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β 1-Integrin Cytoplasmic Subdomains Involved in Dominant Negative Function

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The β 1-integrin cytoplasmic domain consists of a membrane proximal subdomain common to the four known isoforms ("common" region) and a distal subdomain specific for each isoform ("variable" region). To investigate in detail the role of these subdomains in integrin-dependent cellular functions, we used β 1A and β 1B isoforms as well as four mutants lacking the entire cytoplasmic domain (β 1TR), the variable region (β 1COM), or the common region (β 1 Δ COM-B and β 1 Δ COM-A). By expressing these constructs in Chinese hamster ovary and β 1 integrin-deficient GD25 cells (Wennerberg *et al.*, J Cell Biol 132, 227–238, 1996), we show that β 1B, β 1COM, β 1 Δ COM-B, and β 1 Δ COM-A molecules are unable to support efficient cell adhesion to matrix proteins. On exposure to Mn^{++} ions, however, β 1B, but none of the mutants, can mediate cell adhesion, indicating specific functional properties of this isoform. Analysis of adhesive functions of transfected cells shows that β 1B interferes in a dominant negative manner with β 1A and β 3/ β 5 integrins in cell spreading, focal adhesion formation, focal adhesion kinase tyrosine phosphorylation, and fibronectin matrix assembly. None of the β 1 mutants tested shows this property, indicating that the dominant negative effect depends on the specific combination of common and B subdomains, rather than from the absence of the A subdomain in the β 1B isoform.

INTRODUCTION

Integrins are α/β heterodimeric transmembrane cell surface receptors that mediate cell adhesion and migration and also the bidirectional transfer of information across the plasma membrane. These properties are essential in regulating several biological processes, including morphogenesis, immune response, cell growth, differentiation, and survival (Hynes, 1992; Ruoslahti and Reed, 1994).

The cytoplasmic domains of integrin subunits are required for these functions (Hibbs *et al.*, 1991; Yamada and Miyamoto, 1995; Dedhar and Hannigan,

1996). In vitro, the isolated β 1 cytoplasmic domain was shown to bind talin, α -actinin, and paxillin, three cytoskeletal proteins that mediate the anchorage of actin filaments to the plasma membrane (Chen *et al.*, 1995; Otey *et al.*, 1993; Schaller *et al.*, 1995). Binding of the β 1 cytoplasmic sequence to focal adhesion kinase (FAK), a tyrosine kinase specifically localized to focal adhesions, and to the serine/threonine kinase integrin-linked kinase has also been reported (Schaller *et al.*, 1995; Hannigan *et al.*, 1996). In vivo, the β 1 cytoplasmic domain is sufficient for its localization to preformed focal adhesions (LaFlamme *et al.*, 1992; Akiyama *et al.*, 1994) and for the initiation of signaling to FAK (Lukashev *et al.*, 1994). A model has been proposed in which the β 1 subunit cytoplasmic domain

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contains a default signal for interaction with cytoskeletal molecules that is masked by the α subunit cytoplasmic domain (Briesewitz *et al.*, 1993; Ylanne *et al.*, 1993). In response to matrix ligands or to multivalent antibody binding, the inhibitory effect of the α subunit can be released, and integrins can interact with cytoskeletal and signaling molecules (Miyamoto *et al.*, 1995a,b).

Integrins can activate different intracellular signals, including tyrosine phosphorylation of a number of cellular proteins, elevation of the intracellular calcium level, cytoplasmic alkalinization, alteration in phospholipid metabolism, and activation of MAP kinases (Clark and Brugge, 1995; Schaller *et al.*, 1995). The most studied pathway is represented by the activation of the FAK tyrosine kinase (Guan *et al.*, 1991; Burridge *et al.*, 1992). Tyrosine-phosphorylated FAK can function as a docking site for both cytoskeletal proteins, such as paxillin and talin, and signaling molecules, such as Src family tyrosine kinases, Grb2, and phosphatidylinositol-3 kinase (Schaller and Parson, 1994; Burridge and Chrzanowska-Wodnicka, 1996). These interactions are important for coordinating actin cytoskeleton organization and gene expression and, thus, for regulating cell migration, proliferation, differentiation, and survival (Schwartz *et al.*, 1995).

In addition to outside-in signaling, integrins can also transduce signals from the inside to the outside of the cell. The inside-out signaling occurs mainly in the form of regulation of the ligand-binding affinity state (Ginsberg *et al.*, 1992; O' Toole *et al.*, 1994). By acting on the cytoplasmic domain, intracellular signals can modify the conformation of the integrin extracellular domain and, therefore, modify ligand binding capacity (O' Toole *et al.*, 1994).

The amino acid sequences of the β cytoplasmic domain that are important for outside-in and inside-out signaling have been mapped in some detail. Three major sites in the $\beta 1$ cytoplasmic domain, termed cyto-1, 2, and 3, are important for localization of the integrin heterodimer to focal adhesions (Reszka *et al.*, 1992). The cyto-1 site is proximal to the lipid bilayer and partially overlaps with the putative binding sites for FAK, paxillin, and α -actinin (Otey *et al.*, 1993; Schaller *et al.*, 1995). In $\beta 3$ -integrin, a region N-terminal to cyto-1 interacts with the highly conserved sequence GFFKR of the α subunits and is involved in the regulation of ligand-binding affinity (Hughes *et al.*, 1996). The cyto-2 and cyto-3 sites correspond to two NPXY motifs and are also important for regulating the affinity state of the ectodomain (O'Toole *et al.*, 1995).

Interestingly, four isoforms of the $\beta 1$ -integrin exist in humans, differing in their cytoplasmic sequence as well as in their functional properties (Balzac *et al.*, 1994; Fornaro *et al.*, 1995; Belkin *et al.*, 1996). Based on the structural properties of the four splicing variants, the $\beta 1$ cytoplasmic domain can be divided in two

subdomains that we refer to as "common" and "variable" subdomains, respectively. The common subdomain consists of the 25 amino acid residues proximal to the membrane coded for by exon 6 (Altruda *et al.*, 1990; Baudoin *et al.*, 1996) and is shared by all four variants. This region is highly conserved among different β subunits and contains the cyto-1 site (Reszka *et al.*, 1992) and the putative binding sites for FAK, paxillin (Schaller *et al.*, 1995), and α -actinin (Otey *et al.*, 1993). The variable subdomain extending toward the C terminus is coded for by different exons (Baudoin *et al.*, 1996) and characterizes the four different $\beta 1$ isoforms: $\beta 1A$, $\beta 1B$, $\beta 1C$, and $\beta 1D$. The $\beta 1A$ variable subdomain contains cyto-2 and cyto-3 sites, whereas that of $\beta 1B$ is characterized by a unique amino acid sequence lacking both sites. We have shown that these structural features have important consequences for the functional properties of $\beta 1A$ and $\beta 1B$. In fact, whereas $\beta 1A$ is capable of triggering FAK tyrosine phosphorylation and localizing to focal adhesions, $\beta 1B$ lacks both functions (Balzac *et al.*, 1994). Moreover, $\beta 1B$ acts as a dominant negative inhibitor, when expressed in Chinese hamster ovary (CHO) cells, by interfering with cell spreading and migration promoted by endogenous $\beta 1A$ (Balzac *et al.*, 1993, 1994).

To investigate the structural basis of the $\beta 1B$ dominant negative effect further, we prepared different mutants lacking either the $\beta 1$ cytoplasmic domain variable or common region. These constructs, together with $\beta 1B$, were expressed both in GD25 cells, which express $\alpha V\beta 3/5$ and lack $\beta 1$ as a consequence of gene knockout (Wennerberg *et al.*, 1996), and in CHO cells, which express endogenous hamster $\beta 1A$. In these cellular systems we show that $\beta 1B$ acts as dominant negative on adhesive and signaling function of both $\beta 1$ - and $\beta 3/5$ -integrins. Analysis of mutants molecules shows that this dominant negative action is attributable to the unique $\beta 1B$ cytoplasmic domain.

MATERIALS AND METHODS

Antibodies and Reagents

The following antibodies were used: the rat anti-human $\beta 1$ mAb 13 (Akiyama *et al.*, 1989) was a gift from K. Yamada (National Institutes of Health, Bethesda, MD); the activating mouse anti-human $\beta 1$ mAb TS2/16 (Hemler *et al.*, 1984) was obtained from American Type Culture Collection (Rockville, MD); the mouse anti-human $\beta 1$ mAb 12G10 was characterized previously (Mould *et al.*, 1995); the rat anti-mouse $\beta 1$ mAb 9EG7, with human cross-reactivity (Lenter *et al.*, 1993), was a gift from D. Vestweber (ZMBE Technologiehof, Muenster, Germany); the blocking mouse anti-human $\beta 1$ mAb AIIB2 (Werb *et al.*, 1989) was a gift from C. Damsky (Department of Stomatology, University of California, San Francisco, CA); the inhibitory PB1 mAb against hamster $\alpha 5\beta 1$ heterodimer was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA); the blocking anti-mouse αV H9.2B8 mAb (Moulder *et al.*, 1991) was purchased from PharMingen (San Diego, CA); the rat anti-mouse $\alpha 6$ mAb GoH3 (Sonnenberg *et al.*, 1988) was a gift from A. Sonnenberg (The Neederland Cancer Institute, Amsterdam, the Netherlands); the anti-talin mAb 8d4 was obtained

from Sigma (St. Louis, MO); the anti α -actinin mAb 1682 was from Chemicon (Temecula, CA); rabbit polyclonal antisera to human fibronectin and to α V, α 3, and α 5 integrin cytoplasmic domains were produced in our laboratory (Tarone *et al.*, 1984; Defilippi *et al.*, 1992); the FAK 4 polyclonal antibody and the mAb FAK9.2 for FAK immunoprecipitation and Western blotting, respectively, were prepared in our laboratory as described previously (Defilippi *et al.*, 1995); a mouse anti-paxillin mAb and the anti-phosphotyrosine mAb PY20 were purchased from Transduction Laboratories (Nottingham, UK); fluorescein-labeled phalloidin, fluorescein-labeled goat anti-mouse IgG, rhodamine-labeled goat anti-mouse IgG, and rhodamine-labeled goat anti-rabbit IgG were all from Sigma. Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose according to the method of Engvall and Ruoslahti (1977); mouse laminin-1 was obtained from GIBCO BRL (St. Louis, MO); the peptides GRGDSP and GRGESP were synthesized by Drs. L. Lozzi and P. Neri (University of Siena, Siena, Italy).

Integrin β 1 Cytoplasmic Domain Mutagenesis and cDNA Vector Construction

Restriction enzymes, T4 DNA ligase, and a Klenow fragment of DNA polymerase were from New England Biolabs (Beverly, MA); *Taq* DNA polymerase was from Promega (Madison, WI). The SV40-based expression vector pECE (Ellis *et al.*, 1986) containing the β 1A and β 1B cDNA fragments was described previously (Giancotti and Ruoslahti, 1990; Balzac *et al.*, 1993). These plasmids were used to generate four cytoplasmic domain deletion mutants of human β 1-integrin as follows. The *Hind*III cloning site of pECE was eliminated (pECE/ H^{-}), and the 417-bp *Hind*III cDNA fragment, containing the entire cytoplasmic domain and a part of the transmembrane domain of human β 1A, was excised and substituted with either a *Hind*III synthetic oligonucleotide fragment constituting the transmembrane and the cytoplasmic domain up to isoleucine residue 762 or a *Hind*III PCR fragment extending to the end of the β 1 cytoplasmic common subdomain (threonine residue 782), followed by two stop codons, to generate the plasmids pECE/ H^{-} - β 1TR and pECE/ H^{-} - β 1COM, respectively. The internal deletion of the common subdomain (from isoleucine 762 to threonine 782 in the amino acid sequence) of β 1A and β 1B cDNA fragments was obtained by recombinant PCR mutagenesis (Higuchi, 1990). The resulting PCR fragments were used to generate the plasmid pECE/ H^{-} - β 1 Δ COM-A and pECE/ H^{-} - β 1 Δ COM-B. Each mutant cDNA sequence was confirmed by dideoxy sequencing (T7 sequencing kit; Pharmacia Biotech, Upsala, Sweden).

Cells and Transfections

CHO cells expressing human β 1A- or β 1B-integrin isoforms or the relative deletion mutants β 1TR, β 1COM, β 1 Δ COM-A, and β 1 Δ COM-B were obtained by cotransfection with pSV2-neo using the calcium phosphate precipitation method as described (Balzac *et al.*, 1993). The GD25 cell line, derived from β 1-deficient embryonic stem cells (Wennerberg *et al.*, 1996), was transfected with β 1B, β 1A, β 1TR, and β 1COM expression constructs, together with the plasmid pECV6-*hyg* (Belt *et al.*, 1989), by electroporation. Resistant populations were selected in medium containing 300 μ g/ml hygromycin β (Boehringer Mannheim, Mannheim, Germany).

After 10 d of selection, both CHO and GD25-positive cells were sorted for β 1 expression by a modification of the panning method (Margolskee *et al.*, 1993). Briefly, transfected cells were adsorbed to bacteriological dishes coated with 10 μ g/ml sterile-filtered anti-human β 1 mAb TS2/16 in PBS and allowed to attach for 5–30 min at 4°C. Dishes were then rinsed several times with PBS to remove unbound cells, and adherent cells were detached from the substrate by trypsin-EDTA treatment and transferred to tissue culture dishes. To select populations expressing high levels of all β 1 forms used, five cycles of panning in stringent conditions (short time of cell adhesion) were performed.

Integrin Analysis by Flow Cytometry and Immunoprecipitation

The expression level of transfected β 1 integrins and their conformational state was measured by FACS analysis. Cells were suspended in PBS [10 mM phosphate buffer (pH 7.3) and 150 mM NaCl] containing 1 mg/ml BSA. One millimolar $MnCl_2$ was also added when indicated. The cells were then incubated for 1 h at 4°C with saturating concentrations of β 1 monoclonal antibodies followed by fluorescein-labeled goat anti-mouse IgG. After washing, cells were analyzed (5000 per sample) in a FACScan (Becton Dickinson, Mountain View, CA) equipped with 5 W argon laser at 488 nm.

Integrins were immunoprecipitated from cells labeled with ^{125}I as described previously (Rossino *et al.*, 1990; Balzac *et al.*, 1994).

Cell Adhesion and Spreading Assays

Cell adhesion and spreading assays to matrix-coated microtiter plates were performed as described previously (Balzac *et al.*, 1994). To analyze the role of divalent cations in adhesion, cells were plated in "adhesion buffer" [20 mM Tris (pH 7.4), 135 mM NaCl, 5 mM KCl, 2 mM L-glutamine, 1.8 mM glucose, and 1% BSA] containing 1 mM $MnCl_2$, $MgCl_2$, or $CaCl_2$. Where indicated, adhesion was blocked by addition of the β 1 mAb AIIB2 (1:5 dilution of culture supernatant) or 0.5 mg/ml GRGDSP peptide; the GRGESP peptide was also used as control. Cell adhesion was evaluated by colorimetric assay for acid phosphatase activity as described (Defilippi *et al.*, 1991).

Fibronectin Matrix Assembly Assay

Matrix assembly was evaluated by adding exogenous purified human plasma fibronectin (Engvall and Ruoslahti, 1977) to a final concentration of 200 nM for 2 d to confluent cell monolayers grown on glass coverslips in medium containing 1% serum. Where indicated, inhibitory mAb PB1 against hamster α 5 β 1 or mAb H9.2B8 against mouse α V was used (10 μ g/ml). The resulting fibronectin matrix was then visualized by immunofluorescence.

The same protocol was used to quantitate fibronectin assembly using 100 nM ^{125}I -labeled fibronectin (specific activity, 0.08 mCi/nM). A deoxycholate-insoluble fraction was obtained from cell monolayers as described (McKeown-Longo and Mosher, 1985; Wu *et al.*, 1993, 1995). ^{125}I -labeled fibronectin incorporated into the deoxycholate-insoluble extracellular matrix was analyzed by reducing SDS-PAGE (6% running gel) and autoradiography.

Immunofluorescence Microscopy

For immunofluorescence cells were seeded on circular (1-cm-diameter) glass coverslips in 12-well plates and grown for the indicated time. Where indicated, coverslips were coated with 10 μ g/ml human plasma fibronectin. Immunofluorescence staining of paraformaldehyde-fixed cells was performed using a standard protocol (Balzac *et al.*, 1993). A 1:500 dilution of the polyclonal antibody to human fibronectin, a 0.5 μ g/ml solution of paxillin mAb, a 1:200 dilution of the α V cytoplasmic domain polyclonal antibody, and a 10 μ g/ml solution of mAb TS2/16 were used. Bound primary antibodies were then visualized by appropriate rhodamine-labeled secondary antibodies. In some experiments cells were double stained with fluorescein-conjugated phalloidin.

Measurement of Cellular Contractility

Silicone rubber substrata for assessing cellular contractility were made as described previously (Harris *et al.*, 1980; Danowski, 1989). Films were produced by glow discharge polymerization (5 sec, 20 mA). Briefly, 0.5 ml of silicone rubber (dimethyl polysiloxane; viscosity, 10,000–60,000 centistokes; Sigma) was aliquoted into tissue culture dishes and allowed to spread for 24 h. The top of the silicone was then coated with a thin layer of gold-palladium using a cold

sputter coater. The UV glow discharge that occurred during the gold-palladium coating polymerized the silicone rubber. Cells were plated for 1 day on the cross-linked rubber substrata in growth medium with 10% serum, and the presence or absence of wrinkles was examined using an inverted phase-contrast Leitz microscope.

Detection of Phosphotyrosine-containing Proteins

To specifically trigger tyrosine phosphorylation of intracellular proteins mediated by the transfected or endogenous integrins, cells were plated on plastic dishes coated with specific monoclonal antibodies as described (Balzac *et al.*, 1994). Cells were lysed in the presence of phosphatase inhibitors, and FAK was immunoprecipitated as described (Retta *et al.*, 1996). After SDS-PAGE, proteins were transferred to nitrocellulose and processed for Western blotting with the anti-phosphotyrosine mAb PY20 followed by peroxidase-conjugated anti-mouse IgG (Sigma). Bound antibodies were visualized by an ECL detection method (Amersham, Buckinghamshire, UK). After stripping with 2% SDS at 42°C for 1 h to remove bound antibodies, the filter was reprobed with the mAb FAK9.2 to visualize the level of FAK protein.

Coimmunoprecipitation of Proteins Interacting with the $\beta 1$ Cytoplasmic Domain

Transfected CHO cells from confluent culture dishes were suspended by EDTA treatment and incubated with 10 μ g of purified TS2/16 mAb to human $\beta 1$ for 30 min at 4°C on a rotator. Cells were centrifuged (1000 rpm, 3 min), and the pellets were extracted for 3 min on ice with 50 mM PIPES buffer (pH 6.9) containing 0.5% digitonin, 1 mM $MgCl_2$, 1 mM EGTA, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, and 0.5 mM PMSF. Cell extracts were centrifuged (12,000 rpm, 30 min, 4°C), and the resulting supernatants were incubated at 4°C for 45 min with protein G-Sepharose beads. Immunoprecipitates were washed in the same buffer, boiled in SDS sample buffer, and separated by 8% SDS-PAGE. Proteins were transferred onto Immobilon membranes and processed for Western blotting with 8d4 mAb against talin, 1682 mAb against α -actinin, or FAK9.2 mAb against FAK.

RESULTS

Preparation of $\beta 1$ -Integrin Cytoplasmic Domain Variants and Expression in CHO and $\beta 1$ -Null Cells

The cytoplasmic domain of $\beta 1$ -integrin consists of a membrane proximal subdomain, shared by all four $\beta 1$ isoforms (common subdomain), and a distal subdomain toward the C terminus, unique for each isoform (variable subdomain) (Figure 1). To investigate the specific role of these two $\beta 1$ cytoplasmic subdomains, we have generated the following mutants (Figure 1): $\beta 1TR$, lacking the entire cytoplasmic domain; $\beta 1COM$, containing only the common subdomain; and $\beta 1\Delta COM-B$ and $\beta 1\Delta COM-A$, in which the common subdomain has been deleted and the variable B and A subdomains are directly linked to the transmembrane segment. These constructs and the natural $\beta 1B$ and $\beta 1A$ isoforms were expressed in CHO cells (Figure 2A) and in the mouse GD25 fibroblastic cell line, which lacks endogenous $\beta 1$ as a consequence of gene inactivation (Fässler *et al.*, 1995; Wennerberg *et al.*, 1996) (Figure 2, B and C). Previous results showed that human $\beta 1A$ and $\beta 1B$ isoforms correctly associated with endogenous α subunits in CHO cells (Balzac *et al.*

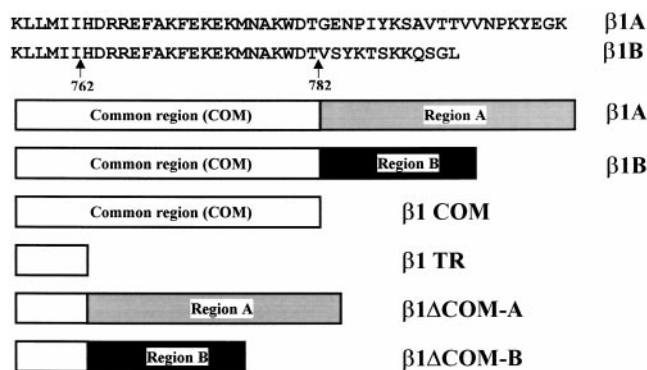


Figure 1. Schematic representation of $\beta 1A$, $\beta 1B$, and cytoplasmic domain deletion mutants prepared for these studies. The amino acid sequences of $\beta 1A$ and $\beta 1B$ cytoplasmic domain are indicated. The $\beta 1COM$ mutant was truncated at threonine 782 and contains only the subdomain common to the different human $\beta 1$ isoforms; the $\beta 1TR$ mutant was truncated at isoleucine residue 762 and lacks the entire cytoplasmic domain; the $\beta 1\Delta COM-A$ and $\beta 1\Delta COM-B$ lack the common subdomain, so that the distal A and B subdomains, respectively, are directly linked to the transmembrane segment. These mutants were prepared by oligonucleotide-mediated and recombinant PCR mutagenesis as described in MATERIALS AND METHODS.

al., 1993, 1994). Identical results were obtained with the four $\beta 1$ mutants described above (Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results). The major integrin complexes at the GD25 cell surface are $\alpha 6\beta 4$ -, $\alpha V\beta 3$ -, and $\alpha V\beta 5$ -integrins (Figure 2C; Wennerberg *et al.*, 1996). Transfection of these cells with the above $\beta 1$ constructs led to surface expression of $\beta 1$ integrin heterodimers with the endogenous $\alpha 3$, $\alpha 5$, and $\alpha 6$ subunits but not with the αV subunit (Figure 2C). A significant amount of $\alpha 5$ was detected at the surface of untransfected GD25 cells, suggesting that a fraction of the molecule can still reach the cell surface in the absence of the β subunit.

Adhesive Properties of $\beta 1$ -Integrin Cytoplasmic Variants

The functional properties of GD25 and CHO cells transfected with either the $\beta 1B$ or $\beta 1A$ isoform or the four $\beta 1$ cytoplasmic domain mutants were evaluated by testing their adhesive properties toward fibronectin and laminin-1. A previous report showed that GD25 $\beta 1$ -null cells adhere to fibronectin via $\alpha V\beta 3$ integrin (Wennerberg *et al.*, 1996). Here we show that GD25- $\beta 1B$ cells have reduced ability to spread on fibronectin and retain a stellate shape with numerous cytoplasmic protrusions (Figure 3A, b). Both untransfected GD25 and GD25- $\beta 1A$ cells, on the other hand, showed normal spreading capacity (Figure 3A, a and c). Differences in spreading capacity between GD25- $\beta 1B$ and

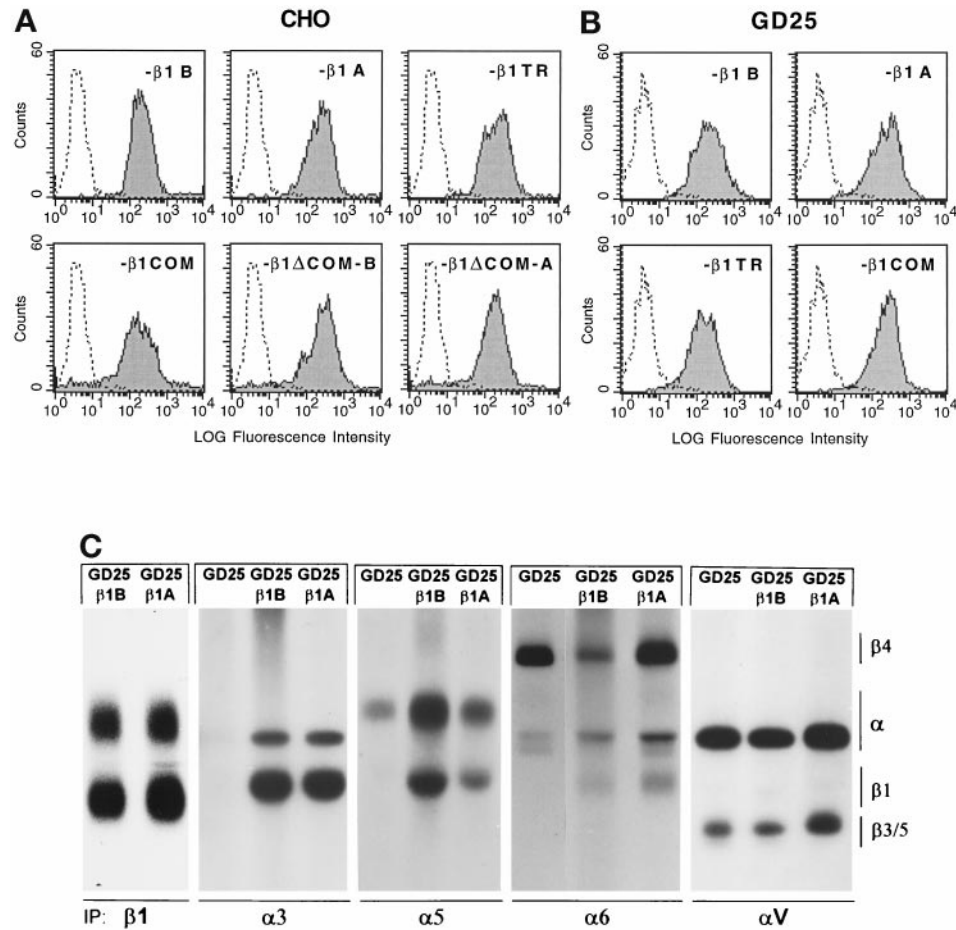


Figure 2. Surface expression of transfected $\beta 1$ variants in CHO and GD25 cells. CHO and GD25 cells were transfected with DNA constructs for human $\beta 1B$ or $\beta 1A$ or for the $\beta 1$ cytoplasmic domain deletion mutants indicated, and positive cells were sorted by panning on $\beta 1$ antibodies as described in MATERIALS AND METHODS. (A and B) To detect surface expression of transfected $\beta 1$ variants, CHO (A) and GD25 (B) transfectants were stained with mAb TS2/16 to human $\beta 1$, followed by fluorescein-labeled anti-mouse IgG, and analyzed by FACS. The level of $\beta 1$ surface expression reported as fluorescence intensity is shown. Untransfected cells were included as a negative control. (C) Integrin heterodimers in ^{125}I -surface-labeled GD25, GD25- $\beta 1B$, and GD25- $\beta 1A$ cells as detected by nonreducing SDS-PAGE and autoradiography of immunoprecipitated integrins with antibodies specific for $\beta 1$, $\alpha 3$, $\alpha 5$, $\alpha 6$, and αV subunits.

GD25 cells were maximal 1 h after plating on fibronectin in serum-free medium.

This result in GD25 cells extends previous data obtained in CHO cells (Balzac *et al.*, 1994) and indicates that $\beta 1B$ is capable of a trans-dominant negative effect toward $\alpha V\beta 3$ integrin. Interestingly, neither $\beta 1TR$ nor $\beta 1COM$ caused reduced spreading when expressed both in GD25 (Figure 3A, d and e) and CHO cells (Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results). Moreover, also the $\beta 1\Delta COM-A$ and $\beta 1\Delta COM-B$ mutants did not interfere with cell spreading (Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results).

We then tested adhesion to laminin-1. GD25 cells do not adhere to laminin-1, allowing the direct evaluation of adhesive properties of $\beta 1$ cytoplasmic domain variants in the absence of functional endogenous integrins. Although expression of $\beta 1A$ restored the ability of GD25 cells to adhere and spread on laminin-1, expression of the $\beta 1B$ isoforms did not (Figure 3B, a–c), indicating that $\alpha/\beta 1B$ heterodimers do not bind efficiently to laminin-1. Lack of adhesion to laminin-1

was also observed in $\beta 1TR$ - and $\beta 1COM$ -transfected GD25 cells (Figure 3C).

To assay whether the adhesive capacity of GD25- $\beta 1B$ cells can be modified, we tested Mn^{++} ions, which are known to increase binding affinity of integrins for their ligands (Gailit and Ruoslahti, 1988). Plating in the presence of 1 mM Mn^{++} restored adhesive capacity to laminin-1 of GD25- $\beta 1B$ (Figure 3, B, b and e, and C) but not of GD25- $\beta 1TR$ and GD25- $\beta 1COM$ cells (Figure 3C), thus indicating that the $\beta 1B$ isoform has unique functional properties with respect to the artificial mutants. Blocking antibodies to human $\beta 1$ (mAb AIIB2) prevented Mn^{++} -dependent adhesion in GD25- $\beta 1B$ cells, indicating that Mn^{++} induces $\beta 1B$ to bind its ligand (Figure 3C). Mg^{++} and Ca^{++} were also tested and found to be ineffective in inducing cell adhesion to laminin-1 (Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results). Interestingly, adhesion of GD25- $\beta 1B$ cells to laminin-1 resulted in poor spreading with respect to GD25- $\beta 1A$ cells (Figure 3B, e and f).

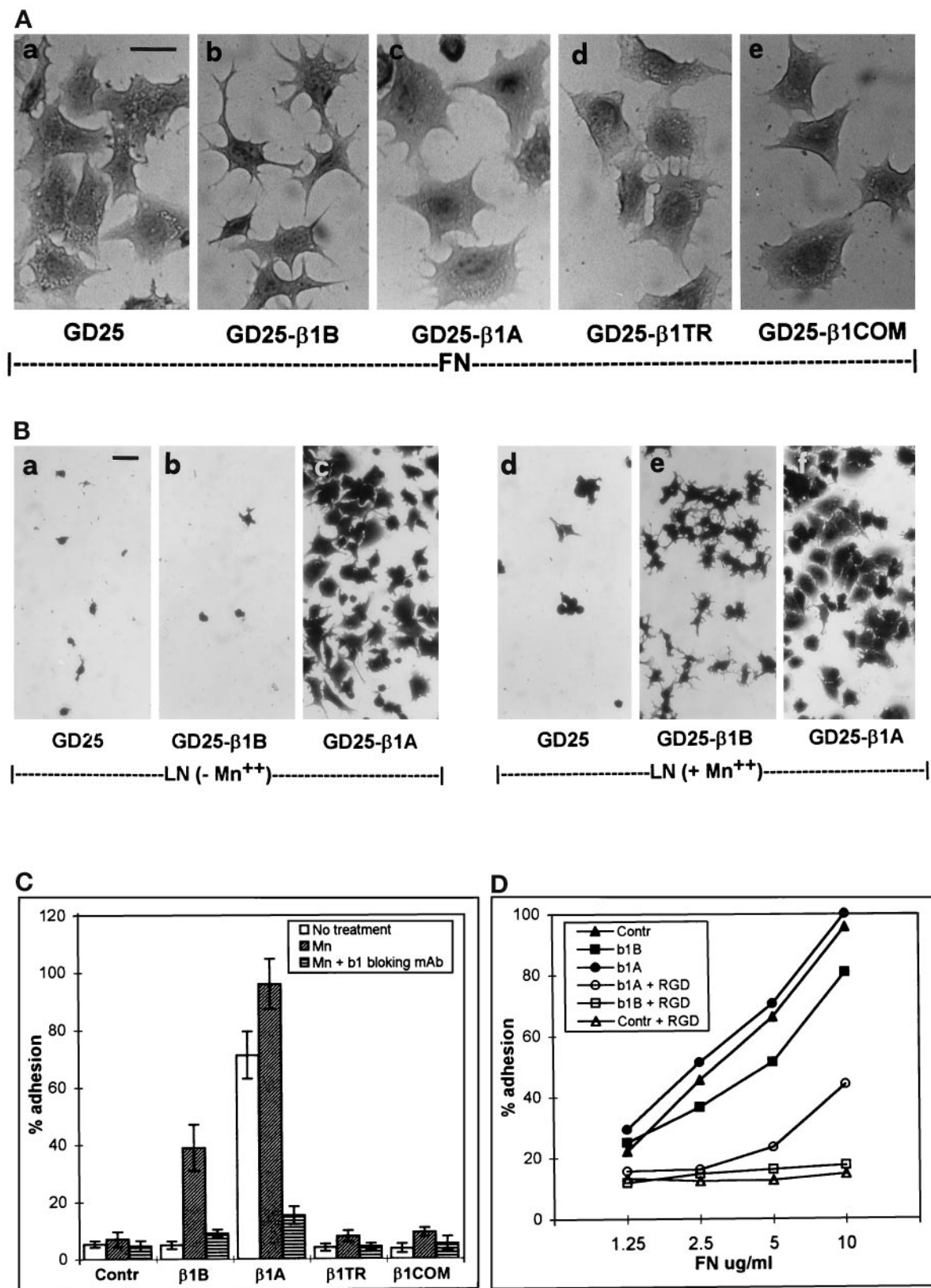


Figure 3.

To test whether lack of adhesion of $\beta 1B$ -transfected cells was restricted to laminin-1, we examined the attachment of these cells to fibronectin-coated dishes. Previous results showed that expression of $\beta 1A$ in GD25 cells leads to altered susceptibility to the Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) peptide (Wennerberg *et al.*, 1996). In agreement with this, although adhesion of GD25 cells to fibronectin was completely inhibited by 500 $\mu\text{g}/\text{ml}$ GRGDSP peptide, only 50% inhibition was observed in GD25- $\beta 1A$ cells (Figure 3D), indicating that transfected $\beta 1A$ modifies adhesive properties of GD25 cells to fibronectin. On the other hand 500 $\mu\text{g}/\text{ml}$ GRGDSP peptide fully inhibited adhesion of GD25- $\beta 1B$ on fibronectin, as in the case of GD25 cells (Figure 3D), indicating that the $\beta 1B$ molecule does not significantly contribute to cell adhesion to fibronectin.

Cytoplasmic Domain Sequences Affect $\beta 1$ -Integrin Ectodomain Conformation

The data discussed above suggest that integrin heterodimers containing the $\beta 1B$ subunit are expressed at the cell surface in a conformation that does not allow efficient binding to matrix ligands. To investigate this possibility further, we used two antibodies, mAb 12G10 and mAb 9EG7, which recognize epitopes expressed only in the ligand-competent and ligand-occupied $\beta 1$ (Lenter *et al.*, 1993; Mould *et al.*, 1995). Expression of these epitopes reflects changes in integrin ectodomain conformation, which can also be induced by exposing $\beta 1$ to Mn^{++} (Bazzoni *et al.*, 1995; Mould *et al.*, 1995). The binding of both 12G10 and 9EG7 mAbs to the $\beta 1$ extracellular domain was always compared with that obtained with mAb 13, which recognizes a constitutive epitope in human $\beta 1$. When

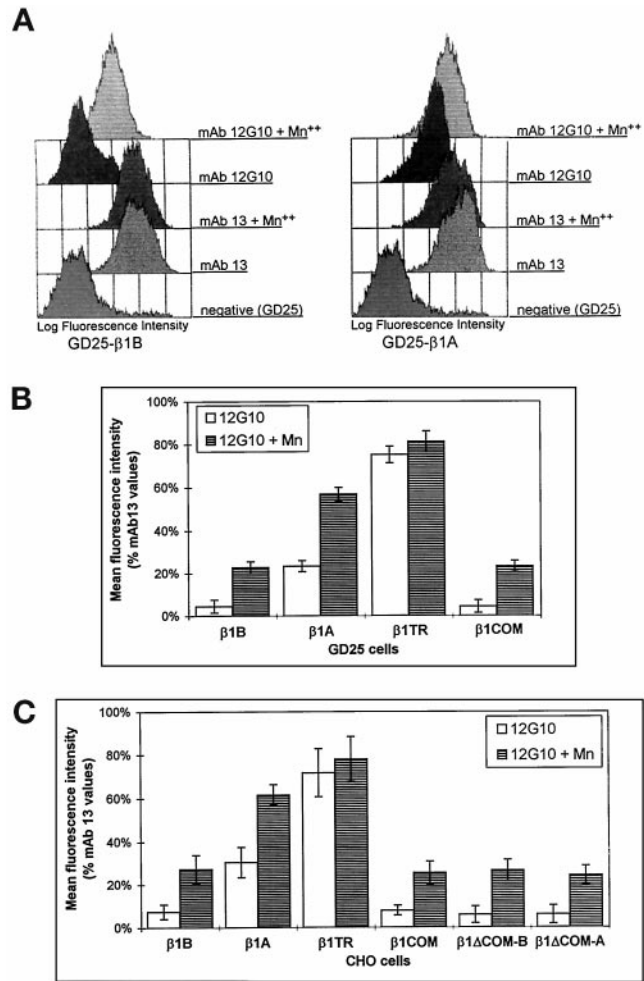


Figure 4. Effect of Mn^{++} on the conformation of $\beta 1B$, $\beta 1A$, and the four mutants as detected by 12G10 mAb. (A) GD25- $\beta 1B$ (left) and GD25- $\beta 1A$ (right) cells were analyzed by flow cytometry for surface expression of the 12G10 mAb epitope in the presence or absence of 1 mM Mn^{++} . As a control, cells were also stained with mAb 13, which recognizes an Mn^{++} -insensitive $\beta 1$ epitope. GD25 cells were used as a negative control. (B and C) The histograms show the level of expression of the 12G10 mAb epitope in $\beta 1$ -transfected GD25 (B) and CHO (C) cells in the absence (open bars) or presence (striped bars) of 1 mM Mn^{++} . Mean fluorescence intensities are expressed \pm SE as percentage of mAb 13 values and represent the mean of five experiments.

Figure 3 (facing page). Adhesion and spreading of $\beta 1$ variant-transfected GD25 cells on fibronectin and laminin-1. Dishes were coated with either fibronectin or laminin-1, and cells were plated in serum-free medium for 1 h at 37°C. (A) The morphology of cells adherent to fibronectin (10 $\mu\text{g}/\text{ml}$ coating) is shown for GD25 (a), GD25- $\beta 1B$ (b), GD25- $\beta 1A$ (c), GD25- $\beta 1TR$ (d), and GD25- $\beta 1COM$ (e). (B) The morphology of the cells plated on laminin-1 (10 $\mu\text{g}/\text{ml}$ coating) in the absence (a–c) and presence of 1 mM Mn^{++} (d–f) is shown for GD25 (a and d), GD25- $\beta 1B$ (b and e), and GD25- $\beta 1A$ (c and f). Similar results were obtained using increasing doses of laminin-1 up to 50 $\mu\text{g}/\text{ml}$. Note the severe reduction of spreading in GD25- $\beta 1B$ cells both on fibronectin and laminin-1. Bars, 25 μm . (C) The histograms show attachment of cells to 10 $\mu\text{g}/\text{ml}$ of laminin-1 in the absence (open bars), in the presence of 1 mM Mn^{++} (slashed bars), and in the presence of 1 mM Mn^{++} plus the $\beta 1$ blocking mAb A11B2 (striped bars). Results are plotted \pm SE as percentage of input cells and are the mean of three experiments. (D) The graph shows cell attachment to increasing doses of fibronectin both in the absence (closed symbols) and in the presence of 500 $\mu\text{g}/\text{ml}$ of GRGDSP peptide (open symbols). Adhesion is expressed as percentage of an equal number of input cells. Symbols used are as follows: GD25 (\blacktriangle and \triangle), GD25- $\beta 1B$ (\blacksquare and \square), and GD25- $\beta 1A$ (\bullet and \circ).

cells were probed with mAb 12G10, virtually no binding was detected on GD25- $\beta 1B$ and GD25- $\beta 1COM$ cells, unless Mn^{++} was present in the medium (Figure 4, A and B). On the contrary, a high level of mAb 12G10 binding was detected on GD25- $\beta 1A$ and GD25- $\beta 1TR$ cells, addition of Mn^{++} causing a further increase (Figure 4, A and B). By comparing the binding values of mAb 12G10 with those of mAb 13, we estimated that only 5–8% of the $\beta 1B$ at the cell surface expressed the 12G10 mAb epitope compared with 25–30% for $\beta 1A$. Similar results were obtained with

mAb 9EG7 (Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results).

Expression of the above $\beta 1$ variants in CHO cells confirmed the data obtained with GD25 transfectants (Figure 4C). Moreover, in the CHO system we also found that $\beta 1\Delta\text{COM-B}$ and $\beta 1\Delta\text{COM-A}$ variants, like $\beta 1\text{B}$ and $\beta 1\text{COM}$, do not express the mAb 12G10 epitope unless exposed to Mn^{++} ions (Figure 4C).

These data show that all of the $\beta 1$ variants analyzed changed the ectodomain conformation after exposure to Mn^{++} ions. Despite this, after Mn^{++} activation only $\beta 1\text{B}$, but none of the artificial $\beta 1$ mutants, was able to support cell adhesion, thus indicating that both the ectodomain conformation and specific cytoplasmic subdomains are required for adhesive function. The adhesive function of Mn^{++} -activated $\beta 1\text{B}$, however, is not comparable to that of $\beta 1\text{A}$, because GD25- $\beta 1\text{B}$ cells cannot reach full spreading (see Figure 3B, e).

$\beta 1\text{B}$ Inhibits Fibronectin Matrix Assembly

The assembly of fibronectin matrix in control and transfected cells was evaluated by immunofluorescence staining of cell monolayers incubated with purified human plasma fibronectin as described in MATERIALS AND METHODS.

GD25 untransfected cells assemble thin fibronectin fibrils on the cell apical surface (Figure 5). This process was inhibited by an αV -blocking antibody (Figure 5, A, a and b, and B), indicating that $\alpha\text{V}\beta 3/5$ integrins mediate matrix assembly in these cells as suggested previously (Wennerberg *et al.*, 1996). Expression of $\beta 1\text{B}$ in GD25 cells resulted in dramatic inhibition of fibronectin matrix assembly (Figure 5A, c), thus indicating that $\beta 1\text{B}$ interferes with αV -containing endogenous integrins. Quantitative data, obtained by measuring ^{125}I -labeled fibronectin incorporation in a detergent-insoluble matrix (Figure 5B), indicated a fivefold reduction in matrix assembly in GD25- $\beta 1\text{B}$ compared with parental cells. Interestingly, the expression of the two mutants $\beta 1\text{COM}$ (Figure 5A, e and f) and $\beta 1\text{TR}$ (Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results) differed from $\beta 1\text{B}$ and did not interfere with matrix assembly. Moreover, expression of $\beta 1\text{A}$ in GD25 cells resulted in increased assembly of fibronectin matrix compared with untransfected cells (Figure 5A, g and h).

To further analyze the $\beta 1\text{B}$ dominant negative effect, we assayed the fibronectin matrix assembly ability of CHO cells expressing the $\beta 1\text{B}$ or $\beta 1\text{A}$ isoform as well as the four $\beta 1$ mutants. As shown in Figure 6, also in CHO cells the expression of $\beta 1\text{B}$ resulted in a dominant negative effect; moreover, none of the other $\beta 1$ constructs showed this ability. Exogenous fibronectin

assembly in these cells is driven by the $\alpha 5\beta 1$ -integrin, because this function was blocked by the anti-hamster $\alpha 5\beta 1$ mAb PB1 (Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results). This result indicates that in CHO cells $\beta 1\text{B}$ interferes with the endogenous $\alpha 5\beta 1$ function.

Thus, the ability of $\beta 1\text{B}$ to block fibronectin matrix assembly in both GD25 and CHO cells indicates that this molecule is able to interfere with different classes of integrins involved in this process.

Expression of $\beta 1$ -Integrin Variants Specifically Affects Focal Adhesion and Stress Fiber Organization

Given the fact that $\beta 1\text{B}$ impaired cell spreading and fibronectin matrix assembly, we also examined its ability to affect focal adhesion organization. As shown in Figure 7a, GD25 cells form αV -containing prominent focal adhesions when plated on fibronectin-coated dishes, consistent with the reported ability of $\alpha\text{V}\beta 3$ to support attachment to fibronectin in these cells (Wennerberg *et al.*, 1996). GD25- $\beta 1\text{B}$ cells plated on fibronectin showed a clear reduction of αV -containing focal adhesions when compared with GD25 cells (Figure 7, a and c). On the contrary, GD25- $\beta 1\text{COM}$ (Figure 7e) and GD25- $\beta 1\text{TR}$ (Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results) cells did not show a reduction in αV -containing focal adhesions.

$\beta 1\text{B}$, $\beta 1\text{COM}$ (Figure 7, d and f), and $\beta 1\text{TR}$ (Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results) molecules were uniformly distributed on the cell surface, as shown by immunofluorescence; thus $\beta 1\text{B}$ displaced αV heterodimers without localizing to focal adhesions.

We then tested whether focal adhesion localization of cytoskeletal proteins, such as paxillin, and actin organization were also affected. As shown in Figure 8, $\beta 1\text{B}$, but not $\beta 1\text{A}$ or $\beta 1\text{COM}$, was able to reduce the number of paxillin-containing focal ad-

Figure 5 (facing page). Exogenous fibronectin matrix assembly in control and transfected GD25 cells in the presence of an anti- αV -blocking antibody. (A) Confluent cell monolayers were cultured for 2 d with 200 nM human plasma fibronectin either in the absence (a, c, e, and g) or in the presence (b, d, f, and h) of the inhibitory mAb H9.2B8 against mouse αV , used to block the endogenous αV integrins. Cells were then fixed for 10 min in 3.7% (v/v) paraformaldehyde in PBS, and the fibronectin in the matrix was stained by a polyclonal antibody followed by a rhodamine-labeled anti-rabbit IgG. Bar, 50 μm . (B) Biochemical evaluation of fibronectin matrix assembly. ^{125}I -labeled fibronectin incorporated into deoxycholate-insoluble matrix of cells either in the absence (–) or in the presence (+) of the αV -blocking H9.2B8 mAb was visualized by SDS-PAGE and autoradiography. The molecular weight of reduced fibronectin is shown.

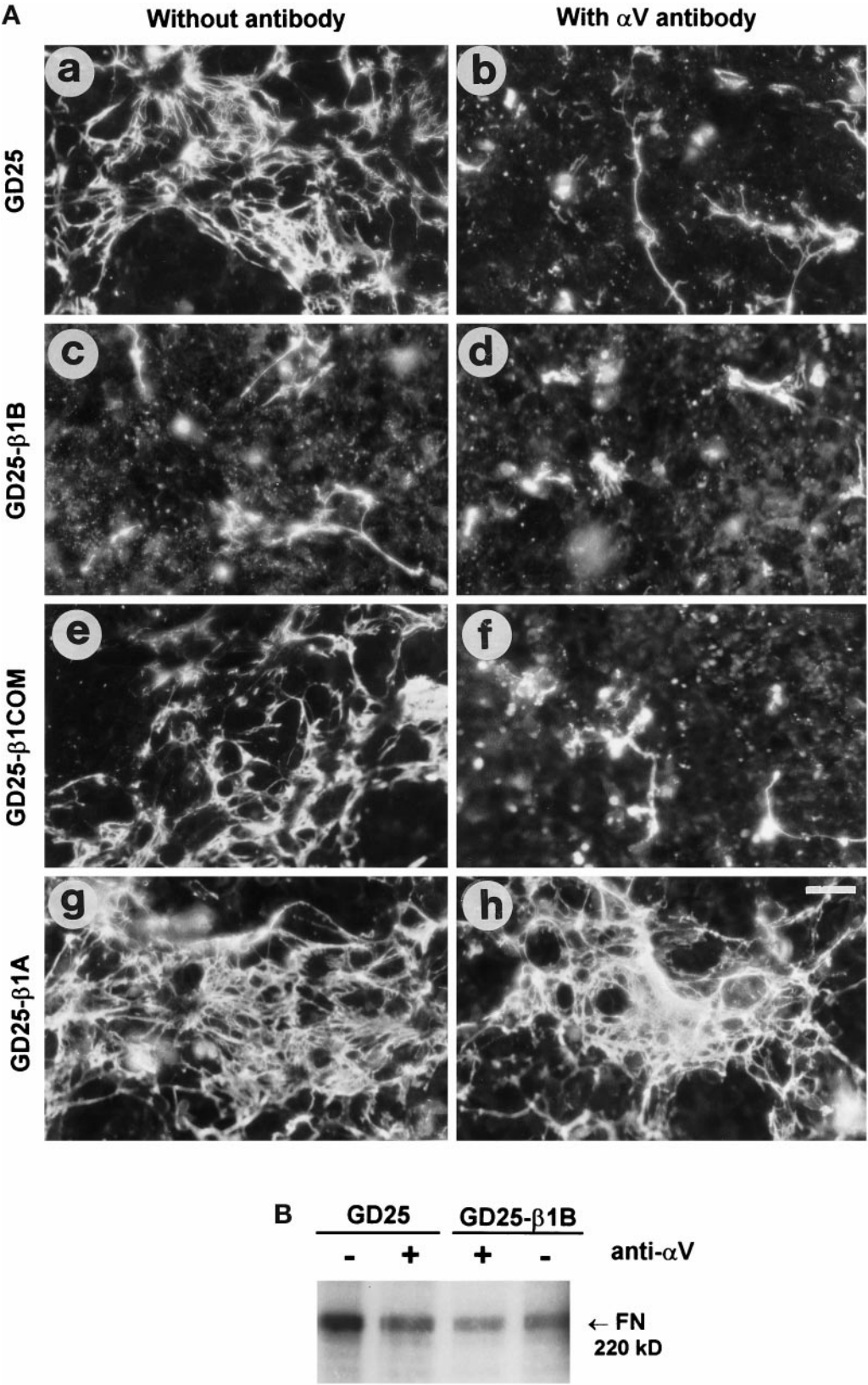


Figure 5.

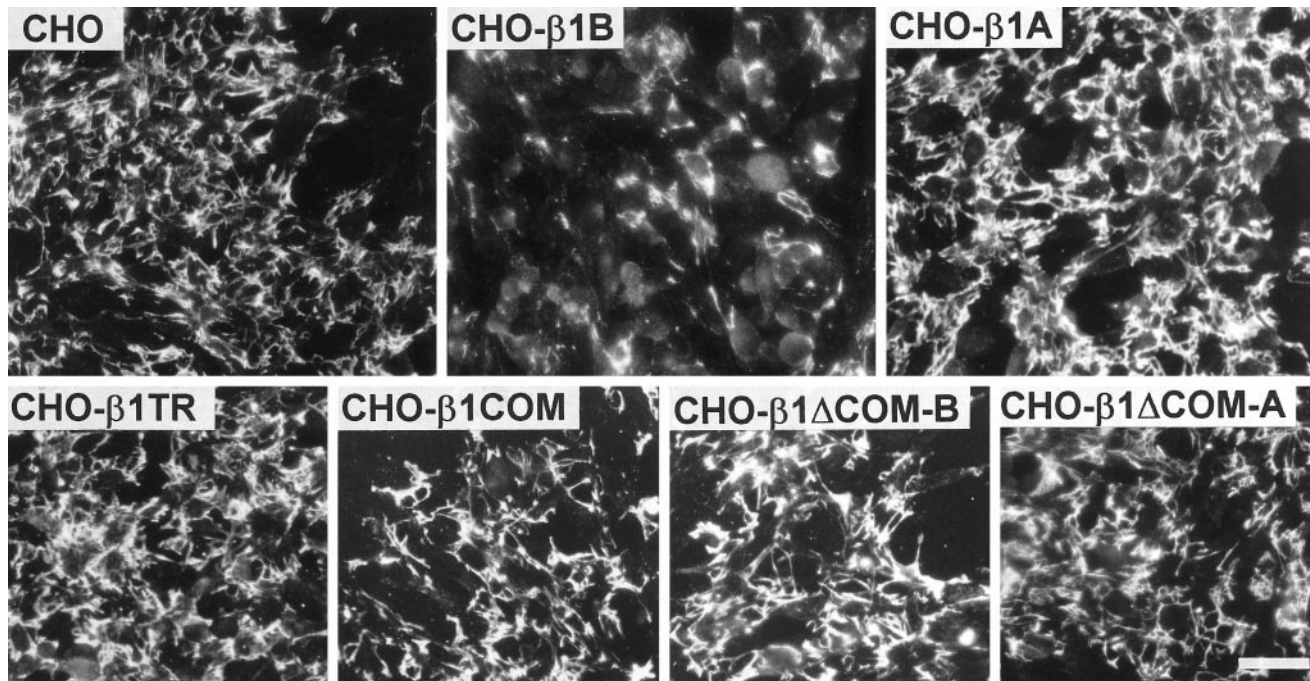


Figure 6. Fibronectin matrix assembly in control and transfected CHO cells. Confluent cell monolayers were cultured for 2 d with 200 nM human plasma fibronectin as described in MATERIALS AND METHODS. Cells were then fixed for 10 min in 3.7% (v/v) paraformaldehyde in PBS, and the fibronectin in the matrix was stained by a specific polyclonal antibody followed by a rhodamine-labeled anti-rabbit IgG. Note the dramatic reduction of fibronectin assembly in β 1B-expressing cells. Bar, 80 μ m.

hesions (Figure 8a–g). Similar results were obtained when we analyzed actin stress fibers. In fact, β 1B-expressing cells showed only residual stress fibers in cell protrusions (Figure 8d), whereas cells expressing the other β 1 constructs did not show significant changes compared with untransfected cells (Figure 8h). The interference of β 1B on stress fiber organization was also supported by analysis of cell contractility by plating cells on silicon rubber films (Harris *et al.*, 1980; Danowski, 1989). As shown in Figure 9, GD25 cells are able to pull on the substrate, inducing wrinkles in the silicon film. On the other hand, no wrinkles were observed in dishes seeded with GD25- β 1B cells. Altogether, these data show a specific dominant negative effect of β 1B on focal adhesion and stress fiber organization.

β 1B Has a Dominant Negative Effect on FAK Tyrosine Phosphorylation

By plating CHO cells on dishes coated with antibodies specific for the transfected human β 1-integrin, we have previously shown that β 1B is unable to trigger FAK tyrosine phosphorylation (Balzac *et al.*, 1994). Given the dominant negative effect of β 1B on cell adhesive functions, we tested the ability of this molecule to interfere with endogenous integrin signaling.

CHO cells expressing different β 1-integrin cytoplasmic variants were plated on dishes coated with anti-hamster β 1, antibody and tyrosine phosphorylation of FAK was evaluated by Western blot. As shown in Figure 10, A and B, endogenous hamster β 1A had a reduced capacity to induce FAK tyrosine phosphorylation in cells expressing β 1B but not in cells expressing human β 1A or the four β 1 cytoplasmic domain mutants (Figure 10C). At the same time, the four β 1 mutants, like β 1B, were not capable of triggering FAK tyrosine phosphorylation to a significant extent (Figure 10D).

Association of Talin and α -Actinin with β 1 Cytoplasmic Domain Variants

To evaluate the ability of different β 1 cytoplasmic domain variants to interact with talin, α -actinin, and FAK, we performed coimmunoprecipitation studies by extracting cells under mild detergent conditions (see MATERIALS AND METHODS). As detected by Western blots, talin was coprecipitated with β 1A but not with β 1B, β 1COM, or β 1TR (Figure 11). On the other hand, α -actinin was coprecipitated with β 1A, β 1B, and β 1COM but not with β 1TR (Figure 11). The amount of α -actinin associated with β 1B and β 1COM immunoprecipitates, however, was reduced compared with β 1A immunoprecipitate.

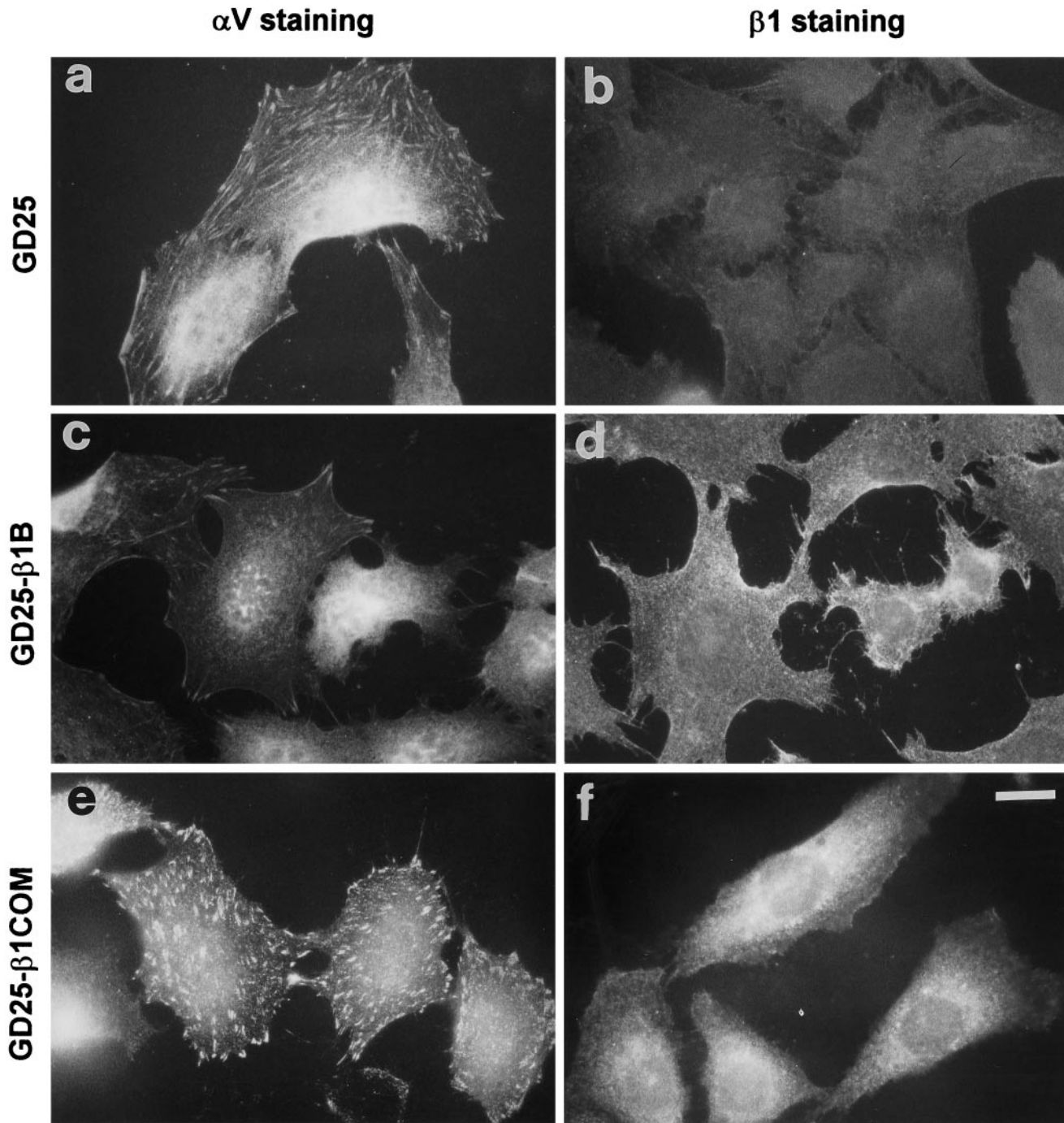


Figure 7. Localization of α V- and $\beta 1$ -integrin subunits to focal adhesion in control and transfected GD25 cells. Cells were plated on 10 μ g/ml fibronectin-coated glass coverslips for 3 h in complete culture medium. Cells were then fixed for 10 min in 3.7% (v/v) paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 and 3.7% formaldehyde in PBS for 5 min, and stained for α V (A, C, E, G) or $\beta 1$ (B, D, F, H) integrins by specific antibodies followed by rhodamine-labeled secondary antibodies. Note the reduction of α V-containing focal adhesion in $\beta 1$ B-transfected cells. Bar, 15 μ m.

FAK was not detected in the immunocomplexes associated with any of the four $\beta 1$ molecules tested

(Retta, Balzac, Ferraris, Belkin, Fässler, Humphries, De Leo, Silengo, and Tarone, unpublished results).

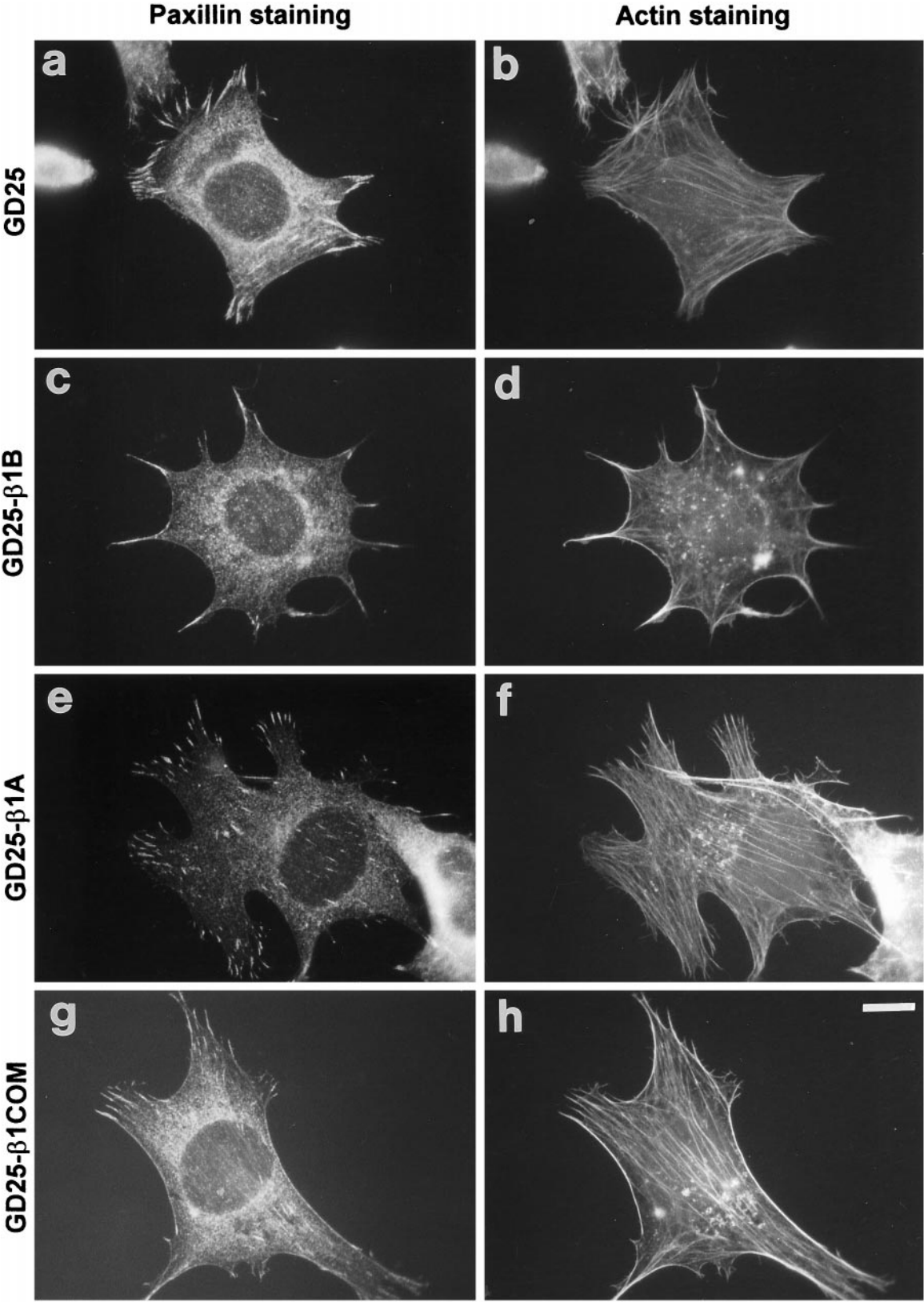


Figure 8.

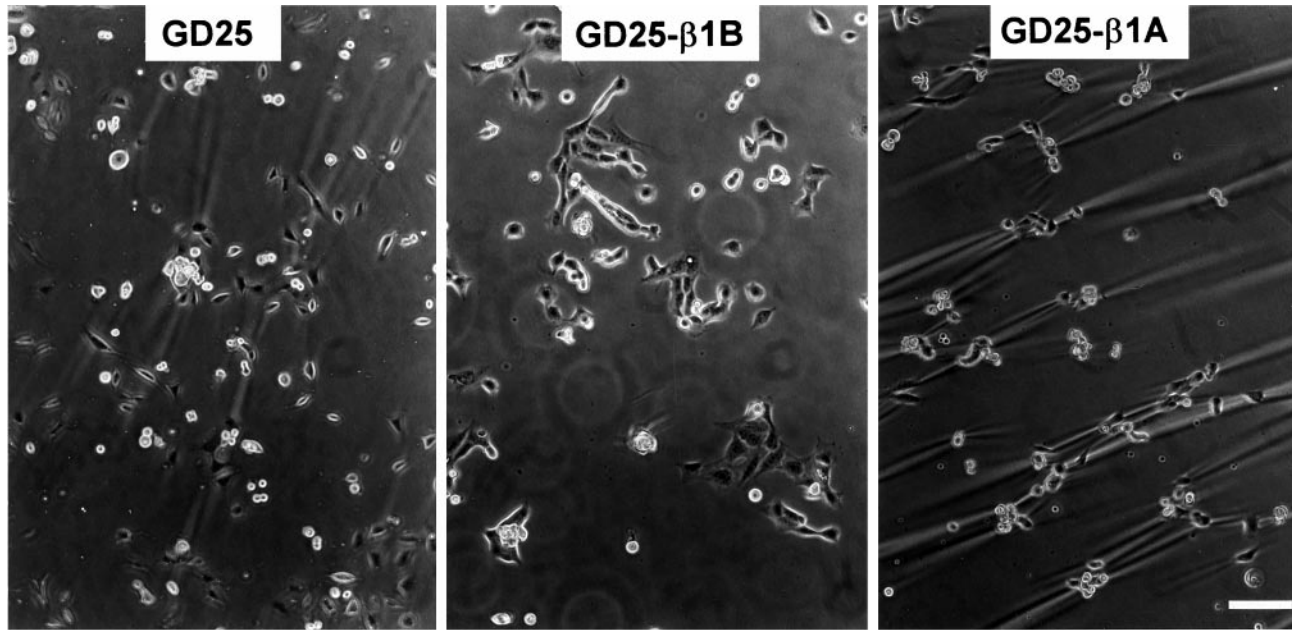


Figure 9. Rubber substrate contractility assay. GD25, GD25- $\beta 1B$, and GD25- $\beta 1A$ cells were plated for 1 d on silicone rubber substrata and photographed under phase contrast. Note the incapacity of GD25- $\beta 1B$ cells to wrinkle the silicon substrate. Bar, 50 μm .

These data show that $\beta 1B$ is incapable of interacting with talin but retains the ability to bind α -actinin. This property is shared with $\beta 1COM$ variants.

DISCUSSION

We have previously shown that $\beta 1B$, an integrin isoform with a distinct cytoplasmic domain, causes reduced cell spreading and migration when expressed in CHO cells (Balzac *et al.*, 1994). In the present study we have examined the role of specific $\beta 1$ cytoplasmic subdomains in determining this dominant negative function. To achieve this aim, we have constructed four deletion mutants (see Figure 1): $\beta 1TR$, lacking the entire cytoplasmic domain; $\beta 1COM$, lacking the cytoplasmic distal subdomain; and $\beta 1\Delta COM-B$ and $\beta 1\Delta COM-A$, in which the distal B and A subdomains are linked to the transmembrane segment by deletion of the common region.

These molecules were expressed both in GD25 cells, which lack the endogenous $\beta 1$ as a consequence of

gene knockout (Wennerberg *et al.*, 1996), and in CHO cells. Adhesion experiments in GD25 cells showed that $\beta 1B$ is unable to support cell adhesion. In fact, GD25- $\beta 1B$ cells, like untransfected GD25 cells, do not adhere to laminin-1, despite correct surface expression of $\alpha 3\beta 1B$ -, $\alpha 5\beta 1B$ -, and $\alpha 6\beta 1B$ -integrin heterodimers. Moreover, $\beta 1B$ -containing heterodimers are not functional in mediating adhesion to fibronectin. On the other hand, $\beta 1A$ supports adhesion of GD25 cells to laminin-1 and fibronectin. $\beta 1TR$ and $\beta 1COM$ behave as $\beta 1B$, being unable to support cell adhesion to laminin-1.

The inability of $\beta 1B$ complexes to mediate cell adhesion can be rescued by treating cells with Mn^{++} ions, which are known to increase binding affinity of integrins for their ligands (Gailit and Ruoslahti, 1988). Mn^{++} treatment, however, did not restore the adhesive function of $\beta 1TR$ - and $\beta 1COM$ -transfected GD25 cells, indicating that the $\beta 1B$ cytoplasmic domain confers specific adhesive function.

Mn^{++} ions affect conformation of the $\beta 1$ ectodomain. This was confirmed by using 12G10 and 9EG7 mAbs, which recognize conformation-specific epitopes in the $\beta 1$ ectodomain. Both mAbs bind very poorly to $\beta 1B$ compared with $\beta 1A$. Addition of Mn^{++} to the medium strongly increases 12G10 and 9EG7 mAb binding to $\beta 1B$. Interestingly, $\beta 1TR$, which does not support adhesion to laminin-1 even in the presence of Mn^{++} ions, constitutively expresses 12G10 and 9EG7 epitopes, indicating that ectodomain conformation is not sufficient to endow $\beta 1$ with adhesive prop-

Figure 8 (facing page). Focal adhesion and stress fiber organization in control and transfected GD25 cells. Cells were plated on 10 $\mu g/ml$ fibronectin-coated glass coverslips for 3 h in complete culture medium. Cells were then fixed for 10 min in 3.7% (v/v) paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 and 3.7% formaldehyde in PBS for 5 min, and double stained for paxillin (A, C, E, G) and F-actin (B, D, F, H) as described in MATERIALS AND METHODS. Note the reduction of stress fibers and paxillin-containing focal adhesions in GD25- $\beta 1B$ cells. Bar, 10 μm .

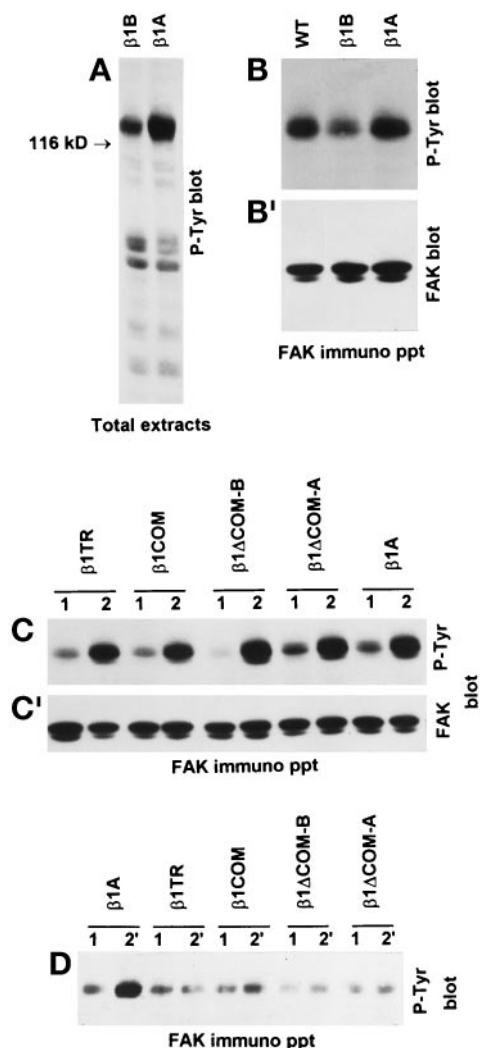


Figure 10. Inhibition of $\beta 1A$ -induced FAK tyrosine phosphorylation by $\beta 1B$ expression. To specifically trigger tyrosine phosphorylation of intracellular proteins mediated by the endogenous or transfected $\beta 1$ integrins, cells were plated on plastic dishes coated with the anti-hamster $\beta 1$ mAb 7E2 (A–C) or with the anti-human $\beta 1$ mAb TS2/16 (D), respectively. (A and B) Tyrosine phosphorylation of total cellular proteins (A) and FAK (B) induced by endogenous hamster $\beta 1A$ was detected by Western blotting with anti-phosphotyrosine mAb PY20. Note reduced tyrosine phosphorylation of 116- to 130-kDa proteins (A) and FAK (B) in $\beta 1B$ -transfected CHO cells. (B') The reprobing of the blot with FAK antibodies is shown. (C) The tyrosine phosphorylation of FAK via endogenous $\beta 1A$ was also evaluated in CHO cells expressing the different cytoplasmic domain mutants. Cells were plated on poly-L-lysine (lanes 1) for control and on 7E2 mAb to endogenous hamster $\beta 1$ (lanes 2). (C') The reprobing of the blot with FAK antibodies is shown. (D) The ability of the different mutants to trigger FAK tyrosine phosphorylation directly was evaluated by plating cells on mAb TS2/16 to the transfected human $\beta 1$ (lanes 2') or poly-L-lysine as control (lanes 1).

erties, but cytoplasmic sequences are also required. On the other hand, the $\beta 1COM$ variant, similarly to $\beta 1B$, expresses 12G10 and 9EG7 epitopes in response

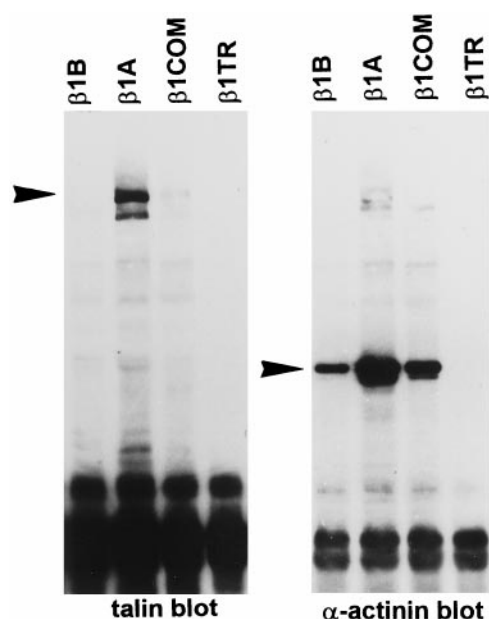


Figure 11. Association of talin and α -actinin with $\beta 1$ cytoplasmic domain variants. CHO cells transfected with $\beta 1B$, $\beta 1A$, $\beta 1COM$, and $\beta 1TR$ integrin variants were incubated in suspension with TS2/16 mAb to human $\beta 1$, and cells were lysated in digitonin-containing buffer. Immunocomplexes were recovered from cell extracts with protein G-Sepharose beads and analyzed by Western blotting with talin antibody. The blot was stripped and reprobed with α -actinin antibody. Arrowheads point to the talin and α -actinin bands.

to Mn^{++} , but it does not support cell adhesion. Thus, the conserved common region of the $\beta 1$ cytoplasmic domain, present in the $\beta 1COM$ mutant, is not sufficient to support cell adhesion but needs to be linked to either the A or B distal subdomain. Although the combination COM + A gives rise to a fully functional molecule, COM + B is only partially functional, because it requires Mn^{++} to support attachment and does not allow cell spreading.

The results presented show that $\beta 1B$ and the four $\beta 1$ cytoplasmic domain mutants $\beta 1TR$, $\beta 1COM$, $\beta 1\Delta COM-A$, and $\beta 1\Delta COM-B$ are incapable of inducing fibronectin matrix assembly. Interestingly, however, $\beta 1B$ shows a strong capacity to inhibit matrix assembly controlled by endogenous integrins, a property that is not shared by $\beta 1TR$, $\beta 1COM$, $\beta 1\Delta COM-A$, and $\beta 1\Delta COM-B$. Fibronectin matrix assembly involves different receptors in CHO and GD25 $\beta 1$ -null cells. Although $\alpha 5\beta 1$ is the major CHO integrin receptor involved in this function, in GD25 cells this role is played by αV -containing integrin complexes. The ability of $\beta 1B$ to inhibit fibronectin matrix assembly in both cell types indicates a dominant negative effect on both $\alpha 5\beta 1$ and $\alpha V\beta 3/\beta 5$ integrins. Work in progress indicates that $\beta 1B$ also inhibits fibronectin matrix assembly in epithelial cells and primary mouse fibro-

blasts, thus showing that this is a general property of β 1B in several cell types.

We also found a strong effect of β 1B on focal adhesion and actin stress fiber organization. In fact, in GD25 cells that adhere to fibronectin via α V β 3, β 1B expression led to a reduced number of α V-containing focal adhesions. In these cells β 1B is uniformly diffuse at the cell surface but does not associate with the α V subunit; thus reduction of α V localization to focal adhesion cannot be explained by competitive displacement. On the other hand, paxillin and F-actin staining showed an overall reduction of focal adhesions and stress fibers in GD25- β 1B cells. This reduction could explain the GD25- β 1B cells' poor spreading on fibronectin as well as the reduced contractility on silicon rubber film. The interference with actin cytoskeleton organization is specific for the β 1B isoform, because neither β 1TR nor β 1COM leads to this effect.

To investigate at a molecular level the dominant negative effects of β 1B, we analyzed the signal transduction ability of endogenous integrins in cells expressing the β 1 cytoplasmic domain variants. The results obtained showed that β 1B clearly exerts a dominant negative effect on the ability of endogenous β 1 to trigger FAK tyrosine phosphorylation, whereas neither β 1A nor the four β 1 mutants showed this effect.

Altogether, these data show that β 1B interferes with adhesive functions of integrins by inhibiting FAK tyrosine phosphorylation, focal adhesion and stress fiber organization, cell spreading, and fibronectin matrix assembly. This dominant negative function is specific to β 1B.

Both the ectoplasmic and cytoplasmic domains of β 1B are functionally altered. We have shown, in fact, that the β 1B extracellular domain is exposed at the cell surface in a conformation that cannot support cell adhesion; in addition, β 1B is defective in two properties depending on the cytoplasmic domain, namely localization to focal adhesions and triggering of FAK tyrosine phosphorylation (Balzac *et al.*, 1994). Functional analysis of the four β 1 cytoplasmic domain mutants presented in this work allows us to conclude that the dominant negative effect does not depend on the altered functional properties of the β 1B ectodomain. In fact, the β 1COM mutant, which is exposed at the cell surface in a conformation that cannot support cell adhesion like β 1B, does not act as a dominant negative molecule. Moreover, the β 1TR mutant, lacking the entire cytoplasmic domain, does not show a dominant negative action. These data indicate that the β 1B cytoplasmic domain is responsible for the dominant negative effect. In addition, the β 1COM and β 1 Δ COM-B mutants indicate that the membrane proximal common and the variable B subdomains alone are not sufficient for this function. We can conclude

that the combination of common and B subdomains is required to confer the dominant negative effect.

Among the possible mechanisms at the basis of the dominant negative function, we can hypothesize that β 1B may act by generating specific negative signals, or it may act by binding and sequestering cytoskeletal or signaling molecules crucial for integrin function. The latter possibility is less likely, as suggested by coprecipitation experiments; in fact, β 1B did not bind talin or FAK. β 1B was able to bind α -actinin, however, but this cannot account for the dominant negative effect, because similar binding was also observed in the case of β 1COM, which does not act as dominant negative.

In conclusion, our data show that the specific linkage of the β 1 cytoplasmic common subdomain with the variable A and B subdomains leads to specific functional properties, suggesting that splicing in the β 1 cytoplasmic domain plays a key role in generating β 1 isoforms that may serve to regulate adhesive functions specifically.

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