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Expression of β1B Integrin Isoform in CHO Cells Results in A Dominant Negative Effect on Cell Adhesion and Motility

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Abstract. The integrin subunit β1B, a β1 isoform with a unique sequence at the cytoplasmic domain, forms heterodimers with integrin α chains and binds fibronectin, but it does not localize to focal adhesion sites (Balzac, F., A. Belkin, V. Koteliansky, Y. Balabanow, F. Altruda, L. Silengo, and G. Tarone. 1993. *J. Cell Biol.* 121:171–178). Here we analyze the functional properties of human β1B by expressing it in hamster CHO cells. When stimulated by specific antibodies, β1B does not trigger tyrosine phosphorylation of a 125-kD cytosolic protein, an intracellular signaling pathway that is activated both by the endogenous hamster or the transfected human β1A. Moreover, expression of β1B results in reduced spreading on fibronectin and laminin, but not on vitronectin. Expression of β1B also results in severe reduction of cell motility in the Boyden chamber assay. Reduced cell spreading and motility could not be accounted for by preferential association of β1B with a given integrin α subunit. These data, together with our previous results, indicate that β1B interferes with β1A function when expressed in CHO cells resulting in a dominant negative effect on cell adhesion and migration.

CELL adhesion and motility requires the coordinated interaction of the fibrous network of extracellular matrix proteins and the intracellular cytoskeleton (Wang et al., 1993) bridged by plasma membrane receptors of the integrin family (Hynes, 1992). Interfering with any of these three elements results in disruption of adhesive cell–matrix interactions. This can be achieved by digestion of extracellular matrix proteins with proteolytic enzymes, by disassembling the actin skeleton with specific drugs or by blocking integrins with specific antibodies. The transmembrane linkage between the extracellular matrix proteins and the cytoskeleton occurs at focal sites on the plasma membrane known as focal adhesions (Burridge et al., 1988; Geiger and Ginsberg, 1991). Immunofluorescence analysis shows that extracellular matrix proteins, integrins, and cytoskeletal proteins, specifically colocalize at focal adhesions (Burridge et al., 1988; Geiger and Ginsberg, 1991; Luna and Hitt, 1992). The molecular interactions between these components, however, is not fully understood. Integrins are heterodimers of β and α subunits consisting of a large extracellular domain, a transmembrane domain, and a short cytoplasmic tail (Hynes, 1992). The extracellular domain of the integrin heterodimers bind to matrix proteins in a cation-dependent manner and in many cases recognize the tripeptide sequence Arg-Gly-Asp (Ruoslahti, 1991). The cytoplasmic tails interact with cytoskeletal elements. Experimental evidence for the interaction of the cytoplasmic domain of β1 integrin with talin and with α-actinin has been reported (Horwitz et al., 1986; Otey et al., 1990). Other molecules may be important in mediating integrin–actin interaction, these include tensin, paxillin, and zyxin (Turner and Burridge, 1991). Moreover, molecules with enzymatic or regulatory functions are localized in focal adhesions and may have a role in integrin-mediated signal transduction; these proteins include specific isoforms of protein kinase C (Jaken et al., 1989), $pp60^{src}$ (Rohrschneider, 1980), as well as p125FAK (Schaller et al., 1992). The latter is a cytosolic tyrosine kinase that is specifically activated when integrins are occupied by their ligands or are bound by specific antibodies (Burridge et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992; Defilippi et al., 1994). The cytoplasmic domain of β1 plays a critical role in the association of integrin with the focal adhesion structure (Reszka et al., 1992) and in the activation of the p125FAK (Guan et al., 1991).

In this study we analyzed the functional properties of β1B, a cytoplasmic domain variant of β1A, where the last 21 COOH-terminal amino acids are replaced by a new sequence of 12 amino acids (Altruda et al., 1990). This form behaves similarly to β1A in terms of fibronectin binding but has a re-

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stricted tissue distribution and does not localize at focal adhesion sites (Balzac et al., 1993). In this study, we show that β1B does not trigger the tyrosine phosphorylation of intracellular proteins, and expression of this molecule in Chinese hamster ovary (CHO)[1] cells results in a dominant negative action on cell adhesion and motility.

Materials and Methods

Constructs and Transfections

Stable transfectants of CHO cells expressing the human integrin β1A or β1B were obtained as described previously (Balzac et al., 1993). Briefly, a 3.5-kb EcoRI fragment of the β1B containing the entire coding sequence was inserted into the EcoRI-cloning site of the SV40-based expression vector pECE (Ellis et al., 1986). The full-length cDNA for the human β1A cloned in the pECE vector was a kind gift of Filippo Giancotti (Giancotti and Ruoslahti, 1990). CHO cells were cotransfected with 20 μg of the plasmid containing β1 cDNA and 2 μg of pSV2-neo (Southern and Berg, 1982), and neomycin resistant clones were selected in HAM's F12 medium with 10% FCS and 800 μg/ml of G418 (GIBCO BRL, Gaithersburg, MD).

Flow Cytometry Analysis

Transfected cells were detached from culture plates by incubation in 5 mM EDTA in PBS (10 mM phosphate buffer pH 7.3, 150 mM sodium chloride) and washed twice at 4°C in PBS with 0.1 mM EDTA and 1 mg/ml of BSA. The cells were then incubated for 1 h at 4°C in the same buffer with saturating concentrations of the monoclonal antibody LM534 to the human β1 integrin (see below). After washing, the cells were incubated 45 min with fluorescein-labeled affinity-purified secondary antibodies (Sigma Chem. Co., St. Louis, MO), and analyzed on the flow cytometer Facs-Star Becton Dickinson (Oxford, UK), equipped with 5 W argon laser at 488 nm. Five thousand cells per sample were analyzed.

Antibodies and Immunoprecipitation of Integrins

The following mAbs reacting with extracellular epitopes common to the two isoforms of human β1 were used: mAb TS2/16 (a gift from Sanchez-Madrid, Hospital de la Princesa, Madrid, Spain); mAb G12 (a gift from Luciano Zardi, Istituto Scientifico Tumori, Genova, Italy) and mAb LM534 (a gift from Filippo Giancotti, Department of Pathology, New York University, NY). None of the above antibodies reacts with hamster β1. mAb 7E2 reacting with hamster integrin β1, but not with the human molecule, was a generous gift of Rudy Juliano (University of North Carolina, Chapel Hill, NC). Antibodies to cytoplasmic sequences of α3, α5, and αV integrin subunits were prepared in our laboratory and previously characterized (Defilippi et al., 1991).

Integrins were immunoprecipitated from cells labeled with ^{125}I. Labeling of membrane proteins with ^{125}I was performed as described previously (Rossino et al., 1990). Briefly, cells were released from culture dishes by 5 mM EDTA treatment in PBS and washed three times by centrifugation. Cells were suspended in PBS containing $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) and labeled with 1 mCi of ^{125}I in the presence of lactoperoxidase (200 μg/ml) and H_2O_2 (0.002%). For immunoprecipitation, labeled cells were extracted for 20 min at 4°C with 0.5% Triton X-100 (BDH Chemicals, Poole, Dorset, UK) in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml aprotinin (all from Sigma Chem. Co.). After centrifugation at 12,000 *g* for 10 min, extracts were incubated with the specific antibodies for 1 h at 4°C with gentle agitation. Soluble immunocomplexes were bound to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) and recovered by centrifugation. After washing, bound material was eluted by boiling beads in 1% SDS (Pierce, Rockford, IL) and analyzed by (6%) SDS-PAGE in the absence of reducing agents. The radioactive proteins were visualized by fluorography with sodium salicilate (Chamberlain, 1979). The amount of antibody necessary to quantitatively precipitate all labeled molecules present in the cell extract was determined in preliminary experiments. The endogenous hamster β1 was immunoprecipitated with mAb 7E2 while mAb LM534 was used for the transfected human β1. The ^{125}I radioactivity present in the immunoprecipitated β1 was determined by cutting the corresponding electrophoretic band and counting in a gamma counter.

Western Blotting

For Western blotting integrin complexes were immunoprecipitated from unlabeled cell extracts with α-specific antibodies. Immunoprecipitated material was separated by SDS-PAGE and transferred to nitrocellulose using a semi-dry apparatus (Novablot, Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The blots were incubated 1 h at 42°C in 5% BSA, washed with TBS (150 mM NaCl, 20 mM Tris-Cl, pH 7.4), and incubated overnight in 10 μg/ml β1 mAbs in TBS, 1% BSA. mAb G12 that specifically reacts in Western blotting with human β1 and mAb 7E2 reacting with hamster β1 were used. The blots were washed three times with TBS, incubated 2 h with peroxidase-conjugated anti-mouse Ig (Sigma Chem. Co.) and washed two times. Bound antibodies were visualized by the chemiluminescent detection method ECL (Amersham, UK). In the experiment shown in Fig. 7, the monoclonal antibodies were directly labeled with ^{125}I by the Cloramine T method. The nitrocellulose filters were first incubated with mAb G12 to visualize the human β1. After stripping with 2% SDS at 42°C for 1 h to remove bound antibodies, the filter was reincubated with mAb 7E2 to visualize the hamster β1. Quantitation of the band intensity was performed by densitometry using a scanner connected to a personal computer (Biomed Instrs., Fullerton, CA).

Adhesion Experiments

Tissue culture microtiter plates were coated by overnight incubation at 4°C with the indicated concentration of purified matrix proteins and post-coated with BSA (Sigma Chem. Co.) for 1 h at 37°C. Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose according to Engvall and Ruoslahti (1977). Vitronectin was purified according to Yatohgo et al. (1988), mouse laminin from EHS tumor was obtained from GIBCO BRL (St. Louis, MO).

Cells at confluence were detached by EDTA treatment in PBS for 10 min, washed twice in serum free DMEM, and plated on the coated tissue culture plates for the indicated times. To eliminate the contribution of protein synthesis and secretion, cells were pretreated 2 h with 20 μM cycloheximide (Sigma Chem. Co.) to prevent protein synthesis, and plated in the presence of 20 μM cycloheximide and 1 μM monensin (Sigma Chem. Co.) to prevent secretion (Defilippi et al., 1992). Plates were rinsed twice with PBS to remove unbound cells, and adherent cells were fixed with paraformaldehyde and stained with Coomassie blue. Cell adhesion was evaluated by reading the absorbance at 540 nm in a microtitre ELISA reader. The correspondence between the optical density and number of cells attached was confirmed in experiments showing that increasing the cell concentration in the original suspension resulted in proportional increase of the optical density.

To measure cell spreading, stained cells were photographed by choosing five random microscopic fields for each sample. The area of 150 cells per sample was determined using a computerized image analysis system (Biomed Instrs.).

Boyden Chamber Migration Assay

Cell migration assay was carried out in Boyden chambers as previously described (Albini et al., 1987). Briefly, CHO cells and transfected clones were resuspended in serum free RPMI medium in the presence of 0.1% of BSA. Cells were then added (2×10^5 cells/ml) to the upper compartment of the Boyden chamber and the lower compartment was filled with either RPMI medium containing 0.1% BSA as control, or with chemoattractants consisting of serum-free conditioned medium from 3T3 cells or of RPMI with 25 μg/ml fibronectin. The chemoattractants were used at concentrations exerting maximal activity as evaluated in preliminary experiments. The two compartments of the Boyden chamber were separated by a polycarbonate filter (8 μm pore size, Nucleopore, Concorezzo, Italy) coated with gelatin (5 μg/ml, Sigma Chem. Co.). Cells were allowed to migrate 6 h at 37°C in a humidified atmosphere containing 5% CO_2. Cells on the upper side of the filter were removed mechanically; cells on the lower surface of the filter were fixed in ethanol, stained with Toluidine blue, and 10 random fields were counted under a microscope at 160×. Each assay was carried out in triplicate and repeated at least three times. In preliminary experiments, the ability of the cells to adhere to the filters was evaluated by fixing and staining the upper side of the filters and observation under the microscope.

1. *Abbreviation used in this paper*: CHO, chinese hamster ovary.

Detection of Phosphotyrosine-containing Proteins

To analyze cell adhesion mediated by integrin monoclonal antibodies, tissue culture plates were first coated overnight at 4°C with 10 μg/ml goat anti-mouse IgG (Sigma Chem. Co.), postcoated with BSA for 1 h at 37°C and incubated with purified mAbs for 2 h at 37°C. The optimal concentration for each mAb was established by measuring cell adhesion.

Cells at confluence were pretreated 2 h with 20 μM cycloheximide (Sigma Chem. Co.) to prevent protein synthesis, detached by EDTA treatment (5 mM) in PBS for 10 min and washed twice in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$. Cells were then resuspended in prewarmed DMEM medium, in the presence of 20 μM cycloheximide and 1 μM monensin (Sigma Chem. Co.) to prevent synthesis and secretion of endogenous extracellular matrix proteins, and plated on tissue culture dishes coated with antibodies to either the endogenous (mAb 7E2) or the transfected (mAb TS2/16) β1 subunit. Cells plated on polylysine-coated tissue culture dishes were used as controls. Cells were spun at the bottom of the dishes by centrifugation at 1,000 RPM for 2 min and incubated for 5 min at 37°C. The cells were washed twice with a stop solution (5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4 in PBS), and detergent extracted in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-Cl, pH 8, 5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 U/ml aprotinin) (all from Sigma Chem. Co.).

Protein concentration was determined in each cell extract by the Bradford protein assay method (BioRad Labs., Hercules, CA, GmbH). Samples containing equal amounts of protein were subjected to 6% polyacrylamide electrophoresis in the presence of SDS (SDS-PAGE) in reducing conditions. Proteins were transferred to nitrocellulose and processed for Western blotting as described above. The mAb PT66 to phosphotyrosine (Sigma Chem. Co.) was used followed by a peroxidase-conjugate anti-mouse IgG (Sigma Chem. Co.).

Results

Expression of β1A and β1B in CHO Cells Transfectants

CHO cells were transfected with the human β1B cDNA under the control of the early SV40 promoter and stable transfectants were isolated. Clones transfected with the human β1A isoform were also selected as control. CHO cells express the endogenous hamster β1A form but do not express β1B as assessed by immunoprecipitation with antibodies directed to the peptide sequence unique to β1B (Balzac et al., 1993).

To determine the expression of β1, membrane proteins were labeled with lactoperoxidase catalyzed radio iodination, and the transfected human and the endogenous hamster β1 proteins were immunoprecipitated from each clone by means of species specific monoclonal antibodies and analyzed by SDS-PAGE (Fig. 1). To quantitate the level of expression, the β1 bands were cut out of the gel and the amount of radioactivity was determined in a gamma counter. One control clone expressing human β1A (β1A-22) and three independent clones expressing β1B (β1B-18, β1B-25, and β1B-50) were selected and analyzed. As shown in Table I, clones β1B-18, β1B-25, and β1B-50 expressed increasing amounts of human β1B; the level ranges from 18 to 50% of the total β1 expressed at the cell surface (endogenous plus transfected) (Table I). The numbers in the clone name indicate the level of surface expression of the transfected protein (see below and Table I). Clone β1B-50 was previously indicated as 18.2 (Balzac et al., 1993). In the control clone β1A-22, the transfected human β1A represents 22% of the total. The ratio of transfected/endogenous β1 in β1A-22 and β1B-25 clones is comparable (Table I), moreover, the amounts of total β1 at the cell surface (endogenous plus transfected) in these clones are comparable to that present on untransfected CHO.

The relative levels of β1 expression in the different clones

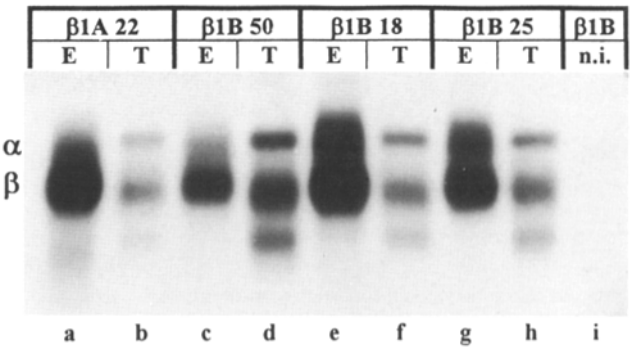

Figure 1. Immunoprecipitation of β1A and β1B in transfected CHO cells. Clones β1A-22 transfected with human β1A and clones β1B-18, β1B-25, and β1B-50 transfected with human β1B were surface radioiodinated and extracted with detergent. The endogenous (*E*) and transfected (*T*) β1 were quantitatively immunoprecipitated, respectively, with mAb 7E2 to hamster β1 and mAb LM534 to human β1. The latter antibody recognizes an extracellular epitope common to β1A and β1B. Lane *i* is material immunoprecipitated with non-immune (*n.i.*) mouse serum. After immunoprecipitation radioactive proteins were separated by SDS-PAGE and visualized by fluorography. α and β1 on the left indicate the positions of the two integrin subunits. A 100-kD band of unknown identity was also immunoprecipitated from the transfected clones with antibodies to the human β1.

were confirmed by flow cytometry analysis which also showed that all clones consist of homogeneous cell population (not shown).

β1B Integrin Does Not Trigger Tyrosine Phosphorylation of a 125-kD Protein

It has recently been shown that interaction of β1 integrin complexes with their ligands or with a specific antibody lead to activation of tyrosine phosphorylation of intracellular proteins (Burridge et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992; Defilippi et al., 1994). We thus tested whether transfected β1A and β1B were equally effective in this respect. To specifically trigger the response mediated by the transfected or by the endogenous β1 subunits, cells were plated on plastic dishes coated with monoclonal antibodies specific for either human or hamster β1. Tyrosine phosphorylation of cellular proteins was determined by Western blotting with phosphotyrosine antibodies. As shown in Fig. 2, in clone β1A-22 both the endogenous and the transfected β1A integrins were equally capable to stimulate tyrosine phosphorylation of a 125-kD cytoplasmic protein. On the

Table I. Surface Expression of β1A and β1B in Transfected Clones

Clones	β1 Endogenous	β1 Transfected	β1 Total
β1A-22	1670*	471 (22% of total)	2141
β1B-25	1570	542 (25% of total)	2112
β1B-18	2233	498 (18% of total)	2731
β1B-50	822	825 (50% of total)	1647

* The data are derived from an experiment shown in Fig. 1. The numbers indicate the counts per minute incorporated in the β1 bands. Values were obtained by cutting the corresponding bans on the SDS gel and counting in a gamma counter.

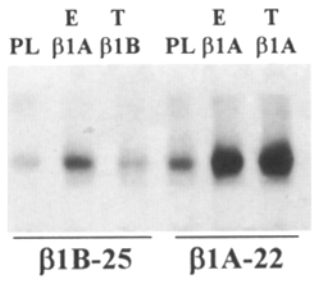

Figure 2. Tyrosine phosphorylation of p125 protein in β1A and β1B transfectants. β1A-22 and β1B-25 cells were plated on dishes coated with mAb 7E2 to the endogenous β1 (*E*) or with mAb TS2/16 to the transfected human β1 (*T*). Controls were obtained by plating cells on polylysine-coated plates (*PL*). After detergent extraction equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose. Proteins containing phosphotyrosine were visualized by means of a specific monoclonal antibody (PT66) and chemiluminescence reaction. The region of the gel in the 96–160-kD range of molecular mass is shown.

other hand, in clone β1B-25, the transfected β1B did not stimulate significantly the tyrosine phosphorylation of the 125-kD protein (Figs. 2 and 3). The endogenous β1A, however, was active (Figs. 2 and 3), showing that the signaling pathway was not altered in this clone. The ability of the transfected human β1A to stimulate tyrosine phosphorylation in clone β1A-22 indicates that the human protein is active in the hamster cells and thus the lack of function of β1B is related to the structural difference between the two integrin isoforms. The same phenomenon was observed in clone β1B-50 (Fig. 3). Immunoprecipitation experiments showed that the 125-kD protein is specifically recognized by monoclonal antibodies to p125FAK (Schaller et al., 1992), a cytosolic tyrosine kinase localized at focal adhesions (not shown).

Expression of β1B Results in Reduced Cell Spreading

To test the adhesive response of the transfectants, cells were plated on dishes coated with antibodies specific for the transfected human protein. This allows it to directly compare

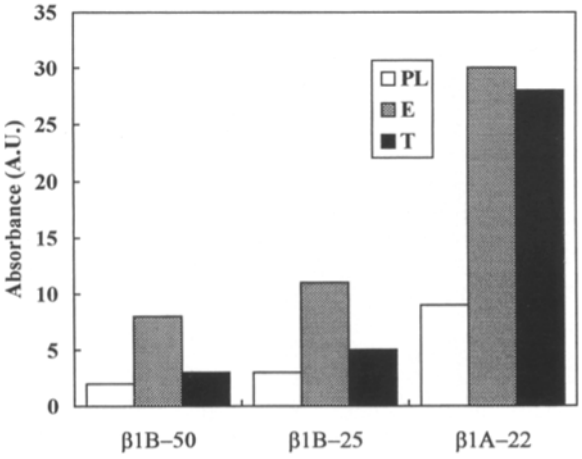

Figure 3. Densitometric analysis of the 125-kD tyrosine-phosphorylated proteins in β1B and β1A transfectants. Cells were allowed to adhere to dishes coated with polylysine (*PL*) or with mAb 7E2 to endogenous β1 (*E*) or mAb TS2/16 to transfected β1 (*T*). The proteins containing phosphotyrosine of the clones β1B-50, β1B-25, and β1A-22 were visualized by Western blotting (see Materials and Methods). Quantitation of the intensity of the band corresponding to the 125-kD phosphoprotein was assessed by densitometry.

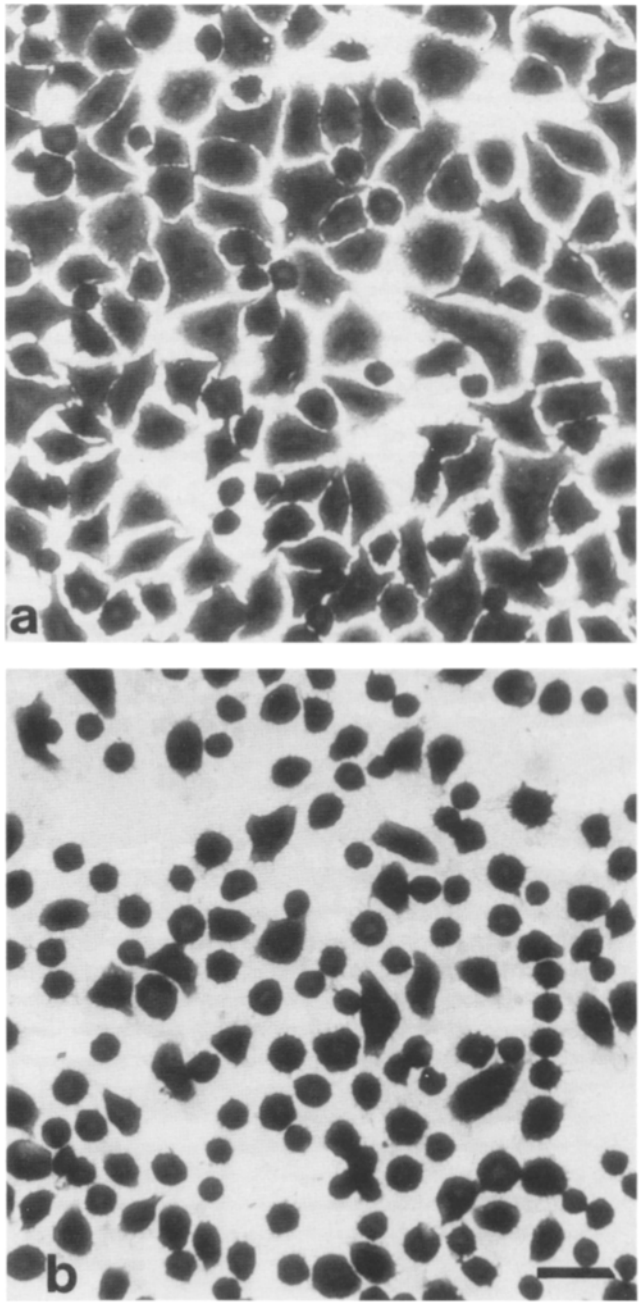

Figure 4. Adhesion of β1A and β1B transfectants to antibody-coated dishes. β1A-22 (*a*) and β1B-50 (*b*) cells were plated on dishes coated with mAb TS2/16 to the transfected human β1 and allowed to adhere for 1 h at 37°C. Cells were photographed after fixation and staining. In *a*, cells adhere to the substratum by means of the transfected β1A while in *b* cells use the transfected β1B. Bar, 100 μm.

adhesion mediated by either the β1B or by the β1A isoform. As shown in Fig. 4, β1A-22 cells adhered and spread normally, while β1B-50 cells failed to spread. Thus, when the β1B isoform is involved in cell-substratum adhesion, cells do not spread normally.

We then tested the adhesive properties of transfected clones on dishes coated with purified matrix proteins. Under these conditions both the endogenous and transfected β1 par-

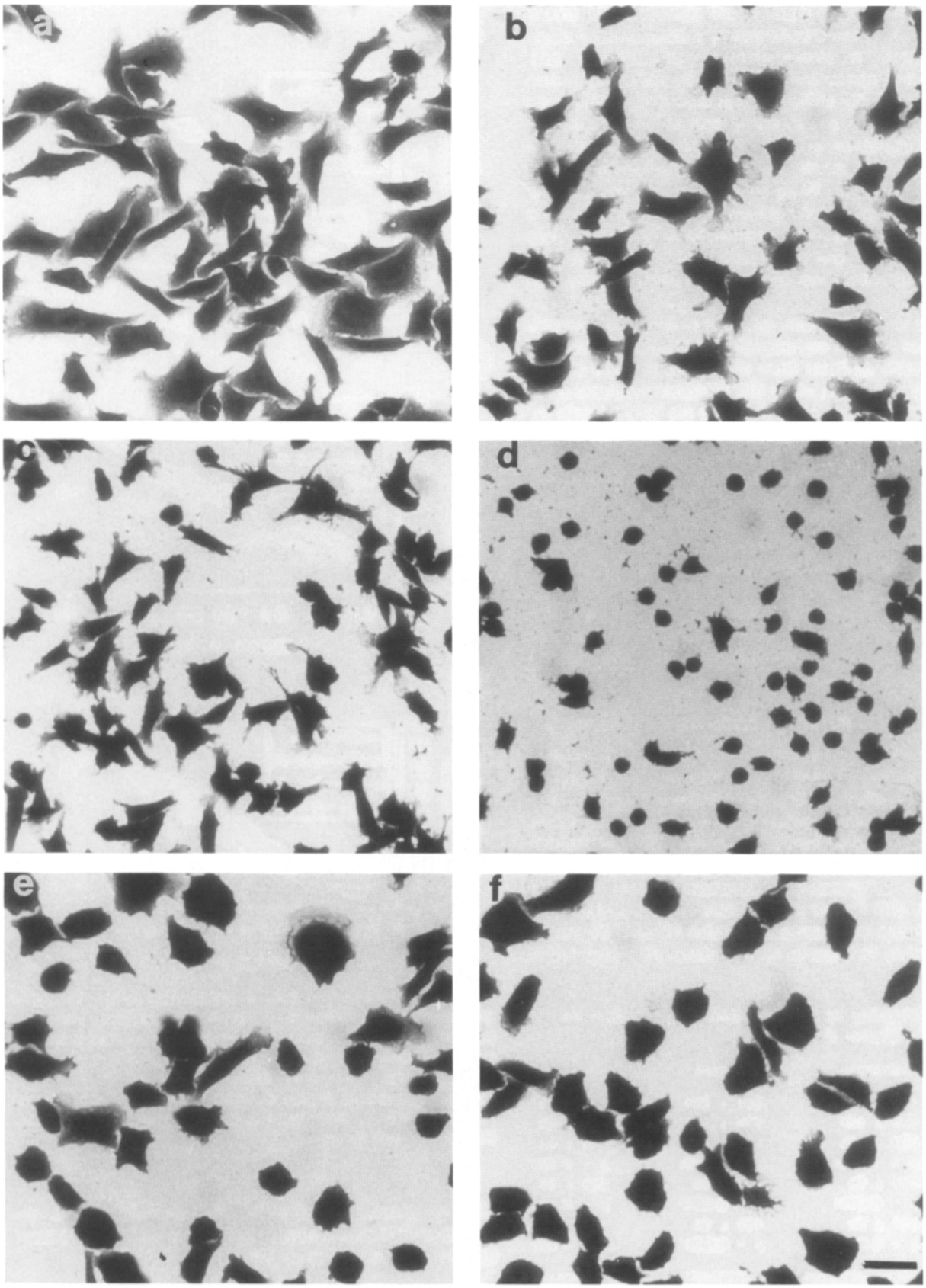

Figure 5. Spreading of β1A and β1B transfectants on purified matrix proteins. β1A-22 (*a*, *c*, and *e*) and β1B-50 (*b*, *d*, and *f*) cells were plated on dishes coated with 2 μg/ml of purified human plasma fibronectin (*a* and *b*); 10 μg/ml of purified mouse laminin (*c* and *d*); and 10 μg/ml of purified human plasma vitronectin. After adhesion for 1 h at 37°C, cells were fixed, stained, and photographed. Bar, 100 μm.

ticipate to adhesion. To quantify cell spreading, the area of the adherent cells was determined by a computerized image analysis system. As shown in Fig. 5 and Table II, β1B-expressing cells spread less on fibronectin compared to cells expressing β1A. The difference was even more pronounced on laminin (Fig. 5, *c* and *d*) and in both cases it was statistically highly significant as determined with the Student's *t* test analysis. Increasing the fibronectin concentration in the coating solution from 2 to 20 μg/ml allowed more spreading of cells but did not abolish the difference between β1B and β1A clones. Cell spreading on vitronectin, on the other hand, was comparable in β1A and β1B transfectants showing that expression of β1B does not lead to a generalized spreading defect.

When the number of cells adherent to dishes coated with fibronectin or laminin was measured, β1B transfectants were not significantly different from untransfected CHO or β1A-22 cells (Fig. 6 *A*). Only clone β1B-50 showed reduced adhesion on both substrates (Fig. 6, *A* and *B*). β1B-50 cells express high levels of β1B compared to the other two clones, suggesting that reduction in adhesion occurs only when β1B expression exceeds a threshold level. The reduced adhesive properties of the β1B-50 clone were also observed by measuring the kinetics of adhesion to a fixed amount of matrix proteins (not shown). On the other hand, all clones including β1B-50 cells adhered normally when plated on vitronectin-coated dishes (Fig. 6 *C*).

In conclusion, expression of β1B leads to a specific reduction in spreading of CHO cells to fibronectin and laminin, but not to vitronectin. In cells expressing the higher level of β1B, cell attachment was also affected.

Expression of β1B Results in Reduced Cell Motility

To evaluate whether the expression of β1B in CHO cells had a consequence on cell motility, we evaluated cell migration through porous filter in a Boyden chamber assay. Cells were plated on top of gelatin-coated porous polycarbonate filters and allowed to migrate toward the bottom surface. Soluble fibronectin or medium conditioned by 3T3 cells were added in the bottom compartment as chemoattractants. As shown in Table III, β1B-25 and β1B-50 cells showed a significant reduction of migration compared to β1A-22 cells. Migration was inhibited by 75% and by 60% in clones β1B-50 and β1B-25, respectively, compared to clone β1A-22. Also in this case, no differences in migration between β1A and β1B transfectants were observed on vitronectin.

Table II. Spreading of β1A and β1B Transfectants on Extracellular Matrix Proteins

Clones	Fibronectin	Laminin	Vitronectin
CHO	565 ± 130*	285 ± 105	570 ± 75
β1A-22	555 ± 128	276 ± 112	580 ± 90
β1B-25	480 ± 106	148 ± 29	574 ± 62
	$p = 0.0084$	$p = 0.0001$	$p = 0.158$
β1B-18	484 ± 96	156 ± 38	568 ± 52
	$p = 0.0081$	$p = 0.0001$	$p = 0.140$
β1B-50	477 ± 85	150 ± 54	555 ± 70
	$p = 0.0088$	$p = 0.0001$	$p = 0.125$

* Cells were allowed to adhere to dishes coated with 2 μg/ml of fibronectin; 10 μg/ml of mouse laminin; 10 μg/ml of vitronectin for 1 h. To measure cell spreading, stained cells were photographed by choosing five random microscopic fields for each sample. The area of 150 cells per sample was determined using a computerized image analysis system. Values represent the mean cell area in arbitrary units ±SD. The *p* value was calculated with a Student's *t* test.

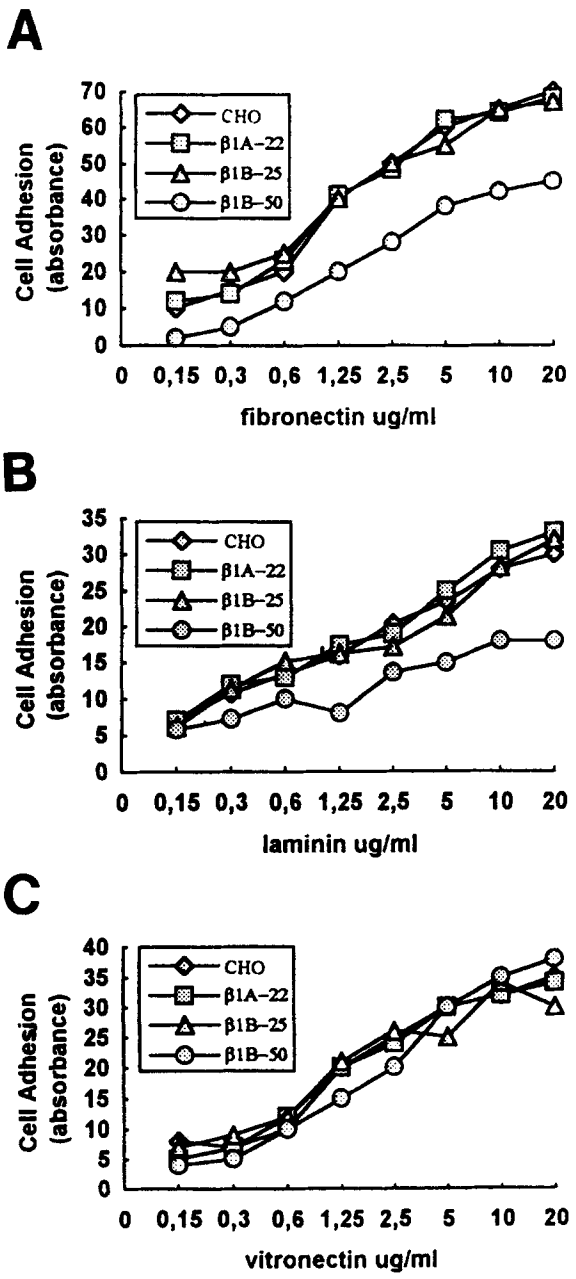

Figure 6. Adhesion of β1A and β1B transfectants on purified matrix proteins. Microtitre plates were coated with increasing concentrations of (*A*) human plasma fibronectin; (*B*) mouse laminin; and (*C*) human plasma vitronectin. Cells were suspended by EDTA treatment and plated in serum free medium for 1 h at 37°C. Cell adhesion was measured by reading the optical density after staining with Coomassie blue. ◇, untransfected CHO; □, β1A-22 cells; △, β1B-25 cells; ○, β1B-50 cells.

Association of β1A and β1B with Hamster Integrin α Subunits

A possible explanation of the adhesive behavior of β1B-transfected cells is that β1B associates with specific integrin

Table III. Migration of β1A and β1B Transfectants in Boyden Chambers

Chemoattractant (lower chamber)	Clones		
	β1A-22	β1B-50	β1B-25
Conditioned medium 3T3‡	122 ± 3*	27 ± 1	36 ± 1
FN 25 μg/ml	92 ± 9	17 ± 4	22 ± 3
VN 50 μg/ml	50 ± 5	50 ± 3	ND
SFM§	8 ± 1	10 ± 2	12 ± 1

* Mean values ± SD of number of cells (per microscopic field) migrated on the lower surface of the porous filter.
‡ Conditioned medium by 3T3 cells.
§ *SFM*, serum free medium.

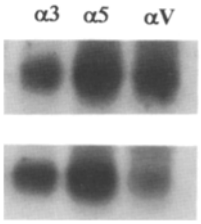

Figure 7. Association of human and hamster β1 with hamster α subunits. β1B-50 cells were immunoprecipitated with α3, α5, or αV antibodies, and the heterodimers were separated by SDS-PAGE and transferred to nitrocellulose by Western blotting. The coprecipitated β1 was detected by incubating the filter with ^{125}I-labeled antibodies specific for the human β1 (mAb G12). After stripping with 1% SDS, the filter was reincubated with ^{125}I-labeled antibodies specific for the hamster β1 (mAb 7E2). Similar results were obtained with cells transfected with human β1A.

α subunits generating a different ratio between integrin complexes normally present at the cell surface of CHO cells. By screening with a panel of α subunit specific antibodies, we determined that CHO cells express α3/β1, α5/β1, and αV; the latter one forms heterodimers with both β1, β3, and β5.

To determine the relative amount of β1A and β1B associated with each α subunit, we performed immunoprecipitation experiments from transfected cells using α3, α5, and αV antibodies; the immunoprecipitated material was run on SDS-PAGE and probed in Western blotting with antibodies to human β1 (mAb G12). The relative amount of the two β1 associated with each α was then determined by densitometry. As shown in Table IV, the relative proportion of the human β1A and β1B present in the three heterodimers was comparable, confirming that the two β1 isoforms did not differ in their association with integrin α chains. We also analyzed whether the human and the hamster β1 molecules showed the same pattern of association with hamster α subunits. The integrin complexes were immunoprecipitated with α-specific antibodies and the associated β1 subunits were detected by Western blotting with the antibodies specific for human (mAb G12) and for hamster β1 molecule (mAb 7E2) as described in the Materials and Methods section. As shown in Fig. 7, the human and hamster β1 subunits displayed similar association with α3 and α5, while a slight preference of the human β1 subunits for αV was observed.

Discussion

The cytoplasmic domains of the integrin β1 and α subunits are highly conserved during evolution (for review see Sastry and Horwitz, 1993), while the primary sequence of the extracellular domains is much less conserved. This indicates a strong evolutive pressure to maintain a given sequence in the cytoplasmic region for the various integrin subunits and suggests that the molecular interactions with cytoskeletal proteins and with proteins of the intracellular signaling machinery pose rigorous structural constraints. Splice variants characterized by new cytoplasmic domain sequences have been identified for several integrin subunits including: β1 (Altruda et al., 1990; Languino and Ruoslahti, 1992), β3 (van Kuppevelt et al., 1989), β4 (Tamura et al., 1990), α3 (Tamura et al., 1991), α6 (Hogervorst et al., 1991; Tamura et al., 1991), and α7 (Collo et al., 1993). On the basis of the considerations discussed above, these variant cytoplasmic sequences are expected to generate specific intracellular signaling and interact with unique cytoskeletal components.

We have identified a β1 variant, β1B, characterized by a distinct cytoplasmic sequence (Altruda et al., 1990). Previous data have shown that β1B form heterodimers with integrin α chains and binds fibronectin, but it does not localize to focal adhesion sites (Balzac et al., 1993). Here we further analyze the functional properties of this variant showing that β1B does not trigger the tyrosine phosphorylation of intracellular proteins and has a dominant negative effect on cell spreading and motility.

β1B is expressed in a tissue specific pattern and is always coexpressed with β1A (Balzac et al., 1993). The coexpression of the two isoforms hampers the functional analysis of β1B in cells that normally express it. To circumvent this problem we transfected human β1B in hamster cells that express only the β1A isoform. In this system it is possible to probe the function of the two isoforms by using species specific antibodies directed to extracellular epitopes and it is, thus, relatively simple to assign functional properties to the transfected molecule.

We have used this approach to show that β1B cytoplasmic variant does not trigger increased tyrosine phosphorylation of the cytoplasmic pl25-kD protein. This molecule is a major component undergoing tyrosine phosphorylation in response to integrin-ligand interaction and it corresponds to the focal adhesion kinase pl25FAK (Burridge et al., 1992; Guan and Shalloway, 1992; Kornberg et al., 1992). The lack of signaling through this pathway was not due to a gross defect of β1B since this variant is exported at the cell surface and forms heterodimers capable of binding extracellular ligands (Balzac et al., 1993). Moreover, the endogenous β1A in the same transfected cells effectively triggers tyrosine phosphorylation, ruling out the possibility that the signaling pathway is nonfunctional in these transfectants. In addition, independent transfectants showed that human β1A isoform is fully functional in signal transduction in the CHO back-

Table IV. Association of Transfected Human β1 Integrins with CHO Cell α Subunits

Clones	Association of transfected β1 with:		
	α3	α5	αV
β1A-22	32*	57	11
β1B-50	30	60	10

* The amount of transfected β1 associated with the endogenous α subunits was determined by densitometry after immunoprecipitation and SDS-PAGE and it is expressed as % of the total transfected protein.

ground. The lack of tyrosine phosphorylation of the p125 protein by β1B is, thus, due to the properties of its cytoplasmic sequence. These results are consistent with data reported by Guan et al. (1991) showing that specific deletion of the COOH-terminal region of the chick β1A, corresponding to the region spliced out in β1B, abrogated the ability to stimulate tyrosine phosphorylation of the 125-kD protein.

The β1B variant was also ineffective in promoting cell spreading when cell adhered to surfaces coated with antibodies specific for this molecule. This is consistent with our previous finding that β1B does not localize at focal adhesions (Balzac et al., 1993) and shows that cell spreading and signaling through tyrosine kinase require an intact β1 cytoplasmic domain.

Our data also show that expression of β1B in CHO cells has a dominant negative effect on cell spreading and motility. The inhibition of these adhesive functions is not due to undesired mutation of the selected clones, since: (*a*) it is common to independent clones; and (*b*) it is restricted to fibronectin and laminin and does not occur on vitronectin. While fibronectin and laminin are recognized by integrins of the β1 class, vitronectin is preferentially recognized by β3 and β5 integrins (for review see Hynes, 1992). Thus, the effect of β1B is restricted to those substrata recognized by β1 class integrins. The β1B is functionally normal as far as the association with α subunits and binding to fibronectin are concerned (Balzac et al., 1993). We also show that β1A and β1B are indistinguishable for the association with α3, α5, and αV subunits expressed in CHO (see Table IV). Preferential association of β1B with a given α subunit would change the ligand specificity of the cells and lead to reduced adhesion to specific ligands. Moreover, comparative analysis of the human and hamster β1 proteins showed that the specie difference did not affect the association with hamster α3 and α5, although a slight preference of the human β1 for αV was detected. This, however, can not explain the distinct reduction of adhesion and migration on fibronectin and laminin. Thus, the dominant negative effect on cell adhesion is most likely explained by the fact that β1B competes with β1A for association with α chains and binding to the extracellular matrix, but it fails to transduce mechanical or biochemical signals inside the cell.

It is interesting to note that significant reduction of cell spreading and migration is observed in cells where β1B represents less than 50% of the total β1 at the cell surface. Whether these effects can be explained solely by the competition of β1B and β1A for heterodimer formation and binding to extracellular ligands is unclear. Other phenomena may contribute to the observed phenotype. β1B may perturb the complex network of low affinity cooperative interactions involved in the integrin-cytoskeleton association leading to an amplified defect in the transduction of mechanical forces across the plasma membrane. Alternatively, β1B may generate specific intracellular signals leading to reduced cell adhesion. These aspects deserve further investigation.

Dominant negative effects have been reported for artificially mutated forms of adhesion receptors. A β1 integrin with a mutation in the extracellular domain that abolished ligand binding was shown to have a dominant negative effect on cell adhesion and spreading (Takada et al., 1992). More recently, a dominant negative effect has been described for a chimeric molecule containing the cytoplasmic domain of the β1 integrin (Lukashev and Pytela, 1993). Expression of a truncated form of N-cadherin, a cell–cell adhesion receptor, consisting of the sole transmembrane and cytoplasmic regions, but lacking the extracellular domain resulted in reduced cell–cell adhesion (Kinter, 1992). The truncated form presumably competes with the native molecule by binding to cytoskeletal proteins necessary to transduce the mechanical force of adhesion. A second example comes from a chimeric membrane protein containing the cytoplasmic domain of desmoglein, a desmosomal cadherin (Troyanovsky et al., 1993). This chimera causes the disruption of desmosomes and detachment of intermediate filaments from the plasma membrane.

All examples discussed above consist of proteins with a functional intracellular segment and an altered or deleted extracellular domain. The data presented here show that an adhesion receptor containing an intact extracellular domain and an altered cytoplasmic region can also cause a dominant negative effect. In the case of β1B, in fact, correct extracellular interactions elicit a peculiar intracellular response that leads to anomalous adhesion. The fact that β1B represents a splice variant raises the interesting question that cells may regulate their adhesive behavior by a splicing mechanism.

The authors are indebted to F. Giancotti, R. Juliano, F. Sanchez-Madrid, and L. Zardi for the generous gift of β1 integrin antibodies. We also thank Rita Palumbo and Daniela Tomatis for helping in some of the experiments.

The work was supported by grants from the Italian Association for Research on Cancer; from the Progetto Finalizzato "Applicazioni Cliniche della Ricerca Oncologica" of the National Research Council, and from the Biomed 1 of the European Community.

Received for publication 22 December 1993 and in revised form 28 June 1994.

References

Albini, A., G. Allavena, A. Melchiorri, F. Giancotti, H. Richter, P. M. Comoglio, S. Parodi, G. R. Martin, and G. Tarone. 1987. Chemotaxis of 3T3 and SV3T3 cells to fibronectin is mediated through the cell attachment site in fibronectin and a fibronectin cell surface receptor. *J. Cell Biol.* 105: 1867–1872.

Altruda, F., P. Cervella, G. Tarone, C. Botta, F. Balzac, G. Stefanuto, and L. Silengo. 1990. A human integrin β1 with a unique cytoplasmic domain generated by alternative mRNA processing. *Gene (Amst.).* 95:261–266.

Balzac, F., A. Belkin, V. Koteliansky, Y. Balabanow, F. Altruda, L Silengo, and G. Tarone. 1993. Expression and functional analysis of a cytoplasmic domain variant of the β1 integrin subunit. *J. Cell Biol.* 121:171–178.

Burridge, C. A., K. Fath, T. Kelley, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487–525.

Burridge, C.A., C. Turner, and L. Romer. 1992. Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.* 119:893–904.

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with water-soluble fluor, sodium salicilate. *Anal. Biochem.* 98:132–135.

Collo, G., L. Starr, and V. Quaranta. 1993. A new isoform of the laminin receptor integrin α7/β1 is developmentally regulated in skeletal muscle. *J. Biol. Chem.* 268:19019–19023.

Defilippi, P., L. Silengo, and G. Tarone. 1992. α6/β1 integrin (lamin-receptor) is down-regulated by tumor necrosis factor β1 and interleukin-1β in human endothelial cells. *J. Biol. Chem.* 267:18303–18307.

Defilippi, P., C. Bozzo, G. Volpe, G. Romano, M. Venturino, L. Silengo, and G. Tarone. 1994. Integrin-mediated signal transduction in human endothelial cells: analysis of tyrosine phosphorylation events. *Cell Adhesion Commun.* 2:75–86.

Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter. 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromise insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell.* 45:721–732.

Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein fibronectin to collagen. *Int. J. Cancer.* 20:1–5.

Geiger, B., and D. Ginsberg. 1991. The cytoplasmic domain of adherens-type junction. *Cell Motil. Cytoskeleton*. 20:1–6.

Giancotti, F. G., and E. Ruoslahti. 1990. Elevated levels of the α5/β1 fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. *Cell*. 60:849–859.

Guan, J. L., and D. Shalloway. 1992. Regulation of focal adhesion associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature (Lond.)*. 358:690–692.

Guan, J. L., J. E. Trevethick, and R. O. Hynes. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120 kD protein. *Cell Regulation*. 2:951–964.

Hogervorst, F., I. Kuikman, A. G. Van Kessel, and A. Sonnenberg. 1991. Molecular cloning of the human α6 integrin subunit: alternative splicing of α6 mRNA and chromosomal localization of the α6 and β4 genes. *Eur. J. Biochem*. 199:425–433.

Horwitz, A., E. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin: a transmembrane linkage. *Nature (Lond.)*. 320:531–533.

Hynes, R. O. 1992. Integrins: versatility, modulation and signalling in cell adhesion. *Cell*. 69:11–25.

Jaken, S., K. Leach, and T. Klauck. 1989. Association of type3 protein kinaseC with focal contacts in rat embryo fibroblasts. *J. Cell Biol*. 109:697–704.

Kinter, C. 1992. Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. *Cell*. 69:225–236.

Kornberg, L., H. S. Earp, J. T. Parsons, M. Schaller, and R. L. Juliano. 1992. Cell adhesion of integrin clustering increases phosphorylation of a focal adhesion associated tyrosine kinase. *J. Biol. Chem*. 267:23439–23442.

Languino, L. R., and E. Ruoslahti. 1992. An alternative form of the integrin β1 subunit with a variant cytoplasmic domain. *J. Biol. Chem*. 267:7116–7120.

Lukashev, M. E., and R. Pytela. 1993. Suppression of cell adhesion by autonomously expressed C-terminal domain of the β1 integrin subunit. *Mol. Biol. Cell*. 4(Suppl.):2352.

Luna, E. J., and A. L. Hitt. 1992. Cytoskeleton-plasma membrane interactions *Science (Wash. DC)*. 258:955–964.

Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. An interaction between α-actinin and the β1 integrin subunit in vitro. *J. Cell Biol*. 111:721–729.

Reszka, A. A., R. A. Hayashi, and A. F. Horwitz. 1992. Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol*. 117:1321–1330.

Rohrschneider, L. R. 1980. Adhesion plaques of Rous Sarcoma virus-transformed cells contain the src gene product. *Proc. Natl. Acad. Sci. USA*. 77:3514–3518.

Rossino, P., P. Defilippi, L. Silengo, and G. Tarone. 1991. Upregulation of integrin α1/β1 in human neuroblastoma cells differentiated by retinoic acid: correlation with increased neurite outgrowth response to laminin. *Cell Regulation*. 2:1021–1033.

Ruoslahti E. 1991. Integrins. *J. Clin. Invest*. 87:1–5.

Sastry, S. K., and A. F. Horwitz. 1993. Integrin cytoplasmic domain: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signalling. *Curr. Opin. Cell Biol*. 5:819–831.

Schaller, M. D., C. A. Borgman, B. S. Cobb, R. R. Vines, A. B. Reynolds, and J. T. Parsons. 1992. pp125FAK, a structurally unique protein tyrosine kinase associated with focal adhesion. *Proc. Natl. Acad. Sci. USA*. 89: 5192–5196.

Southern, P. J., and P. W. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet*. 1:327–341.

Takada, Y., J. Ylänne, D. Mandelman, W. Puzon, and M. H. Ginsberg. 1992. A point mutation of integrin β1 subunit blocks binding of α5/β1 to fibronectin and invasin but not recruitment to adhesion plaques. *J. Cell Biol*. 119:913–921.

Tamura, R. N., H. M. Cooper, G. Collo, and V. Quaranta. 1991. Cell type specific integrin variants with alternative α chain cytoplasmic domains. *Proc. Natl. Acad. Sci. USA*. 88:10186–10187.

Tamura, R. N., C. Rozzo, L. Starr, J. Chambers, L. F. Reichardt, H. M. Cooper, and V. Quaranta. 1990. Epithelial integrin α6/β4: complete primary structure of α6 and variant forms of β4. *J. Cell Biol*. 111:1593–1604.

Troyanovsky, S. M., L. G. Eshkind, R. B. Troyanovsky, R. E. Leube, and W. W. Franke. 1993. Contribution of cytoplasmic domains of desmosomal cadherins to desmosome assembly and intermediate filament anchorage. *Cell*. 72:561–574.

Turner, C. E., and K. Burridge. 1991. Transmembrane molecular assemblies in cell-extracellular matrix interactions. *Curr. Opin. Cell Biol*. 3:849–853.

van Kuppevelt, T. H., L. Languino, J. O. Gailit, S. Suzuki, and E. Ruoslahti. 1989. An alternative cytoplasmic domain of the integrin β3 subunit. *Proc. Natl. Acad. Sci. USA*. 86:5415–5418.

Wang, N., J. P. Butler, and D. E. Ingberg. 1993. Mechanotransduction across the cell surface and through the cytoskeleton. *Science (Wash. DC)*. 260: 1124–1127.

Yatohgo, T., M. Irumi, H. Kasihiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct*. 13:281–292.