

# Modulation of Cell Adhesion by Changes in $\alpha_L \beta_2$ (LFA-1, CD11a/CD18) Cytoplasmic Domain/Cytoskeleton Interaction

By Karlheinz Peter\*<sup>‡</sup> and Timothy E. O'Toole\*

From the \*Department of Vascular Biology, Scripps Research Institute, La Jolla, California 92037; and <sup>‡</sup>Internal Medicine III, University of Heidelberg, 69115 Heidelberg, Germany

## Summary

The integrin  $\alpha_L \beta_2$  (leukocyte function-associated molecule 1, CD11a/CD18) mediates activation-dependent adhesion of leukocytes. The cytoplasmic domains of  $\alpha_L \beta_2$  have been demonstrated to modulate adhesiveness of  $\alpha_L \beta_2$ . Affinity changes of  $\alpha_L \beta_2$  for its ligand or postreceptor events can be responsible for this modulation of adhesiveness. To investigate the possible role of the  $\alpha_L \beta_2$  cytoplasmic domains in postreceptor events we constructed cDNA encoding chimeric proteins with intracellular  $\alpha_L \beta_2$  domains, which are responsible for  $\alpha_L \beta_2$  specific intracellular interactions, and extracellular  $\alpha_{IIb} \beta_3$  (GP IIb/IIIa) domains, which allow the assessment of the receptor affinity state. The cDNA was stably transfected in Chinese hamster ovary cells and chimeric heterodimer formation proven by immunoprecipitations and flow cytometry. The chimeric receptors mediate adhesion to immobilized fibrinogen, and this adhesion is increased by phorbol myristate acetate and abolished by cytochalasin D. However, neither treatment affects the affinity state of the chimeric receptor, suggesting involvement of the cytoskeleton in the regulation of  $\alpha_L \beta_2$  mediated cell adhesion. To exclude the possibility of postoccupancy affinity changes of the chimeric receptors, we locked the receptors into a high affinity state by creating a deletion variant. The region deleted (VGFFK) is highly conserved in integrin  $\alpha$  subunit cytoplasmic domains. Cotransfection of this deletion variant with a  $\beta$  subunit truncation ( $\beta_3 \Delta_{724}$ ) and a triple mutation at 758-760 (TTT to AAA) of  $\beta_2$  abolishes adhesion without changing the affinity state. A single mutation (TTT to TAT) reduces adhesion by half without affinity change. Scanning electron microscopy reveals impaired spreading of these truncated/mutated chimeras. Immunofluorescence microscopy demonstrates a correlation between impaired adhesion and a decrease in the ability to form focal adhesions and to organize the cytoskeleton into stress fibers. These results describe the integrin/cytoskeleton interaction, the organization of the cytoskeleton, and cell spreading as postreceptor events modulating  $\alpha_L \beta_2$  cytoplasmic domain mediated cell adhesion. Furthermore, we demonstrate that the cytoplasmic domain of the  $\beta_2$  subunit, and within it the TTT region, are required for these postreceptor events. Additionally, we present a new approach, using deletion variants to lock integrins in a high affinity state without interfering with the investigated integrin/cytoskeleton interaction. This approach may be generally useful to investigate the role of postreceptor events in integrin-mediated cell adhesion and migration.

The integrin  $\alpha_L \beta_2$  (LFA-1, CD11a/CD18) mediates the activation-dependent adhesion of leukocytes with APCs, cytolytic targets, and endothelial cells (1-5). The augmentation in  $\alpha_L \beta_2$ -dependent adhesion which follows stimulation of these cells by either cross-linking the TCR or by PMA is referred to as increased avidity or adhesiveness (1, 2, 6).  $\alpha_L \beta_2$  adhesiveness is controlled by the cytoplasmic domain of the  $\beta_2$  subunit (7, 8). In particular mutations of a triplet of threonines in the  $\beta_2$  tail profoundly reduce the adhesive-

ness of  $\alpha_L \beta_2$  (8). Altered adhesiveness of  $\alpha_L \beta_2$  could reflect changes in the binding affinity for its counter receptors, the intercellular adhesion molecules (ICAMs)<sup>1</sup> (1, 7, 8). Alternatively, postreceptor events such as integrin/cytoskeleton association, cytoskeletal organization, and cell spreading may regulate the adhesive function of  $\alpha_L \beta_2$ . Both activation-dependent affinity modulation (9-11) and postreceptor events (12-17) are mediated through the integrin cytoplasmic domains.

For another activation-dependent integrin, the platelet

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<sup>1</sup> Abbreviations used in this paper: CHO, Chinese hamster ovary; ICAM, intercellular adhesion molecule.

fibrinogen receptor  $\alpha_{11b} \beta_3$  (GP IIb-IIIa), it could be demonstrated that upon stimulation of platelets this integrin undergoes a conformational change and a marked increase in its ligand binding affinity (18–20). The affinity state of  $\alpha_{11b} \beta_3$  can be assessed by soluble fibrinogen (19–22) or by the mAb PAC-1, which displays a binding region, RYD, similar to the binding region RGD in fibrinogen and thus demonstrates binding characteristics comparable to fibrinogen (9, 10, 18, 21).

In this work, we have evaluated mechanisms by which cytoplasmic domains regulate  $\alpha_L \beta_2$  adhesiveness. Since ICAMs are integral membrane proteins (1, 23), they cannot be used for direct ligand binding studies to assess affinity. To evaluate the role of the cytoplasmic domains of  $\alpha_L \beta_2$  in modulating adhesiveness, we constructed chimeric integrins bearing the cytoplasmic domains of  $\alpha_L \beta_2$  and the transmembrane and extracellular domains of  $\alpha_{11b} \beta_3$ , using the latter as affinity reporter. We identified treatments that enhanced or reduced cell adhesion without changing the affinity state of the chimeric integrin. Moreover, previously described  $\beta_2$  cytoplasmic domain point mutations and truncations that reduce the adhesiveness of  $\alpha_L \beta_2$  (7, 8) also reduced cell adhesion mediated by chimeras, which were permanently locked in a high affinity state by a deletion in the  $\alpha$  subunit. Furthermore, the immunolocalization and spreading ability of the chimeric integrins suggest that interaction of the integrin cytoplasmic domains and the cytoskeleton are involved in the regulation of adhesiveness of  $\alpha_L \beta_2$ .

## Materials and Methods

**Cells.** Chinese hamster ovary (CHO) cells and the human T lymphoma cell line Jurkat were obtained from the American Type Culture Collection (Rockville, MD). Chinese hamster ovary (CHO) cells were maintained in DMEM, 10% FCS (Whittaker M.A. Bio-products, Walkersville, MD), 2 mM L-glutamine, and 1% MEM nonessential amino acids (Sigma Chemical Co., St. Louis, MO). Jurkat cells were maintained in RPMI 1640 (Whittaker M.A. Bio-products), 10% FCS, and 2 mM L-glutamine.

**Antibodies and Peptides.** The  $\alpha_{11b} \beta_3$  complex-specific mAb 2G12 was provided by Virgil Woods (University of California, San Diego, CA). The activation-dependent anti- $\alpha_{11b} \beta_3$  mAb PAC-1 (18) was generously provided by Dr. Sanford Shattil (Hospital of the University of Pennsylvania, Philadelphia, PA). The mAb D57 (anti- $\alpha_{11b}$ ) was produced by Dr. Xiaoping Du (Scripps Research Institute). The mAbs anti-LIBS 6 (anti- $\beta_3$ ), 15 (anti- $\beta_3$ ), and PL98DF6 (anti- $\alpha_{11b}$ ) were generated, isolated, and characterized as described elsewhere (20, 24). TS1/22 (mAb specific for  $\alpha_L$ ) and TS1/18 (mAb specific for  $\beta_2$ ) (25) were kindly provided by Dr. Stanley D'Souza (Cleveland Clinic Foundation, Cleveland, OH).

Polyclonal rabbit antisera were prepared by immunizing rabbits with peptides consisting of the 16 COOH-terminal amino acids of  $\alpha_L$  (NH<sub>2</sub>-KPLHEKDSSEGGKD-COOH) and  $\beta_2$  (NH<sub>2</sub>-KSATTTVMNPKFAES-COOH) coupled to bovine thyroglobulin type I (Sigma Chemical Co.). As a control for the specificity of these antisera to the cytoplasmic domains of  $\alpha_L \beta_2$ , we immunoprecipitated surface-labeled Jurkat cells. As described for  $\alpha_L \beta_2$  (26), we obtained molecular weights of 170,000 and 90,000 on a nonreducing 7% SDS-PAGE (data not shown). The same molecular weights were precipitated with the  $\beta_2$ -specific mAb TS1/18

and TS1/22 (26). Polyclonal rabbit antisera to the cytoplasmic domains of  $\alpha_{11b}$  (989-1008),  $\beta_3$  (742-762), and the extracellular domain of  $\beta_3$  are described elsewhere (9). The synthetic peptide GRGDSP was purchased from Peninsula Laboratories (Belmont, CA).

**DNA Constructs.** The following chimeric integrins were engineered. Depicted in boldface type are amino acids of the transmembrane region of  $\alpha_{11b} \beta_3$  and in roman face type, the cytoplasmic domains of  $\alpha_L \beta_2$ : (a)  $\alpha$  subunit chimera and  $\alpha$  subunit deletion:  $\alpha_{11b} \alpha_L$  (**LLTILV**LAMW **KVGF**FKRNLKEKMEA-GRGVP...),  $\alpha_{11b} \alpha_{L\Delta}$  (the underlined region [VGFFK] of the previous chimera was deleted); (b)  $\beta$  subunit chimera, deletion, and mutations:  $\beta_3 \beta_2$  (**LLITKAL**IHLSDLREYRRFEKEKLSQWN-NDNPLFKSATTTVMNPKFAES),  $\beta_3 \beta_2$  <sub>TAT</sub> (the underlined region was mutated to TAT),  $\beta_3 \beta_2$  <sub>AAA</sub> (the underlined region was mutated to AAA),  $\beta_3 \beta_2$  <sub>Δ724</sub> ( $\beta_3$  was truncated **LLTIHD**).

cDNA clones of  $\alpha_{11b}$  (27) and  $\beta_3$  (28), subcloned in pBluescript II KS (Stratagene, La Jolla, CA), and reverse transcribed cDNA from the human T lymphoma line Jurkat were used as templates for PCR. For the chimeric DNA constructs  $\alpha_{11b} \alpha_L$  and  $\alpha_{11b} \alpha_{L\Delta}$  the sequence 5'-CAG ATA GGA ATC GCG ATG TTG GTG ( $\alpha_{11b}$ : 2216-2240; 27) was used as a sense primer (15 pmol/50  $\mu$ l) and the sequence 5'-GCG TCG ACG TTA ACG GCA GAA TGC AGG CAG AG-3' ( $\alpha_L$ : 3364-3684; 29) as an antisense primer (15 pmol/50  $\mu$ l) with the additional cloning sites Sall and HpaI. A third sense primer with 25 bp up- and downstream of the fusion site was used at 1/20 (0.75 pmol/50  $\mu$ l) of the concentrations of the other two primers. The PCR was performed in a single step reaction with these three primers and two templates, pBluescript  $\alpha_{11b}$  and reverse transcribed Jurkat cDNA. The initial PCR cycles produced an intermediate product (cytoplasmic domain of  $\alpha_L$ ) which is automatically used as a megaprimer in the following PCR cycles (total of 30 cycles). After digestion with BglII and Sall the PCR product was subcloned into pCDM 8 IIb (30) using BamHI and the Sall-compatible XhoI restriction site. For the chimeric DNA construct  $\beta_3 \beta_2$  the sequence 5'-GGC TGG CTG GGA TCC CAG TGT GAG -3' ( $\beta_3$ : 1478-1510; 31) was used as a sense primer (15 pmol/50  $\mu$ l) and the sequence 5'-CCG CTC GAG TGA TCA GTG CTC CTA ACT CTC AGC AA-3' ( $\beta_2$ : 2378-2398; 32) as an antisense primer (15 pmol/50  $\mu$ l) with the additional cloning sites XhoI and BclI. A third sense primer with 25 bp up- and downstream of the fusion site was used at 0.75 pmol/50  $\mu$ l. PCR was performed as described above. After digestion with MluI and XhoI, the PCR product was subcloned into pCDM 8 IIIa (30).  $\beta_3 \beta_2$  <sub>TAT</sub> and  $\beta_3 \beta_2$  <sub>AAA</sub> were constructed by PCR, using  $\beta_3 \beta_2$  as template and the above described sense primer  $\beta_2$ : 2378-2398, and 5'-GGA CTC GAG CTA ACT CTC AGC AAA CTT GGG GTT CAT GAC CGC CGC GGC GCT CTT GAA AAG GGG-3', respectively 5'-GGA CTC GAG CTA ACT CTC AGC AAA CTT GGG GTT CAT GAC CGT CGC GGT GGC GCT CTT GAA AAG GGG-3' as antisense primers. The PCR products were subcloned as described for  $\beta_3 \beta_2$ .  $\beta_2$  <sub>724</sub> was constructed as described elsewhere (9). All PCR products were sequenced using the Sequenase kit™ (United States Biochemical Corp., Cleveland, OH).

**Generation of Stable Cell Lines.** CHO cells were transfected by electroporation (Electroporator model ECM 600; BTX Inc., San Diego, CA) using 370 V, 960  $\mu$ F, and R<sub>3</sub> (timing resistor: 48  $\Omega$ ). 10  $\mu$ g of the  $\alpha$  and  $\beta$  subunit in pCDM8 and 0.5  $\mu$ g pCDM8 with a neomycin resistance gene insert (generous gift of Dr. J. Loftus, Scripps Research Institute) were resuspended in 1.5  $\times$  10<sup>7</sup> cells per 800  $\mu$ l regular cell culture medium. Before and after the electroporation, this suspension was kept at room temperature for 10 min. After 2 d in regular cell culture medium, cells were selected for resistance to 700  $\mu$ g/ml G418 (Genetec; GIBCO-BRL,

Gaithersburg, MD). Cells were then selected for surface expression with mAb D57 on a flow cytometer (FACStar®; Becton Dickinson, San Jose, CA). Individual cell clones were examined on a flow cytometer (FACScan®; Becton Dickinson) with mAbs D57, 2G12, and Ab15 as following: 300,000 cells per 50  $\mu$ l were incubated with 10  $\mu$ g/ml mAb in modified Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO<sub>3</sub>, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 1 mg/ml BSA, and 1 mg/ml dextrose, pH 7.4) for 30 min at 4°C. After one wash in modified Tyrode's buffer, cells were incubated with 1/50 FITC-conjugated goat anti-mouse F(ab')<sub>2</sub> (Tago Inc., Burlingame, CA) for 30 min at 4°C.

**Adhesion of Transfected CHO Cells on Immobilized Fibrinogen.** Fibronectin-depleted fibrinogen was diluted in PBS, pH 7.4, to 20  $\mu$ g/ml and 100  $\mu$ l aliquots incubated in the wells of a 96-well Immulon II plate (Dynatech Laboratories, Inc., Chantilly, VA) overnight at 4°C. After one washing with PBS, the wells were blocked with 100  $\mu$ l aliquots of 10 mg/ml heat-inactivated BSA (fraction V; Calbiochem-Novabiochem Corp., La Jolla, CA) for 1 h at room temperature. Cells were detached with Trypsin-EDTA (Irvine Scientific, Santa Ana, CA) and washed twice in modified Tyrode's buffer. PMA (100 nM; stock in DMSO; Sigma Chemical Co.), cytochalasin D (10  $\mu$ M; stock in DMSO; Sigma Chemical Co.), or as control DMSO incubation was done in a shaker for 30 min at 37°C. 100,000 cells per well were incubated at 37°C or at room temperature for various time periods. The nonadherent cells were washed off with two rounds of pipetting. The residual adherent cells were quantified by a previously described colorimetric assay (33). Briefly, the CHO endogenous cellular acid phosphatase activity was used by adding 100  $\mu$ l of the following substrate/lysis solution to each well: 1% Triton X-100 (Fisher Scientific Co., Pittsburgh, PA), 6 mg/ml *p*-nitrophenylphosphate (Sigma Chemical Co.), in 50 mM sodium acetate buffer, pH 5. After 1 h incubation at 37°C, the reaction was terminated by the addition of 50  $\mu$ l of 1 M NaOH, and read in an ELISA plate reader (Molecular Devices Corp., Menlo Park, CA) with a 405-nm filter. Background values, determined in wells coated with 1% BSA alone, were subtracted from each point. Adherence was expressed as a percentage of the number of cells adhering in the chimera with the intact  $\beta_2$  cytoplasmic domain without any addition. These numbers were corrected for the percentage of surface-expressing cells and compared with a standard curve generated using known numbers of cells.

**PAC-1 Flow Cytometry.** After detachment of the transfected CHO cells with Trypsin-EDTA, cells were washed in modified Tyrode's buffer and incubated with PMA and cytochalasin D as described above. 300,000 cells per 50  $\mu$ l modified Tyrode's buffer were then incubated with PAC-1 ascites (1:500), LIBS 6 (0.75 mg/ml), GRGDSP (10 mM), and biotinylated mAb D57 for 30 min at room temperature. After one washing in modified Tyrode's buffer and resuspension in 50  $\mu$ l, cells were stained with FITC-conjugated anti-mouse IgM (Tago Inc.) and 2  $\mu$ l R-PE-conjugated streptavidin (Molecular Probes Inc., Eugene, OR) for 30 min at 4°C. Cells were examined on a FACScan® with the Lysis II software, and allowed to gate on mAb D 57-positive and (as an internal control) on mAb D 57-negative cells.

**Binding Assays with <sup>125</sup>I-labeled Fibrinogen.** The binding of <sup>125</sup>I-labeled fibrinogen was accomplished as previously described (10, 19). Cells were harvested and resuspended in modified Tyrode's buffer as described above for the adhesion experiments. A typical assay includes 120  $\mu$ l of cells ( $2 \times 10^6$  cells per tube), 40  $\mu$ l of <sup>125</sup>I-labeled fibrinogen (Amersham International, Amersham, Bucks, UK; final concentration, 50 nM), and 40  $\mu$ l GRGDSP (final concentration, 2 mM). After 30 min at room temperature, 50  $\mu$ l aliquots were layered in triplicate on 300  $\mu$ l of 20% sucrose in modified

Tyrode's buffer and centrifuged for 3 min at 12,000 rpm. <sup>125</sup>I-labeled fibrinogen associated with the cell pellet was determined by scintillation spectrometry.

**Surface Labeling and Immunoprecipitations.** Stably transfected CHO cells were surface labeled with <sup>125</sup>I (Amersham, Arlington Heights, IL) by using Iodogen (Pierce, Rockford, IL). 1 mg/ml Iodogen was dissolved in 150  $\mu$ l chloroform in a 14-ml Falcon 2059 tube. After evaporation of the chloroform, Iodogen formed a layer on the bottom of the tube. After two washings with modified Tyrode's buffer, 10<sup>7</sup> cells were added in 500  $\mu$ l modified Tyrode's buffer. The labeling was started with the addition of 1 mCi <sup>125</sup>I and continued for 15 min at room temperature. After three washings in modified Tyrode's buffer and 10 mg/ml KI, cells were solubilized in lysis buffer (modified Tyrode's buffer: 1% Triton X-100, 0.02% NaN<sub>3</sub>, 1 mg/ml *N*-ethylmaleimide, 2 mM Benzamide, 100  $\mu$ M Leupeptin, and 2 mM PMSF). Overnight, cell extracts were immunoprecipitated with preimmune rabbit antiserum, mAb 2G12, polyclonal rabbit antisera directed against the extracellular domains of  $\beta_3$  (anti- $\alpha\beta_3$ ), and polyclonal rabbit antisera directed against the cytoplasmic domains of  $\alpha_{1b}$  (anti- $\alpha_{1b}$ ),  $\beta_3$  (anti- $\beta_3$ ),  $\alpha_L$  (anti- $\alpha_L$ ), and  $\beta_2$  (anti- $\beta_2$ ) at 4°C. The precipitation was carried out by incubation with protein G-coupled Sepharose beads for 2 h at 4°C and a subsequent pelleting by centrifugation. The Sepharose beads containing the antibody-antigen complex were washed three times in the following buffer: modified Tyrode's buffer, 1% Triton X-100, 0.02% NaN<sub>3</sub>, 0.2% SDS, 1% deoxycholic acid, 1 mg/ml *N*-ethylmaleimide, and 2 mM Benzamide. Beads were resuspended in sample buffer, boiled for 3 min, centrifuged, and the precipitated proteins were resolved by SDS PAGE (nonreducing 7% acrylamide gels). The gels were dried and then visualized by autoradiography.

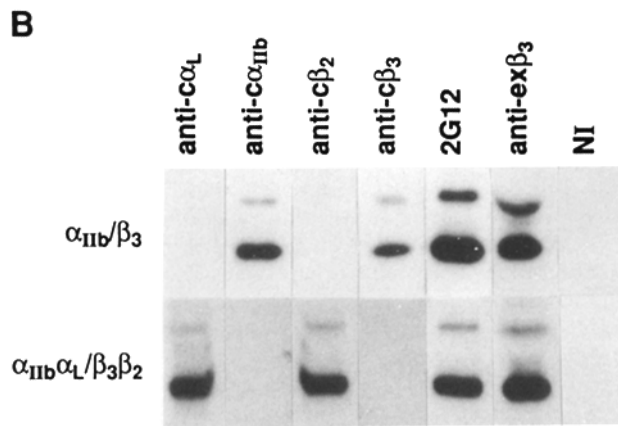
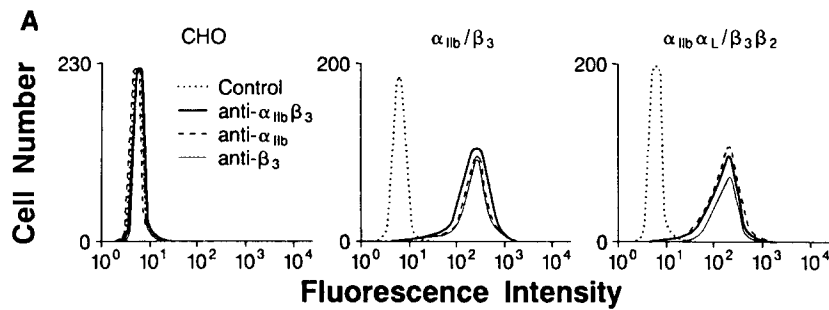
**Scanning Electron and Immunofluorescence Microscopy.** Glass coverslips (12 mm circular, No 1; Fisher Scientific) were coated with 20  $\mu$ g/ml fibrinogen in PBS overnight at 4°C, followed by blocking with 1% BSA (fraction V; Calbiochem-Novabiochem Corp.) for 1 h at room temperature. After detachment and two washes in DMEM, CHO cells transfected with chimeric integrin receptors were allowed to adhere in DMEM medium without FCS for 2 h at 37°C.

For scanning electron microscopy, after one PBS wash, cells were initially fixed in modified Karnovsky's fixative (1% paraformaldehyde, 1.5% glutaraldehyde in 0.1 M Cacodylate buffer, pH 7.4) for 1 h at 4°C, and postfixed in 1% OsO<sub>4</sub> in the same buffer for 1 h at room temperature. Cells were then dehydrated in graded ethanol, critical point dried in Freon 13, and coated with a thin carbon film by a Hummer sputter coater. Preparations were viewed and photographed on a Hitachi S-600 scanning electron microscope.

For immunofluorescence microscopy, cells were fixed with 2% paraformaldehyde and 0.5% Triton X-100 in PBS for 10 min on ice. Cells were washed twice with PBS and incubated with 5  $\mu$ g/ml PL98DF6 (anti- $\alpha_{1b}$ ) for 30 min at room temperature (13, 24). After two washes with PBS, cells were incubated with FITC-conjugated goat anti-mouse F(ab')<sub>2</sub> (Tago Inc.) at 1/50 and rhodamine phalloidin (Sigma Chemical Co.) at 0.1  $\mu$ g/ml for 30 min at room temperature. The coverslips were then washed and mounted in FITC-Guard™ mounting media (Testog Inc., Chicago, IL). Photographs were taken with Kodak Tmax 400 film (Eastman Kodak Co., Rochester, NY) on a Leitz Orthophan microscope with a plan Aplanachromat 100 $\times$  oil immersion objective.

## Results

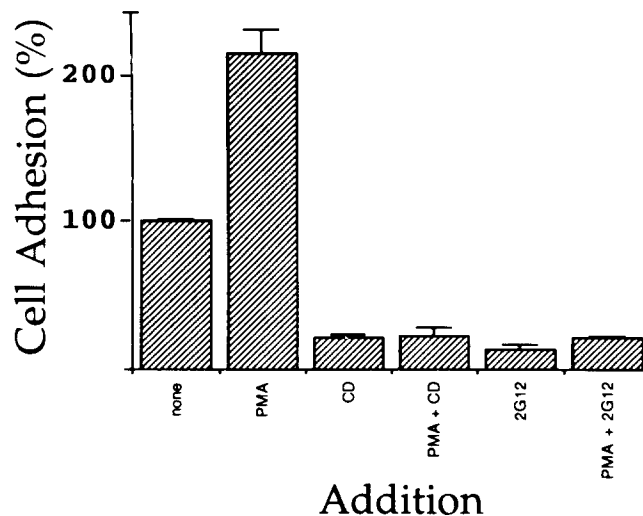
*Alterations in the Adhesiveness of a Chimeric Integrin with  $\alpha_L$   $\beta_2$  Cytoplasmic Domains Are Mediated by Postreceptor Events.*



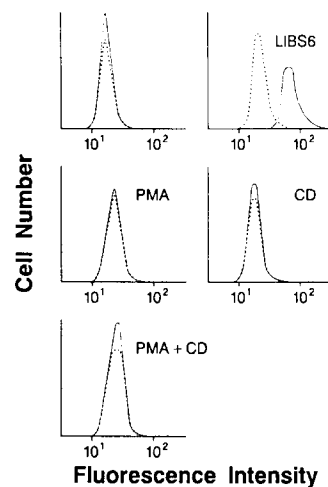
**Figure 1.** Surface expression and verification of the chimeric character of the transfected integrins. (A) Comparison of untransfected CHO cells,  $\alpha_{IIb}/\beta_3$ , and  $\alpha_{IIb}\alpha_L/\beta_3\beta_2$  transfected CHO cells in flow cytometry. Cells were stained with mAb TS1/22 (as negative control), 2G12 (complex-specific anti- $\alpha_{IIb}\beta_3$ ), D 57 (anti- $\alpha_{IIb}$ ), and Ab 15 (anti- $\beta_3$ ), each 10  $\mu\text{g}/\text{ml}$ . The detailed protocol is described in Materials and Methods. (B) Stably transfected CHO cells were surface labeled with  $^{125}\text{I}$ . After solubilization, cell extracts were incubated overnight at 4°C with preimmune rabbit antiserum, mAb 2G12, polyclonal rabbit antisera directed against the extracellular domain of  $\beta_3$  (anti-ex $\beta_3$ ), against the cytoplasmic domains of  $\alpha_{IIb}$  (anti- $\alpha_{IIb}$ ),  $\beta_3$  (anti- $\alpha\beta_3$ ),  $\alpha_L$  (anti- $\alpha_L$ ), and  $\beta_2$  (anti- $\alpha\beta_2$ ). The antibody-antigen complexes were precipitated by incubation with protein G-coupled Sepharose beads for 2 h at 4°C and resolved by electrophoresis on 7% SDS-polyacrylamide gels under nonreducing conditions followed by autoradiography. The detailed procedure is described in Materials and Methods. As examples, immunoprecipitations of the CHO transfectants  $\alpha_{IIb}/\beta_3$  and  $\alpha_{IIb}\alpha_L/\beta_3\beta_2$  are demonstrated with two bands at 95,000 and 140,000 Daltons.

To examine the mechanisms by which cytoplasmic domains of  $\alpha_L\beta_2$  regulate adhesiveness, we fused its cytoplasmic domain to the transmembrane and extracellular domains of  $\alpha_{IIb}\beta_3$ . After the transfection of the cDNA constructs into CHO cells, these chimeric integrins were expressed on the cell sur-

face as detected by staining with mAbs directed against  $\alpha_{IIb}\beta_3$ ,  $\alpha_{IIb}$  and  $\beta_3$  (Fig. 1 A). To verify that the chimeric cDNA constructs were expressed as chimeric proteins, we immunoprecipitated surface-radiolabeled transfected CHO cells with antibodies to the extracellular domains of  $\alpha_{IIb}\beta_3$  and poly-



**Figure 2.**  $\alpha_{IIb}\alpha_L/\beta_3\beta_2$  chimeric receptors constitutively show adhesiveness that can be increased by PMA and decreased by cytochalasin D.  $\alpha_{IIb}\alpha_L/\beta_3\beta_2$  transfected CHO cells were allowed to adhere for 40 min at 37°C onto plastic microtiter wells that were coated with 20  $\mu\text{g}/\text{ml}$  fibrinogen. Cells were preincubated with 100 nM PMA, 10  $\mu\text{M}$  cytochalasin D, 10  $\mu\text{g}/\text{ml}$  mAb 2G12, or no addition for 30 min at 37°C. Adhesion was quantified as described in Materials and Methods. Percent adherence was determined and results expressed as mean  $\pm$  SD of three determinants.



**Figure 3.**  $\alpha_{IIb}\alpha_L/\beta_3\beta_2$  chimeric receptors are in a low affinity state and are activatable with mAb anti-LIBS 6 but not with PMA or cytochalasin D. The binding of the activation-specific mAb PAC-1 to CHO cells stably transfected with  $\alpha_{IIb}\alpha_L/\beta_3\beta_2$  chimeric receptors is examined by flow cytometry. Depicted are histograms with the log of the fluorescence intensity on the abscissa and the cell number on the ordinate. PAC-1 staining as described in Materials and Methods is compared in the presence (dotted line) or absence (solid line) of 2 mM GRGDSP. This peptide inhibits specific binding of mAb PAC-1 and fibrinogen to the extracellular parts of  $\alpha_{IIb}/\beta_3$ . Thus, with super-

imposable histograms in the presence or absence of GRGDSP only unspecific binding is present and thus, the staining is negative. With no addition, the superimposable histograms in the presence or absence of GRGDSP show a constitutively low affinity state. The addition of LIBS 6 causes a high affinity binding of PAC-1 that is inhibited by 2 mM GRGDSP. The addition of 100 nM PMA, 10  $\mu\text{M}$  cytochalasin D, and the combination of PMA and cytochalasin D in the presence or absence of GRGDSP show superimposable histograms and therefore a low affinity state of the chimeric receptor.

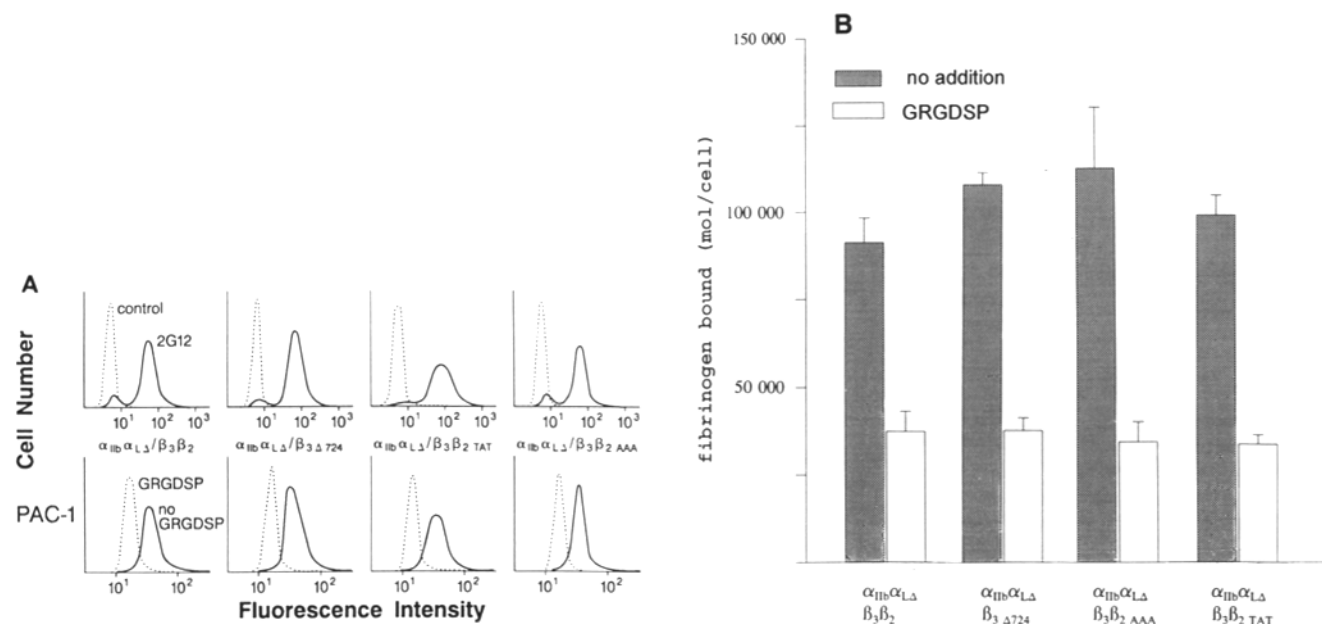
clonal rabbit antisera to the cytoplasmic domains of  $\alpha_L$ ,  $\beta_2$ ,  $\alpha_{IIb}$ , and  $\beta_3$  (Fig. 1 B). The  $\alpha_{IIb} \beta_3$  specific mAb 2G12 and polyclonal antiextracellular domain of  $\beta_3$  (anti-ex $\beta_3$ ), and polyclonal anticytoplasmic domain of  $\alpha_{IIb}$  (anti-c $\alpha_{IIb}$ ) and  $\beta_3$  (anti-c $\beta_3$ ) immunoprecipitated wild-type  $\alpha_{IIb} \beta_3$  from CHO cells. Whereas the monoclonal and polyclonal antibodies to the extracellular domains immunoprecipitated the chimera, the anti  $\alpha_{IIb} \beta_3$  cytoplasmic domain antibodies failed to do so (Fig. 1 B). In contrast, antibodies against the cytoplasmic domains of  $\alpha_L$  and  $\beta_2$  immunoprecipitated the chimera, but not wild-type  $\alpha_{IIb} \beta_3$  (Fig. 1 B). Thus, chimeric proteins containing the extracellular and transmembrane domains of  $\alpha_{IIb} \beta_3$  joined to the cytoplasmic domains of  $\alpha_L \beta_2$  are expressed on the surface of these transfected CHO cells.

To evaluate the adhesiveness of the chimeric receptor, we assessed its capacity to mediate adhesion to immobilized fibrinogen. CHO cells bearing the chimeric receptor constitutively adhered to fibrinogen and this adhesion was mediated by the transfected receptor because it was blocked by a mAb directed against the extracellular domain of  $\alpha_{IIb} \beta_3$  (Fig. 2). PMA addition increased the adhesion of the transfected CHO cells, whereas cytochalasin D treatment reduced it (Fig. 2). The addition of PMA failed to overcome the inhibitory effect of cytochalasin D.

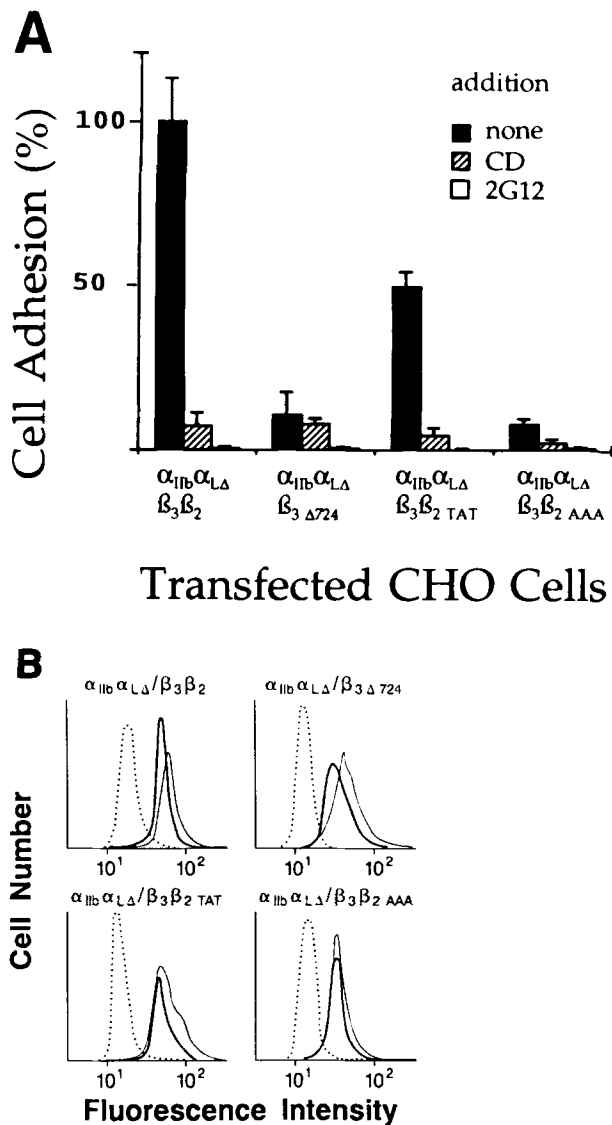
To examine the affinity state of the chimera, we used the binding of mAb PAC-1, a mAb specific for the high affinity

state of  $\alpha_{IIb} \beta_3$  (9, 18, 21). Specific binding of PAC-1 can be inhibited by the ligand mimetic peptide GRGDSP. There was no specific, GRGDSP inhibitable PAC-1 binding to the chimera in the presence or absence of PMA or cytochalasin D. Thus, the chimeric receptor was in a low affinity state that was unchanged by the drugs used to modulate adhesiveness. Nevertheless, the chimeric receptor was functional, since addition of the anti LIBS6 antibody resulted in PAC-1 binding that was specifically inhibited by GRGDSP (Fig. 3). Thus, incubation of the chimera transfected cells with PMA or cytochalasin D changes adhesiveness without changing the affinity state of the chimeric receptor.

**Regulation of Adhesiveness of the Chimeric Receptor by Post-receptor Events.** The foregoing experiments suggest that post-receptor events are responsible for the changes in adhesiveness provoked by cytochalasin D and PMA. Nevertheless, since ligands may activate integrins (22), it is possible that the different treatments influenced the capacity of the chimera to enter the high affinity state after interaction with fibrinogen. To exclude this possibility, we constructed receptors permanently locked in a high affinity state. A complete truncation of the cytoplasmic domain of  $\alpha_{IIb}$  ( $\Delta 991$ ) induces a high affinity state (9), whereas a shorter truncation of  $\alpha_{IIb}$  ( $\Delta 996$ ) results in a low affinity state (10). The difference between these truncations is the retention of a sequence highly conserved in all known integrin  $\alpha$  subunits. We therefore pro-



**Figure 4.**  $\beta$  subunit truncation and mutations in the TTT region of  $\beta_2$  do not change high affinity state of the  $\alpha_{IIb} \alpha_{LD}$  chimeric receptors. (A) (Top) Flow cytometry with  $\alpha_{IIb} \alpha_{LD} / \beta_3 \beta_2$ ,  $\alpha_{IIb} \alpha_{LD} / \beta_3 \Delta 724$ ,  $\alpha_{IIb} \alpha_{LD} / \beta_3 \beta_2$  TAT, and  $\alpha_{IIb} \alpha_{LD} / \beta_3 \beta_2$  AAA transfected CHO cells shows similar surface expression levels by staining with mAb D57 (anti- $\alpha_{IIb}$ ; solid line), mAb 2G12 (anticomplex  $\alpha_{IIb} \beta_3$ ; data not shown), and mAb 15 (anti- $\beta_3$ ; data not shown) compared with a nonspecific mAb binding (dotted line). The selected cell lines have small percentages of nonexpressing cells. (Bottom) PAC-1 staining as described in Materials and Methods is compared in the presence (dotted line) or absence (solid line) of 2 mM GRGDSP. This peptide inhibits specific binding of mAb PAC-1 and fibrinogen to the extracellular parts of  $\alpha_{IIb} / \beta_3$ . The GRGDSP inhibitable binding of PAC-1 demonstrates a high affinity state in  $\alpha_{IIb} \alpha_{LD} / \beta_3 \beta_2$ ,  $\alpha_{IIb} \alpha_{LD} / \beta_3 \Delta 724$ ,  $\alpha_{IIb} \alpha_{LD} / \beta_3 \beta_2$  AAA, and  $\alpha_{IIb} \alpha_{LD} / \beta_3 \beta_2$  TAT transfected CHO cells. As internal control, gating of the nonexpressing cells did not show GRGDSP inhibitable PAC-1 binding (data not shown). No addition (solid line); GRGDSP addition (dotted line). (B) Binding of  $^{125}I$ -labeled fibrinogen to  $\alpha_{IIb} \alpha_{LD} / \beta_3 \beta_2$ ,  $\alpha_{IIb} \alpha_{LD} / \beta_3 \Delta 724$ ,  $\alpha_{IIb} \alpha_{LD} / \beta_3 \beta_2$  AAA, and  $\alpha_{IIb} \alpha_{LD} / \beta_3 \beta_2$  TAT transfected CHO cells reveals a spontaneous binding of fibrinogen that is inhibitable by GRGDSP and the blocking mAb 2G12 (data not shown), indicating a high affinity state of the chimeric receptors. Results are the mean  $\pm$  SD of three determinations.



**Figure 5.**  $\beta$  subunit truncations/mutations and cytochalasin D decrease the adhesiveness without changing the receptor affinity. (A) Transfected CHO cells were allowed to adhere for 20 min at room temperature onto plastic microtiter wells that were coated with 20  $\mu\text{g}/\text{ml}$  fibrinogen. Cells were preincubated with 10  $\mu\text{M}$  cytochalasin D, 10  $\mu\text{g}/\text{ml}$  mAb 2G12, or no addition for 30 min at 37°C. Adhesion was quantified as described in Materials and Methods. Percent adherence was determined and results expressed as mean  $\pm$  SD of three determinations. (B) Incubation with 10  $\mu\text{M}$  cytochalasin D did not change the high affinity state of  $\alpha_{11b}\alpha_{L\Delta}/\beta_3\beta_2$ ,  $\alpha_{11b}\alpha_{L\Delta}/\beta_3\Delta_{724}$ ,  $\alpha_{11b}\alpha_{L\Delta}/\beta_3\beta_2\text{TAT}$ , and  $\alpha_{11b}\alpha_{L\Delta}/\beta_3\beta_2\text{AAA}$ . Flow cytometry with mAb PAC-1 demonstrates GRGDSP inhibitable binding of PAC-1 with or without cytochalasin D. Staining procedures are described in Materials and Methods. (Thick line) No addition; (thin line) cytochalasin D; (dotted line) cytochalasin D and GRGDSP.

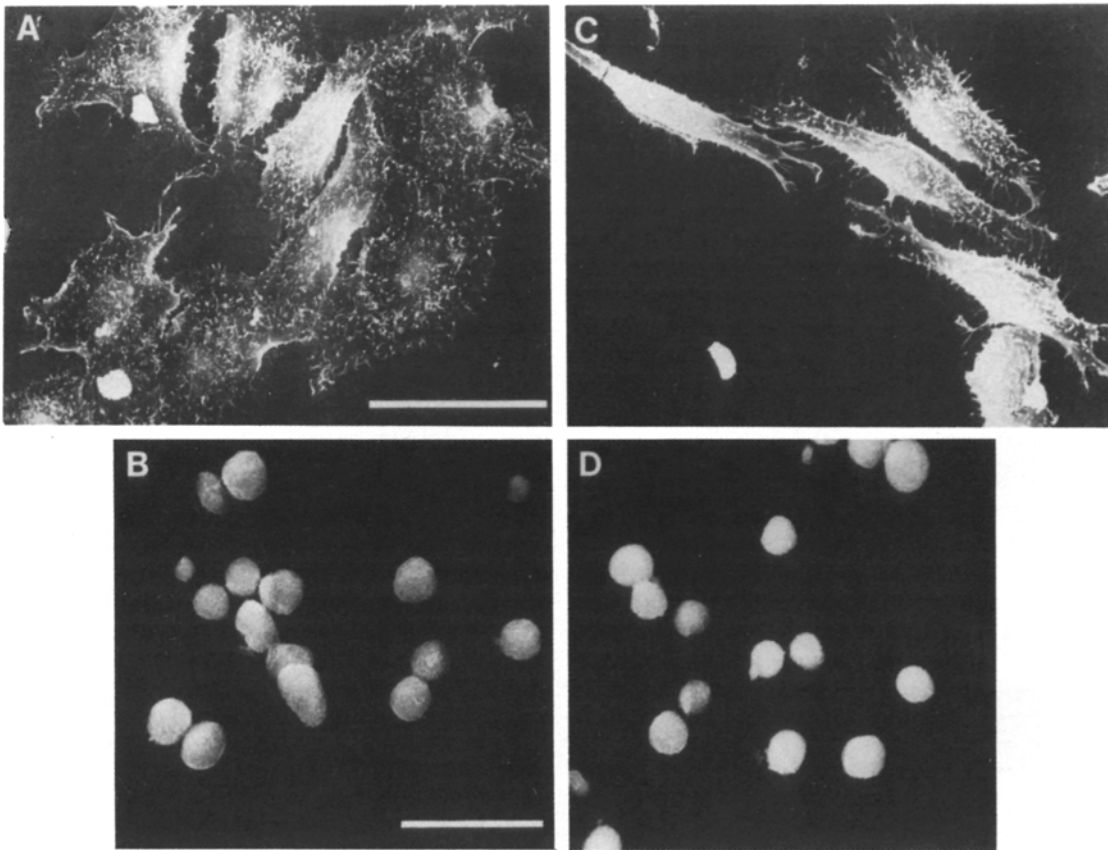
duced a mutation in the  $\alpha_L$  tail by deleting the VGFFK sequence. This mutant,  $\alpha_{11b}\alpha_{L\Delta}$ , when cotransfected with the  $\beta_3\beta_2$  chimera, spontaneously bound the mAb PAC-1 and  $^{125}\text{I}$ -labeled fibrinogen, indicating a high affinity state (Fig. 4). Incubation of  $\alpha_{L\Delta}$  transfected cells with metabolic inhibitors such as  $\text{NaN}_3$  and 2-deoxyglucose do not change the high affinity state (10). Moreover, neither a  $\beta$  subunit trun-

cation ( $\beta_3\Delta_{724}$ ) nor alanine substitutions ( $\beta_2\text{AAA}$ ,  $\beta_2\text{TAT}$ ) diminished the capacity of the chimeric receptor to spontaneously bind mAb PAC-1 or  $^{125}\text{I}$ -labeled fibrinogen (Fig. 4). Thus, the alterations in the  $\beta$  cytoplasmic domain did not influence the high affinity state of the chimeric receptor, and effects of these alterations on adhesiveness could be studied in a receptor fixed in the high affinity state.

When we analyzed the adhesion of these high affinity chimeras to fibrinogen, we found that cytochalasin D completely inhibited the adhesion of the transfected cells to fibrinogen (Fig. 5A). Nevertheless, cytochalasin D did not alter the affinity state (Fig. 5B) as assessed by mAb PAC-1 binding. Truncation mutants and the alanine substitutions are known to profoundly reduce the adhesiveness of  $\alpha_L\beta_2$  (7, 8) and these mutations had similar effects on the adhesiveness of the chimera. Both  $\beta_3\Delta_{724}$  and  $\beta_2\text{AAA}$  virtually abrogated adhesion (Fig. 5A) with no effect on the affinity state (Fig. 4 and Fig. 5B). The alanine point substitution  $\beta_2\text{TAT}$  reduced adhesion by  $\sim 50\%$ , again with no influence on the affinity state (Fig. 5A and B). Thus, cytochalasin D and the cytoplasmic domain mutations in  $\beta_2$  profoundly reduced adhesiveness without influencing the affinity state.

**Effect of  $\beta_2$  Cytoplasmic Domain Mutations on Cell Spreading and Organization of Focal Adhesions.** Postreceptor events that follow the initial integrin-mediated cell contact include the reorganization of the cytoskeleton as manifested by the formation of stress fibers, the localization of integrins at focal adhesions and integrin-mediated cell spreading (12–17, 24, 34–36). The foregoing experiments showed that cytoplasmic domain mutations in the  $\beta_2$  cytoplasmic domain could influence cell adhesion to fibrinogen without changing the affinity state of the integrin. To ascertain that postreceptor events are involved, we assessed the effect of these mutations on cell shape and cytoskeletal assembly. The cells bearing the wild-type  $\beta_2$  chimera combined with the wild-type  $\alpha_L$  or  $\alpha_{L\Delta}$  spread extensively 2 h after plating on fibrinogen-coated coverslips (shown for  $\alpha_{L\Delta}$  in Fig. 6A). In sharp contrast, both the  $\beta_3\Delta_{724}$  and the  $\beta_2\text{AAA}$  showed a complete lack of cell spreading (Fig. 6, B and D). The point mutation of the TTT to TAT resulted in impairment of spreading. Here, most of the cells manifested a spindle shape and failed to spread fully (Fig. 6C). To further document and quantify this difference between the  $\beta_2\text{TAT}$  mutant and the wild-type  $\beta_2$  cytoplasmic domain, we enumerated the shapes of cells bearing each chimera. 64% of cells bearing the wild-type  $\beta_2$  cytoplasmic domain were fully spread, 4% were bipolar, and 32% were rounded ( $n = 566$ ). In contrast, 3% of the cells bearing  $\beta_2\text{TAT}$  were well spread, the majority (55%) manifested bipolar shape, and 41% of cells were rounded ( $n = 385$ ). Thus, the effects of the  $\beta_2$  cytoplasmic domain mutations on adhesiveness correlate with their effects on cell spreading.

As postreceptor events, integrins also initiate the formation of stress fibers and focal adhesions. To assess these parameters, we examined the actin cytoskeleton by rhodamine phalloidin staining and the localization of the chimeric integrin to focal adhesions by staining with an anti- $\alpha_{11b}$  mAb (24). The wild-type  $\beta_2$  cytoplasmic domain, combined



**Figure 6.**  $\beta$  subunit truncation and mutations in the TTT ( $\beta_2$  758–760) region impair spreading ability. Scanning electron microscopy pictures were generated as described in Materials and Methods after cells were allowed to adhere on fibrinogen (20  $\mu\text{g}/\text{ml}$ ) coated glass coverslips. After an incubation period of 2 h at 37°C, CHO cells transfected with  $\alpha_{11b} \alpha_{L\Delta}/\beta_3 \beta_2$  developed a well spread and flattened cell shape (A).  $\alpha_{11b} \alpha_{L\Delta}/\beta_3 \beta_2$  TAT transfected CHO cells showed an impaired spreading and a spindle or triangular shape (C).  $\alpha_{11b} \alpha_{L\Delta}/\beta_3 \Delta 724$  (B) and  $\alpha_{11b} \alpha_{L\Delta}/\beta_3 \beta_2$  AAA (D) transfected CHO cells did not spread at all but remained in a rounded shape. Bars, 30  $\mu\text{m}$ .

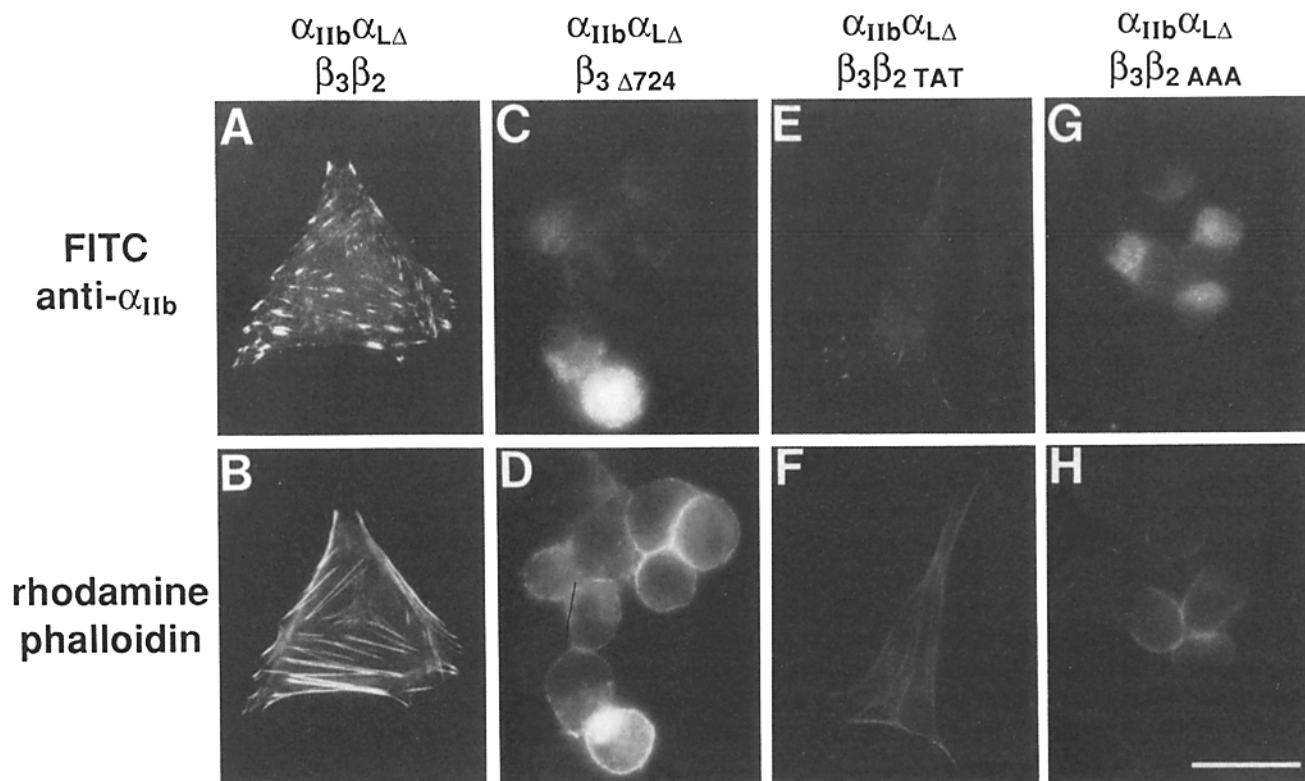
either with the wild-type  $\alpha_L$  or the deleted  $\alpha_{L\Delta}$ , initiated the formation of actin-containing stress fibers and integrin containing focal adhesions (shown for  $\alpha_{L\Delta}$  in Fig. 7, A and B). In sharp contrast, the two mutations that most profoundly affected adhesiveness blocked the capacity to form both stress fibers and focal adhesions (Fig. 7, C, D, G, and H). Moreover, the  $\beta_2$  TAT mutation resulted in a partial impairment of focal adhesion and stress fiber formation (Fig. 7, E and F). Thus, both the changes in cell shape and integrin-mediated cytoskeletal reorganization are impaired by the  $\beta_2$  cytoplasmic domain mutations that reduce adhesiveness. These postreceptor events are impaired even when the receptor is fixed in the high affinity state.

## Discussion

The major findings of this study are: (a) using chimeric  $\alpha_{11b} \alpha_L/\beta_3 \beta_2$  integrins, the cytoplasmic domains of  $\alpha_L \beta_2$  are shown to modulate adhesiveness by postreceptor events independent of integrin affinity changes. (b) The integrin/cytoskeleton interaction, the cytoskeleton organization, and cell spreading are involved in the modulation of adhesiveness mediated by the cytoplasmic domains of  $\alpha_L \beta_2$ . (c)

$\alpha_L \beta_2$  cytoplasmic domains are able to localize integrins at focal adhesions and to organize the actin cytoskeleton to stress fibers. (d) The  $\beta_2$  cytoplasmic domain, and within it the TTT region (758–760), is required for focal adhesion formation, cytoskeletal organization, cell spreading, and consequently for the modulation of adhesiveness. (e) A deletion variant (VGFFK) of the  $\alpha_L$  subunit locks the chimeric integrin in a high affinity state without influencing the described postreceptor events. Since in all known integrin  $\alpha$  subunits this region is highly conserved, similar deletions in other integrins may provide a general tool to evaluate the role of postreceptor events in cell adhesion and migration.

The term adhesiveness describes the ability of an adhesion receptor to mediate cell adhesion. Theoretically, adhesiveness can be regulated by two mechanisms: either by an affinity change of the receptor for its ligand or by postreceptor events. Since the  $\alpha_L \beta_2$  counterreceptors ICAM-1, ICAM-2, and ICAM-3 are integral membrane proteins (1–4) they are not readily available for classical ligand binding studies. Furthermore, the isolation and solubilization of integral membrane proteins has the inherent problem of a potential alteration of their function as ligands. Therefore, using a genetic approach, we engineered chimeric receptors which contain the



**Figure 7.**  $\beta$  subunit truncation and mutations in the TTT ( $\beta$ 2 758–760) region impair focal adhesions formation and cytoskeletal organization. Immunofluorescence pictures were generated as described in Materials and Methods after cells were allowed to adhere on fibrinogen (20  $\mu$ g/ml) coated glass coverslips for 2 h at 37°C. Staining  $\alpha_{Ib} \alpha_{L\Delta}/\beta_3 \beta_2$  transfected CHO cells with mAb PL98DF6 (anti- $\alpha_{Ib}$ ) revealed localization of the transfected chimera in focal adhesion typical structures (A). Staining with rhodamine phalloidin in the same cell demonstrates an extensive cytoskeletal organization in form of stress fibers (B). Beginning and ending of the stress fibers colocalize with the focal adhesion typical stainings of the chimeric receptor.  $\alpha_{Ib} \alpha_{L\Delta}/\beta_3 \beta_2$  TAT transfected CHO cells reveal sparsely distributed and discrete focal contacts (E). Stress fiber formation in these transfectants is decreased and very discrete (F).  $\alpha_{Ib} \alpha_{L\Delta}/\beta_3 \Delta_{724}$  and  $\alpha_{Ib} \alpha_{L\Delta}/\beta_3 \beta_2$  AAA transfected CHO cells are not able to localize the chimeric receptors to focal adhesions and are not able to organize the cytoskeleton to stress fibers (D and H). Bar, 20  $\mu$ m.

extracellular parts of  $\alpha_{Ib} \beta_3$  as an affinity reporter and the cytoplasmic domains of  $\alpha_L \beta_2$  as the region responsible for the proposed postreceptor events. To remove postoccupancy alterations in affinity as a potential confounding factor in our analyses of changes in adhesiveness, we deleted a VGFFK region in the  $\alpha$  subunit and thus, generated a chimeric receptor fixed in a high affinity state. Neither cotransfection with truncation nor pointmutation variants of the  $\beta$  subunit nor treatment with the metabolic inhibitors  $\text{NaN}_3$  or 2-deoxyglucose (10) changed the high affinity state of the deleted integrin. These high affinity mutants allowed us to dissect the role of the postreceptor events in the modulation of cell adhesion, independent of the affinity state of the integrin.

The formation of focal adhesions in cells reflects the clustering of integrins as transmembrane junctions between the extracellular matrix and the cytoskeleton (34), and thus can be considered a postreceptor event. Localization of  $\beta_1$  and  $\beta_3$  integrins to focal adhesions has been repeatedly demonstrated (14, 24, 35, 36). In contrast, to our knowledge this is the first demonstration that the cytoplasmic domains of a  $\beta_2$  integrin can localize the adhesion receptor to

focal adhesions. This implies an association of the actin cytoskeleton with the cytoplasmic domains of  $\alpha_L \beta_2$ . Furthermore, we demonstrate that the extent of localization of a chimeric integrin receptor to focal adhesions correlates with both the spreading ability and the cell adhesion strength. Thus, suggesting that postreceptor events participate in the modulation of adhesiveness of  $\alpha_L \beta_2$ .

We demonstrate that the organization of the cytoskeleton and cell spreading are modulated by the cytoplasmic domains of  $\alpha_L \beta_2$ . PMA and cytochalasin D are known to influence the cytoskeletal organization. Cytochalasin D is a potent inhibitor of actin polymerization (37). PMA induces assembly and reorganization of the actin cytoskeleton (38, 39), and capping or clustering of  $\alpha_L \beta_2$  and colocalized condensation of cytoskeletal elements in leukocytes (40–42). Our experimental finding that cytochalasin D and PMA modulate cell adhesion, independent of the affinity state of the receptor, support the hypothesis that organization of the actin cytoskeleton is an important mechanism for modulating the adhesiveness of  $\alpha_L \beta_2$ . Furthermore, inactivation of the GTP binding protein rho, which regulates the assembly of focal



adhesions and actin stress fibers (43), has been shown to inhibit PMA-induced, LFA-1-dependent lymphocyte aggregation (44). After TCR cross-linking, a direct association of  $\alpha_L \beta_2$  and f-actin could be demonstrated (45). Activation of neutrophils also induces an interaction between  $\beta_2$  and  $\alpha$ -actinin (46). Modulation of adhesiveness independent of affinity changes has also been demonstrated for PMA stimulation of the integrin  $\alpha_5 \beta_1$  (47, 48). Thus, there are several lines of evidence for the participation of the integrin/cytoskeleton interaction and cytoskeletal organization in the regulation of adhesiveness of  $\alpha_L \beta_2$ .

The integrin/cytoskeleton association could modulate cell adhesion by coupling the adhesive forces of the individual integrin/ligand pairs. To break adhesion, all the cytoskeleton-coupled integrin receptors would have to be separated from their ligands simultaneously. This would require more physical force than disengagement of individual integrin/ligand bonds. In our transfected cell lines, the degree of colocalization of the integrins at the ends of the actin stress fibers correlated with the strength of cell adhesion. In the case of  $\alpha_5 \beta_1$ , quantitative measurements of cell adhesion have demonstrated the requirement of cytoskeletal involvement for strong adhesion (49). The reassembly of the actin cytoskeleton after leukocyte stimulation has been demonstrated (50), and by using direct adhesive energy measurements, Tözeren et al. (51) concluded that cytoskeletal structures, anchored to  $\alpha_L \beta_2$ , may contribute to the strength of adhesion of activated T lymphocytes.

Experimental data with other types of adhesion molecules such as cadherins, selectins, and CD44 support the importance of interactions between cytoskeletal proteins and the cytoplasmic domains of adhesion molecules for the modulation of adhesion. For N- and E-cadherin, the direct participation of the cytoplasmic domain in cell adhesion and interaction with the cytoskeleton could be demonstrated (52–54). A truncation of the cytoplasmic domain of L-selectin abolishes leukocyte adhesion and rolling without changing the lectin activity of L-selectin (55). Treatment of cells bearing the wild-type L-selectin with cytochalasin B abolished adhesion as well, without affecting the carbohydrate binding (55). The adhesion molecule CD44 is dependent on its cytoplasmic domain for strong cell adhesion to immobilized hyaluronic acid (56) and for normal cell migration (57).

Hibbs et al. (7, 8) demonstrated that the cytoplasmic domain of  $\beta_2$  (and especially a region of three contiguous threonines at  $\beta_2$  758–760) is important for the regulation of adhesiveness of  $\alpha_L \beta_2$ . In our experiments we found a correlation between the extent of mutation at the identical region (TTT to AAA and to TAT) and the degree of impairment in the integrin/cytoskeleton interaction. The corresponding region in  $\beta_1$  and  $\beta_3$  integrins may have a similar functional importance. Mutants lacking the  $\beta_1$  cytoplasmic domain, or an alternative splice variant, which both exclude the motif VTT ( $\beta_1$  792–794), lose the ability to localize to focal adhesions (15, 16). Recently, a mutation in the cytoplasmic domain of the  $\beta_3$  chain from TST ( $\beta_3$  751–753) to TPT was identified in a variant of Glanzmann thrombasthenia (11),

and additionally was shown to impair interaction of  $\alpha_{11b} \beta_3$  with the cytoskeleton (58). Thus, the TTT region in  $\beta_2$  and the corresponding regions in other integrin  $\beta$  subunits seem to be important for the interaction between the cytoplasmic domain and the cytoskeleton.

Besides the described postreceptor events, affinity changes of the integrin may be responsible for the regulation of adhesiveness of  $\alpha_L \beta_2$ . Two experimental findings argue for the existence of different affinity states of  $\alpha_L \beta_2$ . First, at least two mAbs (mAb24 and NKI-L16) are proposed to bind with different affinities to  $\alpha_L \beta_2$ , dependent on cell maturation, cell activation, and divalent cations (59–62). Second, a competitive binding assay with soluble ICAM-1 proposed an affinity change of a fraction of the  $\alpha_L \beta_2$  integrins (63). Thus, taken together with our experimental findings, modulation of  $\alpha_L \beta_2$  mediated adhesion may involve both, changes in affinity and in the integrin/cytoskeleton interaction, either simultaneously or sequentially. Figdor et al. (60) proposed a model of three activation states of  $\alpha_L \beta_2$ : (a) an inactive state with no exposure of the NKI-L16 epitope; (b) an intermediate activation state with exposure of the NKI-L16 epitope; and (c) an active state defined by cell adhesion or aggregation after TCR cross-linking or PMA stimulation. This model is based on the observation that the expression of the NKI-L16 epitope is a prerequisite, but is not sufficient for  $\alpha_L \beta_2$  mediated cell aggregation (61, 62). This model is particularly attractive, since resting lymphocytes express no or only partially the NKI-L16 epitope and mature lymphocytes express the NKI-L16 epitope. The transition from state 2 to state 3 in Figdor's model can be explained by modulation of the integrin/cytoskeleton interaction. This would imply the acquisition of a high  $\alpha_L \beta_2$  affinity during maturation of lymphocytes and a fast change in adhesiveness during lymphocyte activation due to changes in the cytoskeletal organization and the integrin/cytoskeleton association.

The integrin chimera with the full-length cytoplasmic domain of  $\alpha_L$  and the chimera with the VGFFK-deleted cytoplasmic domain of  $\alpha_L$  did not differ in their ability to mediate cell spreading, to initiate focal adhesions, and to organize the cytoskeleton to stress fibers. This implies that the GFFKR region, which is conserved in all known integrin  $\alpha$  subunits, is essential for the regulation of integrin affinity but that this region is not required to mediate integrin/cytoskeleton interaction, as far as examined by us. Thus, we describe a method to lock an integrin in a high affinity state without changing the investigated interactions with the cytoskeleton. This method may be of general use for all integrins and thus may allow to examine the role of postreceptor events in cell adhesion and migration independent of affinity changes.

In conclusion, our data demonstrate that  $\alpha_L \beta_2$  cytoplasmic domains can modulate integrin adhesiveness independent of affinity changes of the integrin. Integrin/cytoskeleton association, the cytoskeletal organization, and cell spreading are directly modulated by  $\alpha_L \beta_2$  cytoplasmic domains. The  $\beta_2$  subunit cytoplasmic domain, and within it the TTT region, are required for the integrin/cytoskeleton interaction and for efficient cell adhesion. These findings may direct experimental

and therapeutic efforts to influence  $\alpha_L \beta_2$  mediated firm adhesion of leukocytes to endothelium with drugs influencing

cytoskeletal organization or the integrin/cytoskeleton interaction of  $\alpha_L \beta_2$ .

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Address correspondence to Dr. Karlheinz Peter, Innere Medizin III, Universität Heidelberg, Bergheimerstr. 58, 69115 Heidelberg, Germany.

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