Regulation of the VLA Integrin–Ligand Interactions through the β1 Subunit

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Abstract. Integrins from the very late activation antigen (VLA) subfamily are involved in cellular attachment to extracellular matrix (ECM) proteins and in intercellular adhesions. It is known that the interaction of integrin proteins with their ligands can be regulated during cellular activation. We have investigated the regulation of different VLA-mediated adhesive interactions through the common β1 chain. We have found that certain anti-β1 antibodies strongly enhance binding of myelomonocytic U-937 cells to fibronectin. This β1-mediated regulatory effect involved both VLA-4 and VLA-5 fibronectin receptors. Moreover, anti-β1 mAb also induced VLA-4–mediated binding to a recombinant soluble form of its endothelial cell ligand VCAM-1. Non-activated peripheral blood T lymphocytes, unable to mediate VLA-4 interactions with fibronectin or VCAM-1, acquired the ability to bind these ligands in the presence of anti-β1 mAb. The anti-β1–mediated changes in the affinities of β1 integrin for their ligands were comparable to those triggered by different lymphocyte activation agents such as anti-CD3 mAb or phorbol esters. Adhesion of melanoma cells to other ECM proteins such as laminin or collagen as well as that of α2-transfected K-562 cells to collagen, was also strongly enhanced by anti-β1 mAb. These β1-mediated regulatory effects on different VLA–ligand interactions do not involve changes in cell surface membrane expression of different VLA heterodimers. The anti-β1–mediated functional effects required an active metabolism, cytoskeleton integrity and the existence of physiological levels of intracellular calcium as well as a functional Na$^+$/H$^+$ antiporter. β1 antibodies not only increased cell attachment but also promoted spreading and cytoplasmic extension of endothelial cells on plates coated with either fibronectin, collagen, or laminin as well as induced the rapid appearance of microspikes in U-937 cells on fibronectin. Moreover, both β1 integrin and the cytoskeletal protein talin colocalized in the anti-β1 induced microspikes. These results emphasize the central role of the common β1 chain in regulating different adhesive functions mediated by VLA integrins as well as cellular morphology.

INTEGRINS constitute a family of widespread αβ heterodimeric adhesion receptors that mediate cell attachment to extracellular matrix (ECM)[1] proteins as well as cell–cell interactions (29, 57). The very late activation (VLA) antigen subfamily of integrins is composed of a common β1 chain noncovalently associated with one of at least eight different α chains (8, 24, 36). Recent findings of novel associations between α chains and alternative β subunits expand the VLA molecular and functional repertoire (7, 24, 26, 60).

The VLA members functionally behave as receptors for different ECM proteins. Thus, the integrins VLA-2, VLA-5, and VLA-6 are prototype receptors for collagen type I, fibronectin and laminin, respectively (48, 55, 61). Other VLA heterodimers such as VLA-1 and VLA-3 can bind more than one ECM component (19, 31, 35). Very recently, VLA-3 has been also implicated in epidermal cell–matrix contacts by interacting with a novel ligand, termed epiligrin (13). VLA-4 is involved in both cell–cell and cell–ECM adhesive interactions (5, 11, 27, 47, 62), since it can bind fibronectin in an RGD-independent manner, and is also able to interact with a cell surface molecule, termed VCAM-1, on activated endothelial cells (18).

The α chains of VLA integrins were thought to confer the specificity for the recognition of the different ligands, whereas the common β1 chain was suggested to be involved in signal transduction (22, 65). Recently, it has been found that both α and β chains contribute to both ligand and binding events (6, 33, 52, 63, 66). Previous studies have documented that cellular activation increases the affinity of different integrin proteins for their corresponding ligands (17, 53,

1. *Abbreviations used in this paper:* cAMP, cyclic adenosine monophosphate; COL, collagen; ECM, extracellular matrix; ELAM-1, endothelial leukocyte adhesion molecule-1; EPA, N-ethyl,N-propyl amiloride; FN, fibronectin; HUVEC, human umbilical vein endothelial cells; LFA-1, leukocyte function associated antigen-1; LN, laminin; PKC, protein kinase C; PMA, phorbol myristate acetate; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1; VLA, very late activation antigen; VN, vitronectin.

54, 64). The involvement of the β2 chain of leukocyte integrins in regulating the affinity of these interactions has been postulated (21). Furthermore, evidence indicating that the ligand affinities of β1 integrins can be modulated on developing retinal neurons through the β1 subunit has been recently reported (42).

In this study, we have investigated the possible role of the common β1 subunit of VLA integrins in the regulation of adhesive functions of different heterodimers. We have found that anti-β1 mAb can promote binding of distinct VLAαβ heterodimers to both ECM and cellular ligands.

Materials and Methods

Cells and Cell Cultures

U-937 myelomonocytic cell line was obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 5% FCS (Flow Laboratories), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Flow Laboratories). A375 melanoma cell line (34) was grown in MEM (Flow Laboratories) supplemented with 10% FCS, 10 mM Hepes buffer, essential aminoacids, vitamins, and antibiotics (Flow Laboratories).

Human umbilical vein endothelial cells (HUVEC) were obtained as described (15). Briefly, umbilical vein was cannulated, washed, and incubated with 0.1% collagenase P (Boehringer Mannheim GmbH, Mannheim, Germany) for 20 min at 37°C. Cells were put into flasks (Nunc, Roskilde, Denmark) and cultured in M199 medium (Flow Laboratories) supplemented with 20% newborn calf serum, 50 μg/ml of endothelial cell growth supplement, 100 μg/ml heparin from porcine intestinal mucose (Sigma Chemical Co., St. Louis, MO), 50 U/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml fungizone. Cells within two passages were used. Cells were split 1:3 and detached with a solution of 0.05% Trypsin and 0.02% EDTA (Flow Laboratories) before using.

Peripheral blood T cells were obtained from heparinized venous blood of normal voluntary donors. After Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) centrifugation, peripheral mononuclear cells were depleted of adherent cells by two steps of adherence incubation in plastic flasks (Costar, Cambridge, MA) at 37°C for 2 h. T cells were purified by passage through a nylon wool column. T cell-enriched population contained >90% CD3+ and <5% CD20+ and CD14+ cells.

α2- and α4-transfected K-562 cells have been previously reported (18) and were grown in RPMI 1640 medium supplemented with 5% FCS, 2 mM L-glutamine, 50 μg/ml gentamicin, and with 1.5 mg/ml Geneticin (G-418; Gibco Laboratories, Grand Island, NY).

Reagents

Staurosporine, Quin-2a, Cytochalasin B, 2 DO-glucose, PMA, and the Ca²⁺ ionophore A23187 were purchased from Sigma. N-ethyl,N-propyl amiloride (EPA) was a gift from Merck (Merck & Co. Inc., Darmstadt, West Germany).

mAbs, F(ab')₂ and Fab Fragments

Anti-β1 chain Lia1/2, Lia 1/5 and Alex 1/4 mAb and anti-CD43 TP1/36 mAb were obtained in our laboratory and their characteristics will be described elsewhere. The anti-β1 TS2/16, A-1A5, P4C10 and K20 (3, 12, 25); the anti-α1 TS2/7 (25), the anti-α2 P1E6 (12) and 12F1 (46), the anti-α3 P1B5 (61), the anti-α4 chain HP2/1 (50), the anti-VCAM-1 4B9 (51) and the anti-HLA-A, B W6/32 (4) mAb have been previously described. The anti-α5 SAM-1 and the anti-α6 GoH3 (55) were purchased from Central Laboratory of Blood Transfusion Service (Netherland Red Cross, Amsterdam, The Netherlands).

Anti-β1 mAb were purified using affinity chromatography on protein A-Sepharose columns while (F(ab')₂ and Fab fragments were prepared as previously reported (44).

Cell Attachment Assays

Plasma fibronectin (FN), 38 (FN38), and 80 (FN80) kD proteolytic fragments were kindly provided by Dr. A. García-Pardo (Centro de Investigaciones Biológicas, Consejo Superior Investigaciones Cientificas, Madrid).

Collagen (COL), type I and type IV, laminin (LN), and fibrinogen (Fg) were purchased from Sigma Chemical Co. Recombinant soluble VCAM-1 (rsVCAM-1) and ELAM-1 (rsELAM-1) were purified by immunoaffinity chromatography from conditioned medium of CHO cells stably transfected with a truncated cDNA for VCAM-1 (38) and ELAM-1, respectively (37). The adhesion assays were essentially performed as previously described (15). Briefly, 96-well flat-bottomed plates (Titertek; Flow Laboratories) were coated overnight at 4°C with different substrates diluted in PBS with divalent cations. Thereafter, the plate was washed once with PBS and then saturated with 1% BSA 1 h at 37°C. The plate was washed twice with PBS and cells (preincubated with different mAb or medium as control) were added in serum free medium and incubated for 30 min at 37°C unless otherwise indicated. To quantify cell attachment, the plate was washed twice with PBS, cells were fixed with a mixture of acetone/methanol 1:1 and dyed with violet crystal 0.5%. Then, absorbance at 540 nm was measured in an ELISA detector (Pasteur Laboratories, Paris, France) and optical density was found to be practically a linear function of number of cells by a calibration curve (optical density vs number of cells) made for each cell type used in our assays. There was a linear relation in a range from 5×10^3–1.5×10^5 cells (0.013–0.230 OD units) for U-937 and K-562 cells; from 4×10^3–1.2×10^5 cells (0.013–0.270 OD) for A375 cells, and from 3×10^4–4.5×10^5 cells (0.020–0.290 OD) for T lymphocytes. To calculate percentage of attachment, basal adherence to BSA (cell binding to BSA-coated wells was constant enough for each cell type and was always <2%) was substracted from attachment values obtained and the final results expressed as percent control (attachment in mAb-free wells as 100%). The assays were performed in triplicate. Total cellular input was calculated by spinning wells with original number of cell aliquots, then fixing, staining, and measuring OD.

FACS Analysis

Fluorescence flow cytometry analysis was performed on a FACScan cytofluorometer (a registered trademark of Becton Dickinson, Mountain View, CA). A375 melanoma cells were detached with a solution of 0.05% Trypsin and 0.02% EDTA (Flow Laboratories) and incubated at 4°C with 100 μl hybridoma culture supernatants, followed by washing and labeling with fluorescein isothiocyanate-labeled goat anti-mouse Fc specific fragment of Ig. The rat GoH3 mAb anti-α6 was developed by using as second reagent a mixture of the mouse anti-rat Ig mAb RG7/7.6 and RG7/9.1, specific for rat kappa chain (58). Data were collected in a logarithmic scale and percentage of positive cells was obtained by substracting the background given by the negative control mouse myeloma P3X63.

Indirect Immunofluorescence

After the induction of cell adhesion to FN-coated coverslips with different reagents, cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. The cells were permeabilized with 0.2% Triton X-100 in Tris-buffered saline (TBS: 50 mM Tris-HCl, 150 mM NaCl, 0.1% NaN₃, pH 7.6) for 3 min. Cells were stained with the primary antibody diluted in TBS for 1 h at 37°C, rinsed in TBS and stained with a 1/50 dilution of the appropriate secondary antibody. Immunostaining for β1 integrin was performed using the Alex 1/4 mAb diluted 1:4 in TBS. Polyclonal rabbit anti-talin was kindly provided by Dr. K. Burridge (University of North Carolina, Chapel Hill, NC) and diluted 1:300. The secondary antibodies used were: FITC goat anti-rabbit IgG (Cappel Laboratories), and RITC goat anti-mouse IgG (Chemicon, Teruecula, CA). For double-label studies, cells were simultaneously stained with a mixture of the fluorescent secondary antibodies. After a final wash, the coverslips were rinsed in deionized water to remove salts and mounted in Gelvatol (Monsanto, St. Louis, MO). The cells were observed using a IM-35 photomicroscope (IM-35; Carl Zeiss, Oberkochen, Germany) with a × 100 oil immersion objective and photographed on TMAX 400 film (Eastman Kodak Co., Rochester, NY) processed to 800–1,600 ASA with TMAX developer (Eastman Kodak Co.).

Results

Enhancement of Cell Binding to Fibronectin Promoted by Anti-β1 Antibodies

To study the possible regulatory role of the common β1 subunit of VLA integrins in cell binding to different ECM components, we first examined the functional effects of different anti-β1 mAb on the attachment of myelomonocytic
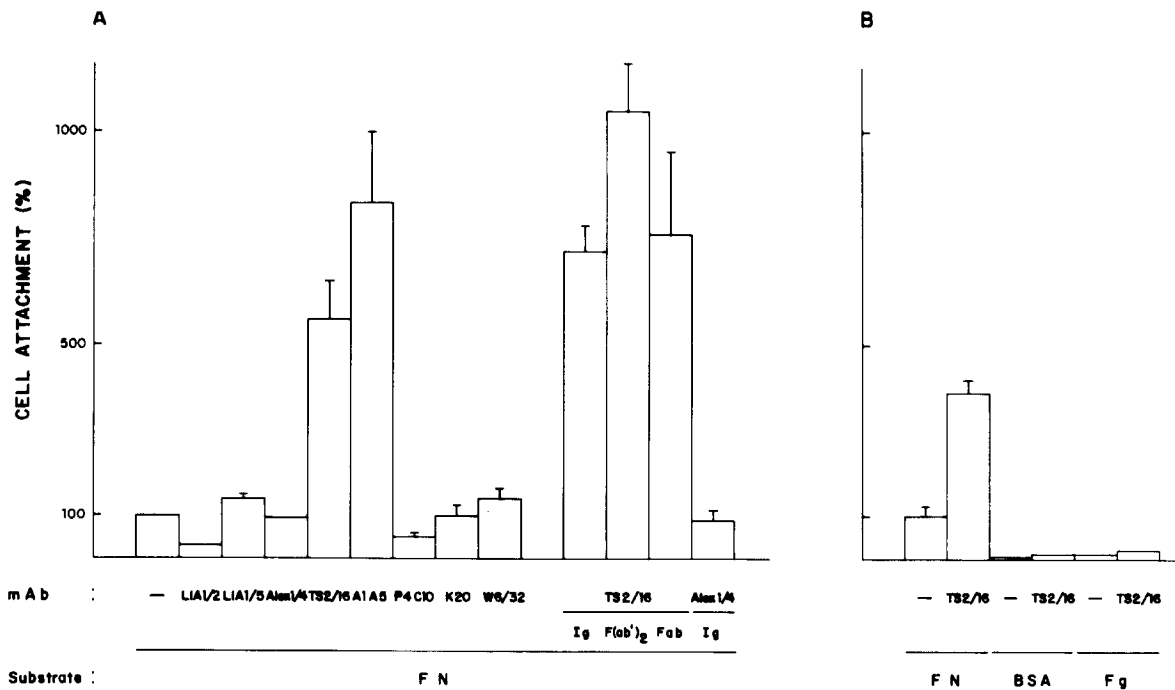
Figure 1. Effect of anti-$\beta 1$ mAb on the attachment of U-937 cells to plasma fibronectin. U-937 cells were preincubated with different anti-$\beta 1$ mAb (*Lia 1/2, Lia 1/5, Alex 1/4, A-1A5, P4C10, K20, and TS2/16*) and anti-HLA-A,B mAb (*W6/32*) as well as with 1 μg/ml of purified Ig, F(ab')2 and 10 μg/ml of Fab fragments of anti-$\beta 1$ TS2/16 mAb. The different anti-$\beta 1$ mAb were assayed at a dose of 1 μg/ml. Anti-$\beta 1$ TS2/16 and Alex 1/4 mAb were assayed in a wide range of doses (from 0.1 to 50 μg/ml) and their effects were similar to the ones shown in the figure. Then, cells were assayed for attachment to 10 μg/ml of plasma FN for 30 min as described in Materials and Methods (Fig. 1 *A*). Control without mAb represents 6% of total cellular input. U937 were also assayed (preincubated or not with 1:10 dilution of the anti-$\beta 1$ TS2/16 mAb) on plates coated with 10 μg/ml of plasma FN, 1% BSA, and 500 μg/ml of Fg (Fig. 1 *B*). A representative experiment out of seven independent ones is shown.

U-937 cells to plasma fibronectin. As shown in Fig. 1 *A*, the anti-$\beta 1$ Lia1/2 and P4C10 mAb inhibited cell binding to FN, whereas no significant effect was exerted by other anti-$\beta 1$ mAb such as Lia1/5, Alex 1/4, or K20. Interestingly, two anti-$\beta 1$ TS2/16 and A-1A5 mAb (IgG1 and IgG2b subclasses, respectively) induced a strong enhancement of cell binding

to FN. These two anti-$\beta 1$ mAb have been previously described as defining an identical or closely related epitope on $\beta 1$ chain (25), which is either functionally (Lia 1/2, Lia 1/5, and Alex 1/4) or topographically (K20) distinct from those defined by other anti-$\beta 1$ antibodies (data not shown). The magnitude of this enhancing effect was in a range of six- to
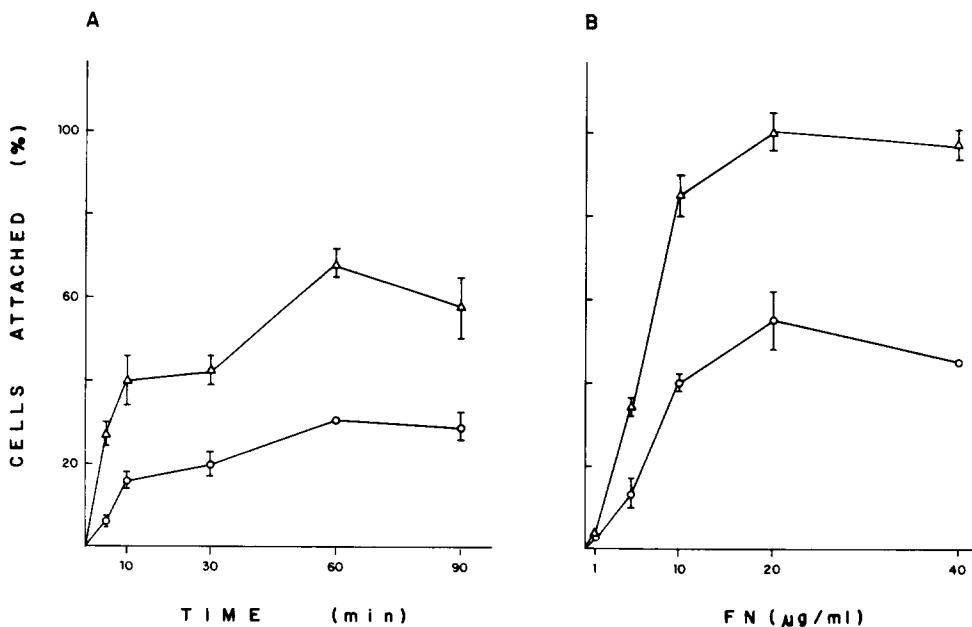

Figure 2. Kinetics and dose-dependence of the anti-$\beta 1$–enhanced binding of U-937 cells to plasma fibronectin. U-937 cells were incubated in the presence ($\triangle$) or in the absence ($\bigcirc$) of 1 μg/ml of purified anti-$\beta 1$ TS2/16 mAb and then assayed for binding onto plates coated with 10 μg/ml of plasma FN for different times of incubation (*A*) or assayed at several doses of applied plasma FN during 30 min (*B*). Percentage of attached cells respect total cellular input is shown. A representative experiment out of three independent ones is shown.
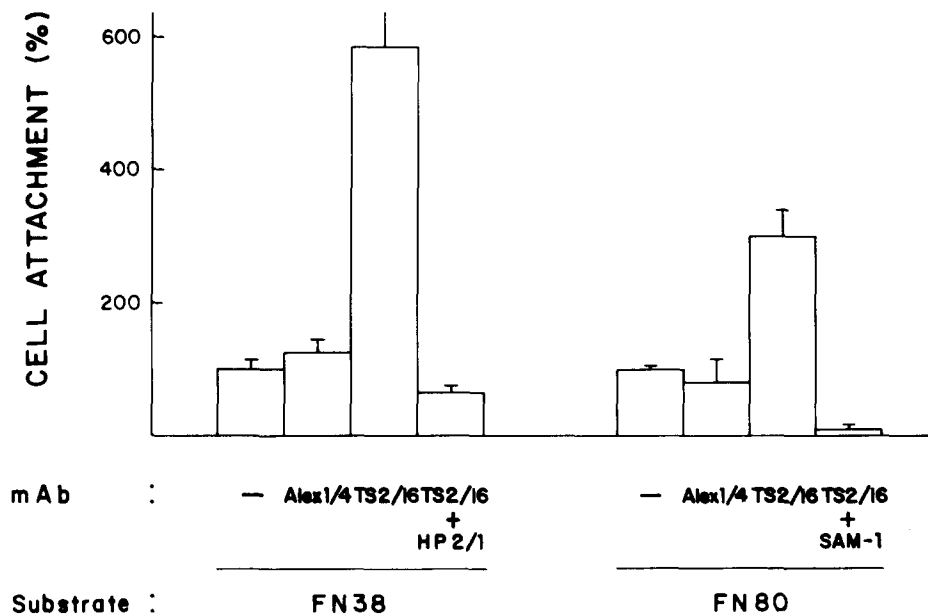
Figure 3. Anti-β1 mAb enhance the binding of U-937 cells to both 38 and 80 kD proteolytic fragments of fibronectin. U-937 cells were preincubated with 1 µg/ml of purified Ig of the indicated anti-β1 mAb either alone or combined with 1 µg/ml of the anti-α4 (HP2/1) or anti-α5 (SAM-1) mAb. Then, cells were assayed for attachment to plates coated with 10 µg/ml of FN38 or 1 µg/ml of FN80. Control without mAb represents 5 and 25% of total cellular input on FN38 and FN80, respectively. A representative experiment out of three independent ones is shown.

CELL ATTACHMENT (%)

mAb : — Aα1/4 TS2/16 TS2/16 + HP2/1   — Aα1/4 TS2/16 TS2/16 + SAM-1

Substrate : FN38   FN80

eightfold above the basal level of binding obtained either in the absence of antibody or in the presence of the anti-HLA-A, B mAb as control (Fig. 1 A). This adhesion assay was performed seven different times and the enhancing effect was always observed although some variability in the level of anti-β1–mediated enhancement of U-937 cells attachment to FN was detected. The effect of anti-β1 TS2/16 mAb appears

CELL ATTACHMENT (%)

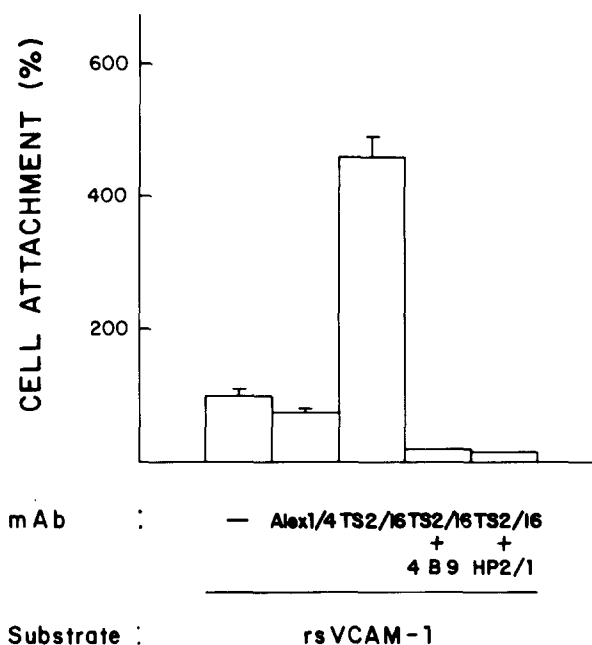mAb : — Aα1/4 TS2/16 TS2/16 TS2/16 + + 4B9 HP2/1

Substrate : rsVCAM-1

Figure 4. Anti-β1 mAb enhance the binding of U-937 cells to the VLA-4 cellular ligand VCAM-1. U-937 cells were preincubated with 1 µg/ml of purified Ig of the indicated anti-β1 mAb either alone or combined with anti-α4 HP2/1 or anti-VCAM-1 4B9 mAb. Then, cells were assayed for attachment to plates coated with 5 µg/ml of recombinant soluble VCAM-1 (rsVCAM-1). Control without antibody represents ~8.5% of total cellular input. A representative experiment out of three independent ones is shown.

to be substratum specific since no significant induction of cell attachment was observed to plates coated with either fibrinogen or seroalbumin (Fig. 1 B).

The upregulatory effect of anti-β1 mAb was isotype and Fc independent and could be observed with purified Ig as well as with both divalent F(ab')2 and monovalent Fab fragments of the TS2/16 mAb (Fig. 1 A). Furthermore, β1-mediated enhancement of cell attachment occurred very rapidly, within 5-10 min after anti-β1 mAb addition (Fig. 2 A), and was dependent on the concentration of coated FN, with maximal induction in the range of 5-10 µg/ml of FN (Fig. 2 B).

Involvement of VLA-4 and VLA-5 Integrins in β1-regulated Cell Binding to FN

Since U-937 cells have been previously shown to bear both VLA-4 and VLA-5 types of fibronectin receptors (20), we explored whether both integrin members could be implicated in β1-enhanced cell binding to FN. The anti-β1 TS2/16 mAb enhanced cell attachment to two distinct fibronectin proteolytic fragments of 38 and 80 kD, containing binding sites for VLAα4 and α5, respectively (Fig. 3). Moreover, β1-mediated cell binding to FN38 or FN80 kD was virtually abrogated by the simultaneous cell treatment with blocking anti-α4 HP2/1 or anti-α5 SAM-1 mAb, respectively (Fig. 3). These results clearly demonstrate that β1-regulated cell binding to fibronectin involves both α4 and α5 integrin fibronectin receptors.

Upregulation of VLA-4–mediated Cell Binding to the Endothelial Ligand VCAM-1

We next investigated the regulatory effect of anti-β1 TS2/16 mAb on U-937 cell binding to plates coated with a recombinant soluble form of VCAM-1, a cytokine-inducible endothelial cell ligand for the VLA-4 integrin. As shown in Fig. 4, a remarkable increase in cell attachment to VCAM-1 was observed after cell treatment with anti-β1 TS2/16 mAb. Cell binding to VCAM-1 could be inhibited by either anti-VLA-4
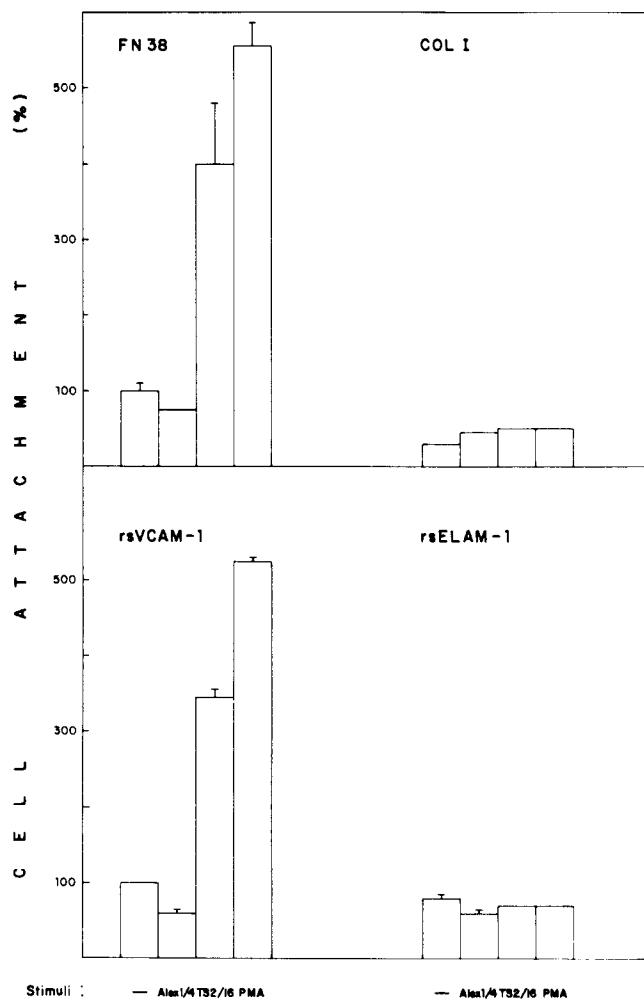
Figure 5. Anti-β1 mAb promote specific attachment of resting peripheral blood T lymphocytes to 38-kD proteolytic fragment of fibronectin and rsVCAM-1. Purified peripheral blood T cells were preincubated with 1 μg/ml of purified Ig of anti-β1 TS2/16 and Alex 1/4 mAb, or 50 ng/ml of PMA. Then, cells were assayed for binding to plates coated with 20 μg/ml FN38, 5 μg/ml rsVCAM-1, 20 μg/ml COL I, or 5 μg/ml rsELAM-1. Control without mAb represents 11.25 and 10.6% of total cellular input on FN38 and rsVCAM-1, respectively. The percentage of adhesion to COL I and rsELAM-1 is referred to those obtained with FN38 or rsVCAM-1, respectively. A representative experiment out of three independent ones is shown.

or anti-VCAM-1 mAb (Fig. 4). These results clearly indicate, that mAb to β1 can upregulate cell binding of VLA-4 to its VCAM-1 counterreceptor on endothelial cells as well as to fibronectin.

Anti-β1 mAb Promote Attachment of Resting T Lymphocytes to Both VLA-4 Ligands

The functional activity of integrins is regulated during lymphocyte activation. Thus, resting peripheral blood T cells which are unable to bind to VCAM-1 and FN ligands, acquire these VLA-4-mediated capacities after treatment with different stimulating agents (54, 64).

The treatment of purified T lymphocytes with anti-β1 TS2/16 mAb promoted a strong enhancement of T cell interactions with FN38 and VCAM-1 (Fig. 5). This enhancing

effect was comparable in magnitude to that triggered by activating agents such as phorbol esters (Fig. 5) or anti-CD3 mAb and was VLA-4 mediated since it could be virtually abrogated by anti-VLAα4 mAb (data not shown). The enhancing effect of anti-β1 TS2/16 mAb was also observed on T lymphocyte binding to the FN80 proteolytic fragment. The β1-mediated increment of T cell attachment to FN80 was threefold above control and was inhibited by anti-α5 mAb (data not shown).

This β1-mediated regulatory effect was specifically exerted on VLA-integrin-ligand interactions since no effect was observed on lymphocyte binding to either an unrelated endothelial cell adhesion ligand, the selectin ELAM-1, or to collagen, an ECM protein whose specific receptor (VLA-2) is not detected on resting lymphocytes (25) (Fig. 5).

β1-mediated Increase of Cell Interactions with Other ECM Proteins

To assess whether the observed regulatory effects of anti-β1 mAb could be also extended to other distinct VLA-mediated adhesive interactions, we studied the attachment of the A375 melanoma cells, which bear both VLA-2 and VLA-6 receptors for collagen and laminin, respectively (56), to either collagen type I or laminin in the presence of the anti-β1 TS2/16 mAb. As observed in Fig. 6 A, binding to both collagen and laminin was notably increased by the incubation with anti-β1 mAb. Moreover, β1-mediated cell binding to laminin involved the VLA-6 heterodimer since it was specifically blocked by simultaneous cell incubation with the anti-VLAα6 GoH3 mAb (Fig. 6 A). A similar enhancing effect of anti-β1 mAb was also observed on T lymphocyte binding to LN and was mediated by VLA-6 as deduced from the blocking effect of anti-α6 GoH3 mAb. An enhancement of A375 cell binding to FN, whose receptor in these cells has been previously described (40), was also observed after treatment with anti-β1 mAb (data not shown). In contrast, we cannot conclude that VLA-2 was involved in β1-mediated A375 cell binding to collagen since no inhibitory effect was exerted by the anti-VLAα2 P1E6 mAb used (Fig. 6 A), thus implying that other collagen receptor beside VLA-2 could be additionally involved in this particular cell-ECM interaction. These enhancing effects of anti-β1 mAb do not appear to involve changes in A375 cell surface membrane expression of different VLA heterodimers as demonstrated by quantitative flow cytometry analyses in A375 melanoma cells (Table I). As shown, A375 cells expressed low amounts of VLA-6 (Table I), as previously reported (56), that account for cell binding to LN, as demonstrated by inhibition with anti-α6 GoH3 mAb (Fig. 6 A). Furthermore, the functional effects of anti-β1 TS2/16 mAb were also observed in A375 cells previously treated with inhibitors of either protein or RNA synthesis (data not shown).

To further substantiate the possible regulatory role of anti-β1 mAb on VLA-2-collagen interaction, we studied the attachment of K-562 cells transfected with a cDNA encoding α2 chain to type I collagen. The enhancing effect of anti-β1 TS2/16 mAb was observed and it could be virtually abrogated by anti-α2 P1E6 mAb indicating that the function of the VLA-2 heterodimer could be also regulated through the β1 chain (Fig. 6 B). As control of specificity, binding of α4-K-562-transfected cells to both COL and FN38 were also included (Fig. 6 B).

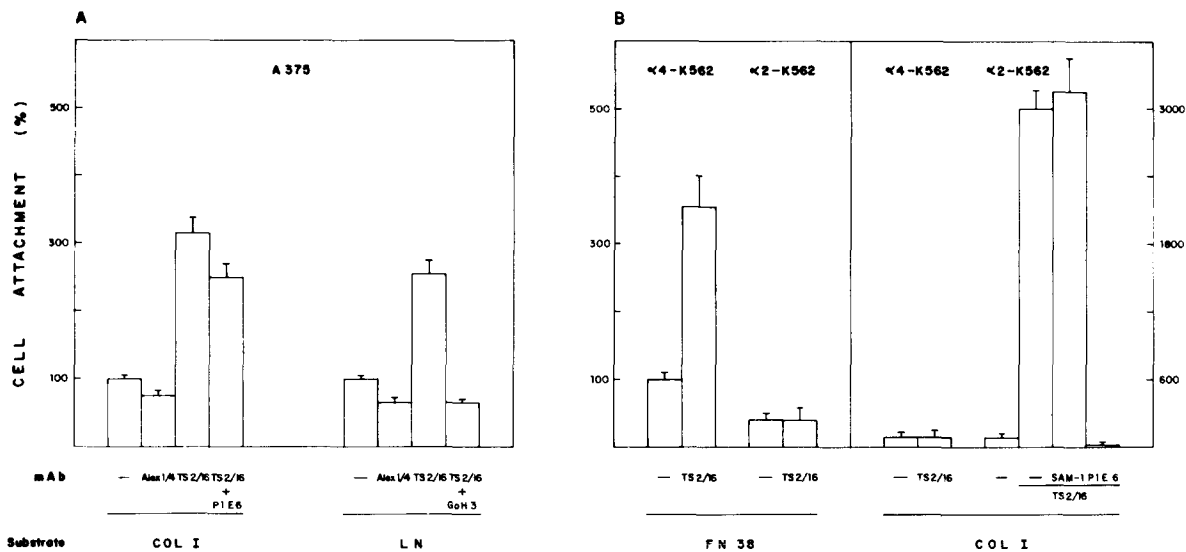Arroyo et al. Regulation of VLA Integrin–Ligand Interactions

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Figure 6. Anti-β1 mAb-mediated attachment of A375 melanoma cells to collagen type I and laminin and of α2-transfected K-562 to collagen type I. (*A*) A375 cells were detached and then preincubated with 1 µg/ml of indicated anti-β1 mAb either alone or combined with a 1:10 dilution of anti-α2 P1E6 or 1 µg/ml of anti-α6 GoH3. Then, cells were assayed for attachment to plates coated with 5 µg/ml of collagen type I or 10 µg/ml of laminin. Control without antibody represents 10 and 8% of total cellular input on collagen and laminin, respectively. A representative experiment out of three independent ones is shown. (*B*) α2- and α4-transfected K-562 were preincubated with 1 µg/ml of purified Ig of anti-β1 TS2/16 mAb either alone or combined with anti-α5 SAM-1 or anti-α2 P1E6. Then, cells were assayed for attachment to plates coated with 10 µg/ml of FN38 and/or with 10 µg/ml of collagen type I. Control without antibody represents about 6.25 and 1% of total cellular input for α4-transfected on FN38 and for α2-transfected K-562 on collagen, respectively. A representative experiment out of three independent ones is shown.

Metabolic Requirements of β1-mediated T Lymphocyte Adhesion

When the requirements of β1-mediated functional effects were investigated in T cells, we found that this process required an active metabolism, cytoskeleton integrity, physiological levels of intracellular Ca^{2+}, and a functional Na^+/H^+ antiporter as shown by inhibition experiments. Thus, incubation of T cells at 4°C or pretreatment of cells with different agents such as 2 DO-glucose plus sodium azide, cytochalasin B, Quin-2a, or EPA prevented the enhancing effects of anti-β1 TS2/16 mAb on T cell adhesion to FN (Table II). These reagents also inhibited the adhesion induced by other stimuli such as the Ca^{2+} ionophore A23187 and PMA indicating that intact cellular metabolism is needed for the induction of cell adhesiveness. In contrast, pretreatment of T cells with staurosporine, an specific inhibitor of PKC, did not inhibit the β1-mediated regulatory effect, whereas it specifically abrogated PMA-mediated adhesion (Table II).

We also studied possible intracellular signals involved in anti-β1 TS2/16 mAb-enhancing effect. No changes in intra-

cellular levels of cAMP and Ca^{2+} were observed after treatment of T lymphocytes with anti-β1 TS2/16 mAb (data not shown). Only minor alterations of intracellular pH (an increase ∼0.03–0.06 pH after treatment of T lymphocytes with TS2/16 mAb) could be detected, in agreement with results reported in a recent study using the anti-β1 TS2/16 mAb (52).

Changes in Cell Morphology and Cytoskeletal Organization Induced by anti-β1 mAb on Endothelial and U-937 Cells

The regulatory effect of the anti-β1 on the attachment of an adherent cell type such as HUVEC to either FN, LN, and COL type IV was morphologically analyzed. The anti-β1 TS2/16 mAb not only increased the number of cells attached to these ECM proteins but most interestingly, it induced a dramatic increase in cellular spreading. Thus, endothelial cells treated with anti-β1 TS2/16 mAb displayed an extended morphology showing an increased number of cytoplasmic projections (Fig. 7, *A*, *B*, and *C*), compared to cells bound in the absence of anti-β1 mAb (Fig. 7, *D*, *E*, and *F*). A rapid

Table I. Effect of Anti-β1 mAb on Cell Surface Antigen Expression of A375 Melanoma Cells

Stimuli	CD43	HLA-A,B	α1	α2	α3	α4	α5	α6	β1
					Antigen expression				
					Percent positive cells (MFI)				
–	0(299)	82(637)	1(307)	57(466)	54(466)	62(388)	69(443)	44(528)	81(548)
TS2/16	0(306)	80(646)	0(354)	55(452)	51(449)	60(390)	67(461)	35(490)	77(504)

A375 melanoma cells were preincubated either with medium or with Fab fragment of anti-β1 TS2/16 mAb at 10 µg/ml for 1 h at 37°C and then assayed for antigen expression as described in Materials and Methods. mAbs used were: TP1/36 (CD43), W6/32 (HLA-A,B) TS2/7 (α1), 12F1 (α2), P1B5 (α3), HP2/1 (α4), SAM-1 (α5), GoH3 (α6), and TS2/16 (β1). Percentage of positive cells and mean of fluorescence intensity (MFI) in logarithmic scale are shown.

Table II. Metabolic Requirements for β1-mediated Adhesion to Fibronectin of T Lymphocytes

Stimuli			Cell treatment				
	–	4°C	2 DO-glucose + sodium azide	Cyto-chalasin B	Quin-2a	EPA	Stauro-sporine
			Percent cell attachment				
–	5	0	0	0	2	0	3
TS2/16	15	1	3	2	3	3	20
PMA	48	2	2	3	17	2	10
A23187	15	0	1	1	3	1	9

T lymphocytes were preincubated with different agents for 30 min at 37°C (with the exception of control and 4°C samples) at the following doses: 2 DO-glucose 50 mM + sodium azide 0.1%, cytochalasin B 20 µM, Quin-2a 20 µM, N-ethyl,N-propyl amiloride (EPA) 50 µM, and staurosporine 200 nM. The different treatment did not significantly affect cellular viability as assessed by trypan blue staining. Then, cells were stimulated for 30 min at 37°C with no stimuli, the anti-β1 TS2/16 mAb at 10 µg/ml, PMA 50 ng/ml or A23187 1 µM, and then assayed for attachment on FN-coated plates (20 µg/ml). Percentage of cells attached was calculated. A representative experiment out of three is shown.

clustering and organization of focal adhesions were also observed (data not shown). These results suggested a possible signalling through β1 chain to interactive cytoskeletal components.

Next, we analyzed morphological changes induced in U-937 cells by anti-β1 TS2/16 mAb, compared to other agents such as the Ca^{2+} ionophore A23187 or PMA. As observed in Fig. 8, a dramatic change in U-937 cellular morphology was observed after anti-β1 TS2/16 mAb treatment. After the induction of adhesion by anti-β1 mAb, U-937 cells which are normally round, extend many long thin protrusions known as microspikes or filopodia (Fig. 8 E). These β1-mediated morphological changes are distinct from those induced in U-937 cells by PMA; with this treatment instead of microspikes, cells extend thin sheetlike processes known as lamellipodia (Fig. 8 D). When anti-β1 mAb and PMA were used in combination, greater cell spreading in the form of lamellipodia extension could be observed (Fig. 8 F). By contrast, cells remained unaltered after treatment with the Ca^{2+} ionophore A23187 or the anti-β1 Alex1/4 mAb included as control (Fig. 8, C and B, respectively). These data point out the possibility of different pathways for the transduction of adherent signals in U-937 cells.

Kinetics studies indicated that the morphological changes induced in U-937 cells by anti-β1 TS2/16 mAb were time dependent (Fig. 9, left), correlating with the kinetics of the enhancing effects triggered by anti-β1 mAb on U-937 cell adhesion to FN (Fig. 2 A). The appearance of microspikes could be observed as early as 5 min after TS2/16 mAb addition. Furthermore, both the β1 integrin and the cytoskeletal protein talin colocalized in the TS2/16-induced microspikes (Fig. 9, right), suggesting that anti-β1 regulatory effect on cell adhesion could be associated with organization of cytoskeleton. These results emphasize the central role of the common β1 chain in the regulation of different adhesive functions of VLA integrins as well as its implication in controlling cell shape.

Discussion

Our results support a pivotal role of VLA β1 chain in the regulation of adhesion receptor functions of different VLA heterodimers. We have found that certain β1 antibodies can promote VLA-mediated cell binding to both ECM and cellular ligands. Recent studies have described several antibodies against integrin α subunits that induce changes in receptor-ligand interactions (5, 11, 23, 32, 59). Herein, we have identified two antibodies, TS2/16 and A-1A5 specific for the β1 chain of VLA integrins, that induce an enhancement of VLA receptor-ligand binding affinities. The engagement of distinct β1 epitopes seems to modulate in a different manner VLA-mediated adhesion functions. Hence, TS2/16 mAb, which increased cell attachment to different ECM ligands, is also able to inhibit VLA-4-mediated cell-cell aggregation (11). Conversely, the Lia 1/2 and P4C10 mAb inhibit cell attachment to ECM, and other β1 antibodies, Alex 1/4, Lia 1/5, and K20, did not affect cell attachment. Thus, these results clearly show that the upregulatory effect of anti-β1 mAb appears to be site specific and document the existence of a differential pattern of functional involvement associated with each β1 epitope.

The anti-β1–enhanced cell binding appears to be isotype and Fc independent and can be also triggered by both divalent and monovalent Fab fragments. The β1 regulatory effect is promoted in a dose dependence of ligand and does not require de novo protein synthesis since it follows a very rapid kinetics and could be observed in the presence of inhibitors of protein and RNA synthesis. Moreover, the β1-mediated-enhanced adhesion does not involve any associated change in cell surface expression of the different VLA heterodimers. This is similar to the regulatory effects previously described with either antibodies specific for some integrin α subunits or specific ligands (5, 11, 16, 23, 32, 59). Taken together, these results suggest that anti-β1 mAb could function by increasing the affinity of different VLA αβ1 heterodimers for their respective ligands.

The enhancement of affinity promoted by anti-β1 mAb is not restricted to a unique member of VLA integrins nor to a single substrate. The β1-regulatory effects have been observed with at least VLA-2, VLA-4, VLA-5, and VLA-6 heterodimers and with ECM proteins (FN, COL, and LN) as well as cellular ligands (VCAM-1). These effects have been shown to be substratum specific because they are only promoted on substrates known to be ligands of VLA integrins.

It has been previously reported that the ligand affinity of several integrins from the β2 (LFA-1, Mac-1) (2, 17), β3 (gpIIb/IIIa) (16, 45), and more recently from the β1 family (VLA-4, VLA-5, and VLA-6) (53, 54) can be regulated by different stimuli. We report herewith that the interaction of VLA integrins with their ligands can be also regulated through the common β1 chain. The anti-β1–mediated regulatory effects are exerted on different cell types including the U-937 myelomonocytic cell line, melanoma cells, α2- and α4-transfected K-562 cells, HUVEC, and normal resting peripheral blood T cells. In resting lymphocytes, the anti-β1 mAb promoted VLA-4-mediated cell attachment to its two ligands, VCAM-1 and the FN38 proteolytic fragment containing CS-I region as well as to FN80 and LN (ligands for VLA-5 and VLA-6, respectively), in a similar manner to that described with activating agents such as phorbol esters or anti-CD3 mAb (53, 54).

The β and α chains of integrins could contribute to both binding of ligands, as demonstrated for β3 subunit (6), and
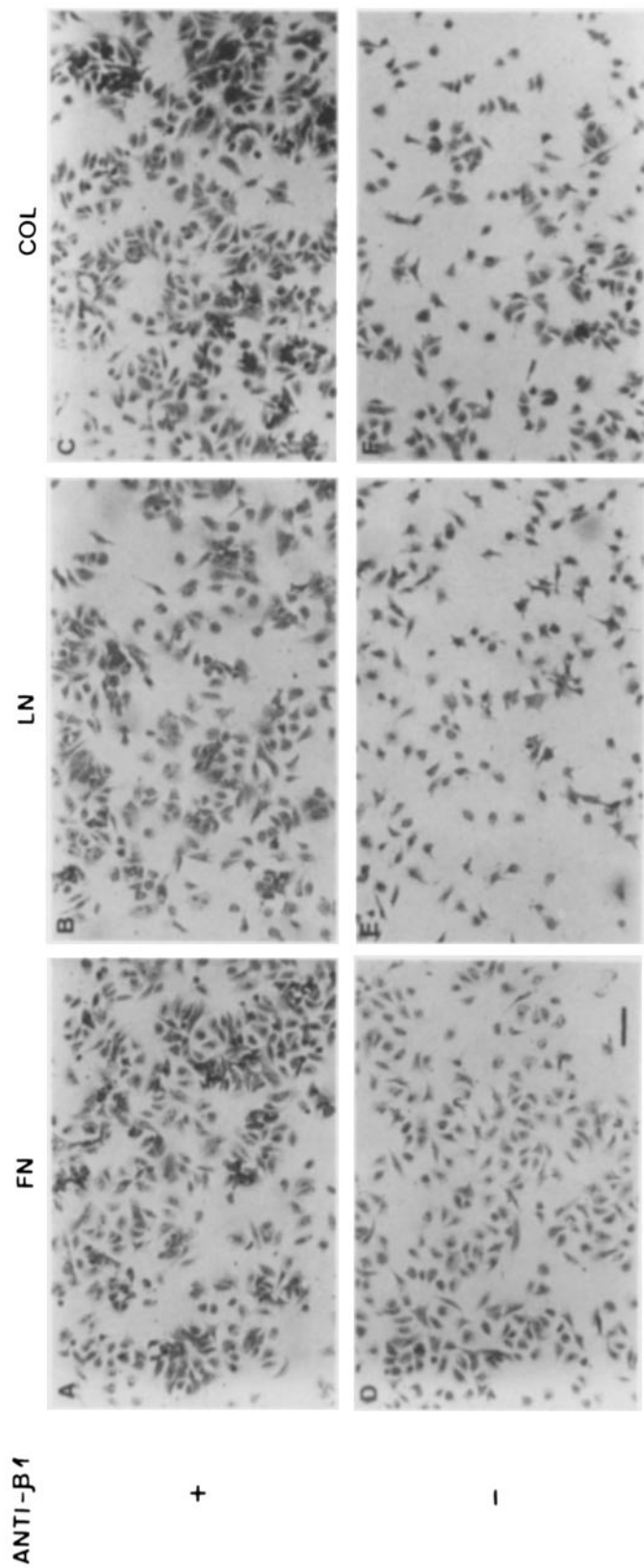
Figure 7. Morphological changes on endothelial cells promoted by anti-β1 mAb on attachment to FN, LN, and COL IV. HUVEC were preincubated in the presence (*A*, *B*, and *C*) or in the absence (*D*, *E*, and *F*) of the anti-β1 TS2/16 mAb and assayed for attachment to 7 μg/ml of FN, 30 μg/ml of laminin, or 1 mg/ml of collagen type IV as described in Materials and Methods. Photomicrographs showing morphological changes were taken after 30 min. Bar, 150 μm.
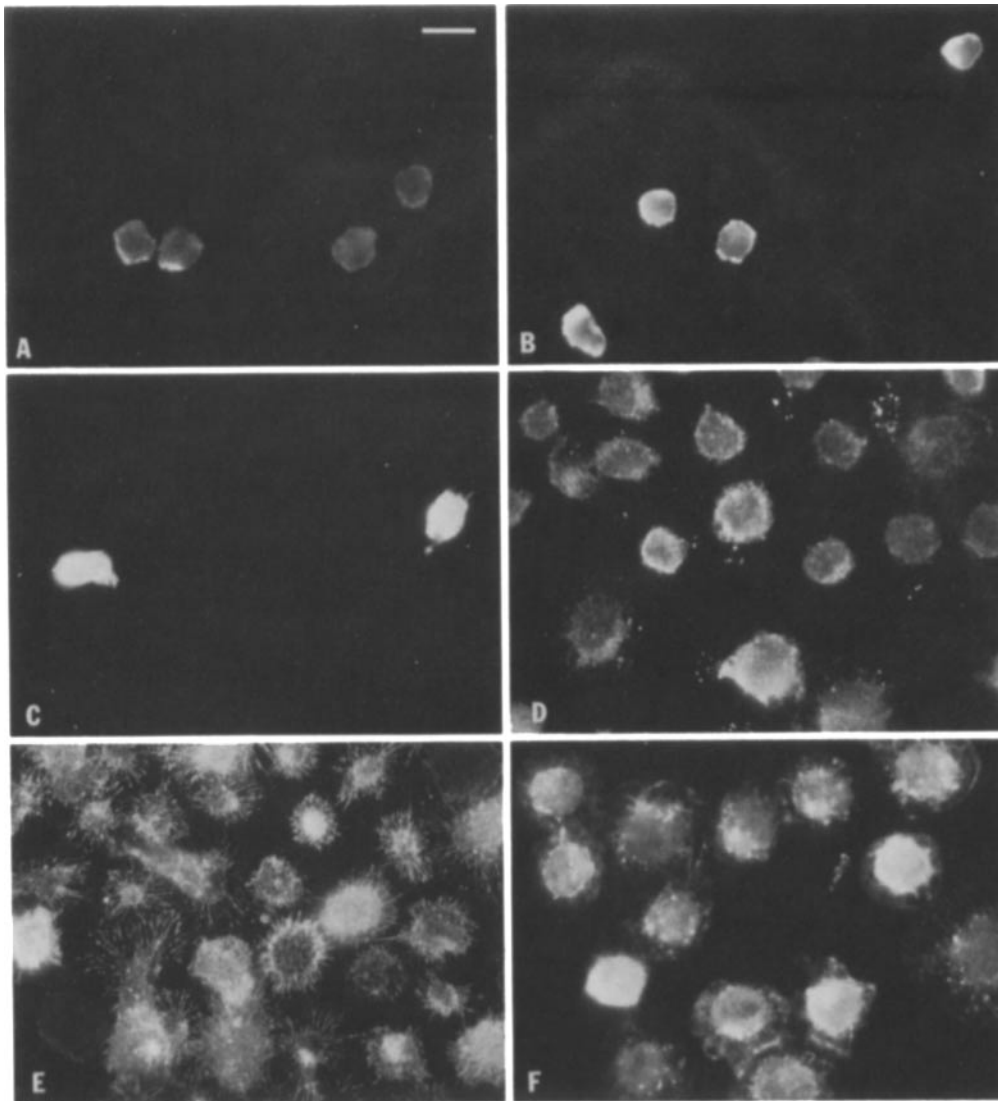
Figure 8. Morphological changes induced in U-937 cells by different reagents that promote the adhesion to FN. Cells were treated for 2 h with the following reagents: no treatment (*A*), 1 μg/ml Alex 1/4 anti-β1 mAb (*B*), 1 μM A23187 calcium ionophore (*C*), 50 ng/ml PMA (*D*), 1 μg/ml TS2/16 mAb (*E*), or a mixture of 20 ng/ml PMA plus 1 μg/ml TS2/16 mAb (*F*). Then cells were fixed and stained by immunofluorescence for β1 integrin with anti-β1 Alex 1/4 mAb. Bar, 20 μm.

the transduction of intracellular signals (33, 52, 63, 66). Recently, the β1 chain has been also shown to regulate different cellular functions since anti-β1 mAb induce comitogenic proliferative signals on T lymphocytes (65) and increase attachment of neuronal cells to laminin and collagen (42). These observations are in line with results reported here, supporting a central role of β1 chain in regulating VLA-mediated interactions.

Possible mechanisms accounting for the β1-mediated enhancement of VLA–ligand interactions include: (*a*) a conformational change on the αβ heterodimer as a result of the binding of anti-β1 mAb resulting in a higher affinity form of the integrin that enables the interaction with ligand(s). This putative conformational change affects the α chain since the anti-β1–mediated effect could be abrogated by specific anti-VLAα antibodies. In fact, conformational changes mediated by either anti-integrin mAb or ligand binding have been previously reported for the LFA-1, Mac-1, and gpIIb/IIIa integrins (2, 16, 17, 45, 59); and (*b*) the binding of anti-β1 mAb may trigger intracellular signals. In this regard, it has been recently described that insoluble FN as well as antibodies specific for β1 chain activate the Na^+/H^+ antiporter (52).

Moreover, clustering of β1 chain integrins also increased protein tyrosine phosphorylation (33). Whether these intracellular signallings are accounting for our observations on β1-mediated VLA–ligand adhesive interactions still remains unclear. When we explored intracellular signals triggered on T lymphocytes by anti-β1 TS2/16 mAb, no changes in second messengers such as intracellular cAMP and calcium levels were detected. By contrast, a small increase in intracellular pH could be detected under the same conditions, in agreement with previous reported data (52). However, our data using inhibitors of different signal transduction pathways point out to a general requirement of an intact metabolism for regulation of cell adhesiveness by different stimuli instead of ascribing a specific mechanism for β1-mediated effects. It is also conceivable that both putative mechanisms, i.e., modification of associated α chains and signal transduction, might be acting in concert.

Evidence suggesting a role for VLA-5 FN receptor in regulating cell spreading and migration, matrix assembly, and cytoskeletal organization has been previously described (1, 41). In this context, it is important to remark on our observations concerning the changes induced by anti-β1 TS2/16

Figure 9. Time-course of morphological changes promoted by anti-β1 mAb on the attachment of U-937 cells to FN and participation of cytoskeletal proteins in these morphological changes. (*Left*) U-937 cells were incubated in the absence (*A*) or in the presence (*B*, *C*, and *D*) of the anti-β1 TS2/16 mAb for different periods of time: 5 min, 30 min and 2 h (*B*, *C*, and *D*, respectively), on FN-coated coverslips. Cells were fixed and stained by immunofluorescence for β1 integrin with anti-β1 Alex 1/4 mAb. (*Right*) U-937 cells were cultured for 2 h in the presence of TS2/16 mAb on FN-coated coverslips. Cells were fixed, permeabilized, and stained by double-label immunofluorescence for β1 integrin with anti-β1 Alex 1/4 mAb (*A*) and for talin (*B*). Note that the integrin and the talin colocalize at the microspikes. Bar, 20 μm.
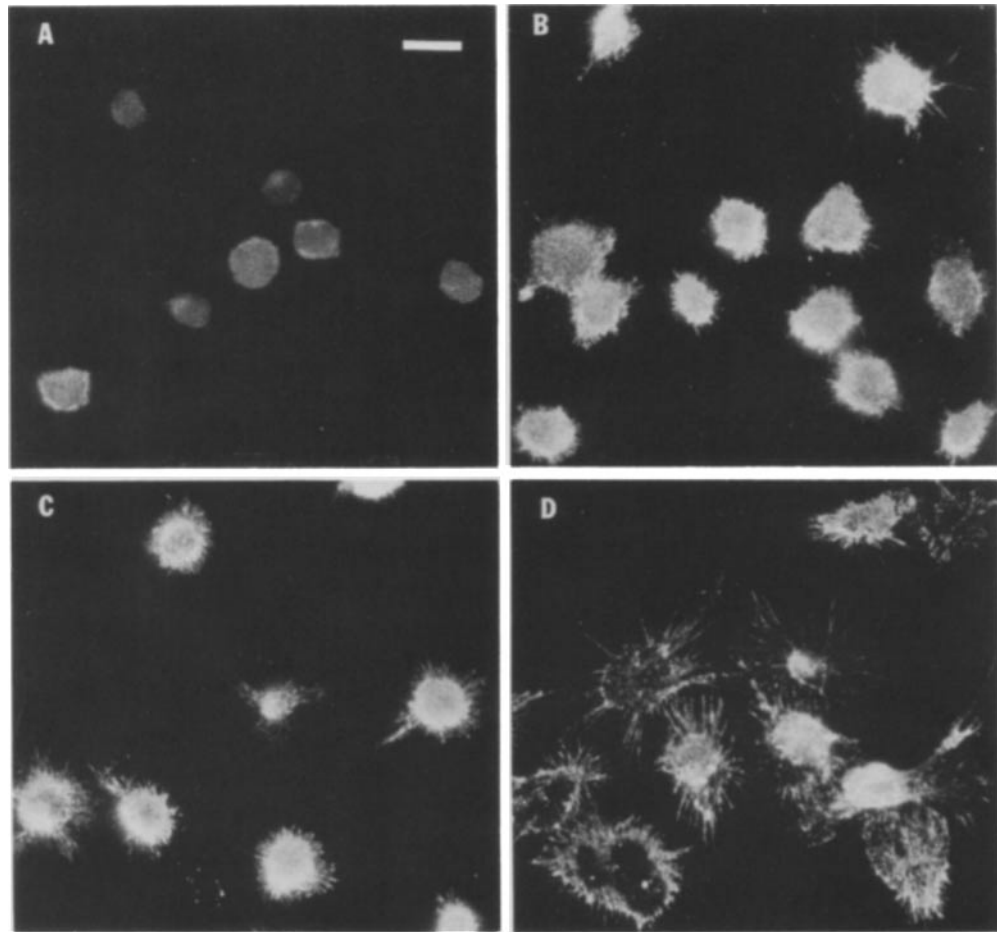
mAb on both cell morphology and organization of cytoskeleton. The interaction of the cytoplasmic tail of β1 chain with the cytoskeletal proteins talin and α-actinin involved in organization of actin microfilaments as well as in the regulation of cellular shape and motility has been previously documented (9, 10, 28, 43). Our data showing the colocalization of both β1 integrin and talin at the β1-induced cellular microspikes indicate a regulatory role of β1 chain in cell shape. Moreover, both β1-mediated effects, enhancement of cell adhesion and morphological changes, display similar kinetics and thus, they appear to happen coordinately. On the other hand, the β1-mediated induction of microspikes (filopodia) on U-937 cells is clearly different from morphological changes, mainly involving lamellipodia formation, triggered by other stimuli as phorbol esters. Although both anti-β1 TS2/16 mAb and phorbol esters induced cell adhesiveness, they appear to exert their effects through distinct mechanisms as evidenced by both the observed morphological differences and the selective inhibitory effect of protein kinase C specific inhibitors in phorbol ester–mediated cellular adhesiveness.

It is worth emphasizing the essential role that the β1 chain plays in regulating the affinities of VLA integrins for their ligands. This fact could be of physiological relevance in the regulation of the adhesive and tissue infiltrating properties of leukocytes as well as the metastatic capacities of tumor cells. For example, the regulation through β1 chain of VLA-4 interaction with its counterreceptor VCAM-1 on activated endothelial cells could be of crucial importance for binding and migration of leukocytes at sites of tissue inflammation. Furthermore, it also might be of relevance in regulating the metastatic properties of some tumors such as melanoma cells, since this receptor–counterreceptor pair represents the only adhesion pathway used by melanoma cells to adhere to endothelium in vitro (39, 49).

Finally, our finding that anti-β1 mAb enhance both cell attachment and spreading and cytoplasmic extension of endothelial cells to different ECM proteins such as COL, LN, and FN could be also physiologically important. It might represent a mechanism to regulate and modify the process of tissue repair that takes place in injured vessels. There is increasing evidence that the cell migration that takes place during tissue repair such as wound healing depends on integrin-mediated interactions (14, 30). Therefore, β1-regulated increases in endothelial cell adhesion to ECM proteins may allow for a better attachment, migration, and differentiation, thus minimizing the exposure of subendothelium to platelets and procoagulant factors that could provoke thrombotic processes.
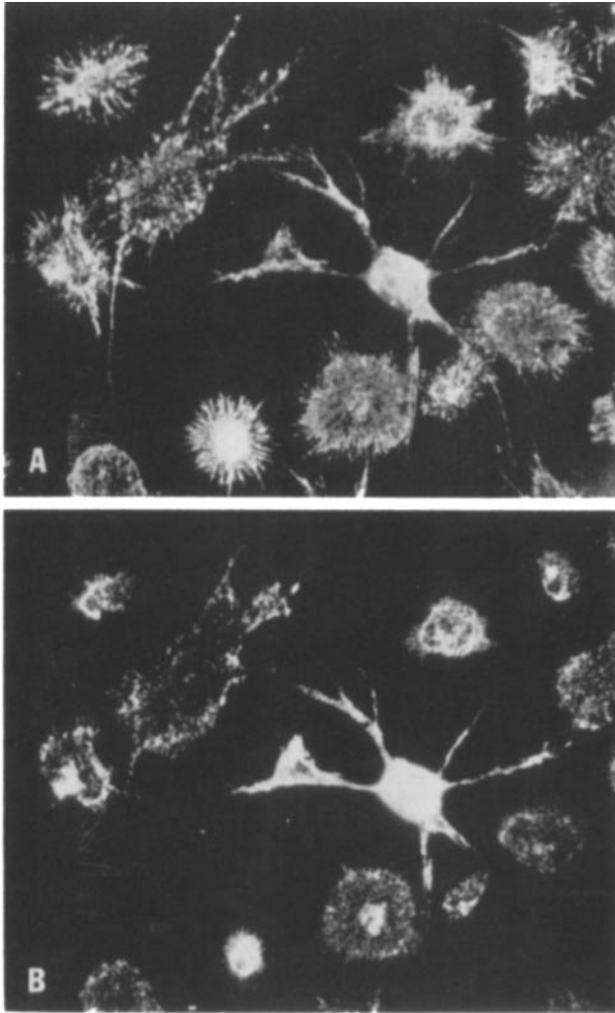
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