFA-1 into clusters. Others have demonstrated that the NPTY motif within the \( \beta_2 \) cytoplasmic domain is important for localization of the integrin into focal contacts (Reszka et al., 1992; Mauro and Dixon, 1994; O'Toole et al., 1995). Interestingly, this motif is absent in the \( \beta_2 \) and \( \beta_2 \) cytoplasmic domain (Figure 2), which may explain the absence of a clustered LFA-1 distribution on the wild-type \( \alpha_4 \beta_2 \) and the chimeric \( \alpha_4 \beta_2/\beta_2 \) 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 cooperate for strong adhesion.

It has been demonstrated that integrins can associate with cytoskeletal components (α-actinin and talin), particularly through the \( \beta \)-chain, and thereby regulate the cell surface distribution of the integrin (Burn et al., 1988; Pavalko and LaRoche, 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 \( \alpha_4 \beta_2/\Delta724 \) transfectants. Furthermore, it may well be that clustering of integrins on the cell surface colocalizes important kinases essential for proper signal transduction (Miyamoto et al., 1995). Not only is the intracellular conformation or association with regulatory proteins affected by clustering of integrins on the cell surface but also the extracellular conformation is altered, as evidenced by enhanced L16 epitope expression when the \( \beta_2 \) cyto-
plasmic domain was deleted or replaced for the \( \beta_1 \) 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 \( \beta \)-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 \( \Pi \)MA-induced signaling. Furthermore, \( \Pi \)MA can activate \( \beta_1 \) integrins on K562, whereas it failed to activate the lymphocyte-specific \( \beta_1 \) and \( \beta_2 \) integrins in K562, suggesting that \( \Pi \)MA activates \( \beta_2 \) and \( \beta_5 \) integrins through lymphocyte-specific elements that are absent in the nonlymphocytic cell line K562.

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