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Muscle β 1D Integrin Reinforces the Cytoskeleton–Matrix Link: Modulation of Integrin Adhesive Function by Alternative Splicing

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Abstract. Expression of muscle-specific β 1D integrin with an alternatively spliced cytoplasmic domain in CHO and GD25, β 1 integrin-minus cells leads to their phenotypic conversion. β 1D-transfected nonmuscle cells display rounded morphology, lack of pseudopodial activity, retarded spreading, reduced migration, and significantly enhanced contractility compared with their β 1A-expressing counterparts. The transfected β 1D is targeted to focal adhesions and efficiently displaces the endogenous β 1A and α v β 3 integrins from the sites of cell–matrix contact. This displacement is observed on several types of extracellular matrix substrata and leads to elevated stability of focal adhesions in β 1D transfectants. Whereas a significant part of cellular β 1A integrin is extractable in digitonin, the majority of the transfected β 1D is digitonin-insoluble and is strongly associated with the detergent-insoluble cytoskeleton.

Increased interaction of β 1D integrin with the actin cytoskeleton is consistent with and might be mediated by its enhanced binding to talin. In contrast, β 1A interacts more strongly with α -actinin, than β 1D. Inside-out driven activation of the β 1D ectodomain increases ligand binding and fibronectin matrix assembly by β 1D transfectants. Phenotypic effects of β 1D integrin expression in nonmuscle cells are due to its enhanced interactions with both cytoskeletal and extracellular ligands. They parallel the transitions that muscle cells undergo during differentiation. Modulation of β 1 integrin adhesive function by alternative splicing serves as a physiological mechanism reinforcing the cytoskeleton–matrix link in muscle cells. This reflects the major role for β 1D integrin in muscle, where extremely stable association is required for contraction.

INTEGRINS are a large family of transmembrane heterodimeric receptors that play a key role in cell adhesion to extracellular matrix (Hynes, 1992). Integrin receptors serve a dual purpose, linking extracellular matrix to the actin cytoskeleton and providing bidirectional transmission of signals between the extracellular matrix and the cytoplasm (Schwartz et al., 1995; Yamada and Miyamoto, 1995; Burridge and Chrzanowska-Wodnicka, 1996). At least two major actin-binding proteins, talin and α -actinin, are thought to interact directly with the cytoplasmic domain of several β subunits, providing a link to the actin cytoskeleton (Horwitz et al., 1986; Otey et al., 1990; Hemler et al., 1994). Among integrins, β 1 is typically the most abundant and ubiquitously expressed subunit associated

with a number of α subunits to form distinct heterodimers. These interact with a variety of extracellular matrix and cell adhesion molecules (Hynes, 1992). The entire structure of the β 1 integrin cytoplasmic domain is critical for integrin–cytoskeleton interaction (Hayashi et al., 1990; LaFlamme et al., 1992; Reszka et al., 1992; Ylanne et al., 1993; Lewis and Schwartz, 1995).

Integrin functions within the cell can be regulated at different levels. These include cell type–specific biosynthesis of certain integrin heterodimers, maturation and processing of the receptors, as well as their transport to the cell surface (Hynes, 1992). Another level of control of integrin function is through regulation of the ligand-binding affinity of integrins on the cell surface. This type of regulation involves conformational changes within integrins. The conformational state of the extracellular domains (activation) of integrins is regulated via their cytoplasmic tails and is referred to as inside-out signaling (Ginsberg et al., 1992;

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O'Toole et al., 1994; Schwartz et al., 1995). Thus, deletions or mutations of certain residues in the cytoplasmic domains of α and β subunits can either increase or inhibit the ligand-binding activity of integrin receptors (Takada et al., 1992; O'Toole et al., 1994, 1995). The activation state of integrins can also be controlled by some lipid metabolites (Hermanowski-Vosatka et al., 1992; Smyth et al., 1993) and small GTP-binding proteins (Zhang et al., 1996; Hughes et al., 1997). Finally, functional properties of integrin receptors can be modulated by alternative splicing involving their cytoplasmic tails.

So far, four cytoplasmic domain variants of the $\beta 1$ integrin subunit have been described. Besides the major $\beta 1A$ isoform, characteristic for all known cell types except red blood cells and terminally differentiated striated muscles, two minor cytoplasmic domain isoforms of $\beta 1$ integrin, $\beta 1B$ and $\beta 1C$, have been characterized (Altruda et al., 1990; Languino and Ruoslahti, 1992). Although their functions remain uncertain, it has been speculated that $\beta 1B$ can serve as a negative regulator of cell adhesion during development, whereas $\beta 1C$ can strongly inhibit cell growth (Balzac et al., 1993, 1994; Meredith et al., 1995). The alternatively spliced sequences of $\beta 1B$ and $\beta 1C$ have no homology to the major $\beta 1A$ isoform and are unable to localize to cell-matrix adhesion sites apparently because of impaired interaction with the actin cytoskeleton (Balzac et al., 1993; Meredith et al., 1995). Interestingly, $\beta 1B$ and $\beta 1C$ variants have been found only in humans, whereas the fourth $\beta 1$ isoform, $\beta 1D$, is highly conserved at least throughout vertebrate evolution, suggesting an important role for this muscle-specific variant (van der Flier et al., 1995; Zhidkova et al., 1995; Baudoin et al., 1996; Belkin et al., 1996).

$\beta 1$ integrin is localized at junctional structures of striated muscles (Bozyczko et al., 1989). Expression of the $\beta 1$ integrin subunit as well as the ligand occupation of $\beta 1$ -containing heterodimers is essential for myodifferentiation and the formation of sarcomeric cytoarchitecture (Menko and Boettiger, 1987; Volk et al., 1990). Integrin-mediated cytoskeleton-matrix linkage has to be distinct in muscle cells because of high tensile forces transmitted across the membrane and enhanced stability of muscle adhesive structures. This implies a modified function for $\beta 1$ integrin in muscles. This function is now attributed primarily to $\beta 1D$ cytoplasmic domain variant, which is a major $\beta 1$ isoform that completely displaces $\beta 1A$ integrin in differentiated striated muscles (Belkin et al., 1996). Its cytoplasmic domain is highly homologous to that of $\beta 1A$, including conservation of both NPXY motifs involved in the regulation of ligand-binding affinity (Tamkun et al., 1986; Argraves et al., 1987; Zhidkova et al., 1995; van der Flier et al., 1995). $\beta 1D$ accumulates at all major cell-matrix adhesion sites both in skeletal muscle fibers and cardiomyocytes. $\alpha 7\beta 1D$ is a predominant integrin in adult skeletal and heart muscle tissues (Belkin et al., 1996). However, other α subunits, including $\alpha 5$ and $\alpha 6A$, can pair with $\beta 1D$ in developing heart muscle (Brancaccio et al., 1997). The data obtained so far lead to the suggestion that $\beta 1D$ integrin plays a crucial role in linking the subsarcolemmal cytoskeleton to the surrounding extracellular matrix in muscle tissues (Belkin et al., 1996; Fassler et al., 1996).

Upon transfection into nonmuscle cells, $\beta 1D$ is targeted to focal adhesions, proving that the muscle-specific iso-

form of $\beta 1$ integrin is able to interact with the nonmuscle cytoskeleton as well (Belkin et al., 1996). To get an insight in the functional properties of this integrin, we have expressed human $\beta 1D$ and $\beta 1A$ cytoplasmic domain isoforms in CHO cells and the mouse GD25 cell line. GD25 cells lack endogenous $\beta 1$ integrin as a consequence of gene inactivation (Wennerberg et al., 1996). Here we report that the expression of $\beta 1D$ integrin in nonmuscle cells leads to a conversion of cellular phenotype. The observed alterations in cell morphology, inhibition of spreading and motility, as well as an increase in the ligand-binding affinity, fibronectin matrix assembly and contractility, are caused by an enhanced association of $\beta 1D$ integrin with both the actin cytoskeleton and extracellular matrix ligands. $\beta 1D$ -mediated enhancement of actin-membrane attachment is, at least in part, due to a higher affinity interaction of this integrin with the focal adhesion protein, talin. The altered structure of the $\beta 1D$ cytoplasmic domain causes a conformational change of its ectodomain via inside-out signaling mechanisms, leading to activation of ligand binding. Reinforcement of the cytoskeleton-matrix association by $\beta 1D$ reflects a key role for this integrin as a cytoskeleton-matrix linker, strengthening adhesive structures in muscle tissues.

Materials and Methods

Antibodies and Reagents

The following antibodies against $\beta 1$ integrin were used in this study: TS2/16 mAb to human $\beta 1$ subunit, which activates ligand binding by $\beta 1$ -containing heterodimers was a gift from Dr. M. Hemler (Dana-Farber Cancer Institute, Boston, MA) (Hemler et al., 1984; Arroyo et al., 1992); function-blocking P4C10 mAb against human $\beta 1$ integrin (Carter et al., 1990) was from GIBCO BRL (Gaithersburg, MD); 102DF5 mAb against human $\beta 1$ integrin (Ylanne and Virtanen, 1989); 12G10 mAb, which reacts with activated (high affinity conformation for ligand binding) human $\beta 1$ (Mould et al., 1995); 9EG7 mAb reacting with ligand-, Mg^{2+} -, Mn^{2+} -induced, Ca^{2+} -inhibited epitope on human $\beta 1$ integrin subunit (Bazzoni et al., 1995); A1A5 mAb against human $\beta 1$ integrin (Hemler et al., 1984), conjugated with fluorescein and rabbit polyclonal antibody against human $\beta 1$ integrin (Belkin et al., 1990). 7E2 mAb against hamster $\beta 1$ integrin and inhibitory PB1 mAb against intact hamster $\alpha 5\beta 1$ heterodimer were generous gifts from Dr. R. Juliano (University of North Carolina, Chapel Hill, NC) (Brown and Juliano, 1985, 1988). Isoform-specific antibodies against $\beta 1A$ and $\beta 1D$ integrins were described earlier (Belkin et al., 1996).

Blocking anti-mouse αv H9.2B8 mAb (Moulder et al., 1991) was obtained from PharMingen (San Diego, CA). Rabbit polyclonal antibodies against αv , $\alpha 3$, and $\alpha 5$ cytoplasmic domains were described earlier (Defilippi et al., 1992; Balzac et al., 1994). mAb 8d4 against talin was obtained from Sigma Chemical Co. (St. Louis, MO) and mAb 1682 against α -actinin was from Chemicon International, Inc. (Temecula, CA). Rabbit polyclonal antibody against platelet myosin II, cross-reacting with nonmuscle myosin, was a gift from Dr. R. Adelstein (National Institutes of Health, Bethesda, MD). Rabbit polyclonal antibody against human plasma fibronectin (Fn) was provided by Dr. L.B. Chen (Dana-Farber Cancer Institute).

Human plasma Fn was from GIBCO BRL. Recombinant, 12-kD cell-binding Fn fragment corresponded to the tenth Arg-Gly-Asp-containing (cell-binding), type III Fn repeat. Cytochalasin D was from Sigma Chemical Co.. Digitonin was purchased from Sigma Chemical Co. and purified by dissolving in water, filtering, and lyophilization before use. ^{35}S -Translabel and methionine- and cysteine-free medium were from ICN Biomedicals Inc. (Costa Mesa, CA). $Na^{125}I$ and $[^{32}P]$ orthophosphate were from Dupont-NEN (Boston, MA).

Expression Constructs, Transfection, and Cell Culture

Human full-length cDNAs encoding $\beta 1A$ integrin or $\beta 1D$ integrin in SV40-based expression vector pECE (Ellis et al., 1986), were transfected into CHO cells or $\beta 1$ -minus GD25 cell line (Wennerberg et al., 1996), and

transfectants were selected as described (Belkin et al., 1996). More than 95% of cells in each population expressed human $\beta 1$ integrin; the expression levels of the transfected $\beta 1A$ and $\beta 1D$ integrins were very similar and comparable to the level of the endogenous hamster $\beta 1$ integrin subunit in CHO cells and close to the levels of the endogenous αv and $\beta 3$ integrins in GD25 transfectants. CHO transfectants were cultured in Ham's F12 medium with 10% FBS, and GD25 transfectants were cultured in DME plus 10% FBS.

Morphological Analysis and Spreading

For analysis of cell phenotype, CHO transfectants were plated and cultured for 1 d on Fn-coated dishes. Phase-contrast photographs of live $\beta 1A$ -CHO and $\beta 1D$ -CHO cells were taken on an inverted microscope. The outlines of randomly chosen cells not in contact with other cells were analyzed by the computer Tracer V1.0 software (Dunn and Brown, 1986). The spread area, cell perimeter, and two morphometric parameters of cell shape, cell dispersion and elongation, were calculated as characteristics of cell spreading and polarization. For analysis of the time course of spreading, $\beta 1A$ - and $\beta 1D$ -transfected CHO and GD25 cells were plated in serum-free medium on Fn, laminin, vitronectin, or on immobilized mAb TS2/16 to human $\beta 1$ integrin (Balzac et al., 1994; Belkin et al., 1996). After specific periods of time, cells were fixed with formaldehyde, stained with Coomassie brilliant blue (Balzac et al., 1994), and then photographed.

Measurements of the Ligand-Binding Affinity

The binding of the ^{125}I -labeled Fn(III)10 fragment to $\beta 1A$ -CHO, $\beta 1D$ -CHO, $\beta 1A$ -GD25, and $\beta 1D$ -GD25 cells in suspension was quantified as described (O'Toole et al., 1990; Wu et al., 1995). Since CHO cells express the endogenous $\alpha 5\beta 1$, and $\beta 1$ -minus GD25 cells express $\alpha v\beta 3$ as a major Fn-binding integrin (Wennerberg et al., 1996), inhibitory mAbs PB1 against hamster $\alpha 5\beta 1$ or H9.2B8 against mouse αv were used for CHO and GD25 cells, respectively. In some experiments, blocking P4C10 mAb against human $\beta 1$ integrin was used in combination with either PB1 mAb (for CHO cells) or H9.2B8 mAb (for GD25 cells). Cells (0.2 ml of 5×10^6 cells/ml) in Tyrode's buffer were incubated with specified concentrations of ^{125}I -labeled Fn(III)10 fragment (sp act 0.12 mCi/nM) for 30 min at 37°C either alone or in the presence of $10 \mu\text{g/ml}$ of purified TS2/16 mAb, which activates human $\beta 1$ integrins. Coincubation with an excess of unlabeled Fn(III)10 fragment (0.5 mg/ml) was used to determine and subtract the nonspecific background binding. $50\text{-}\mu\text{l}$ aliquots were layered on 0.3 ml of 20% sucrose in Tyrode's buffer and centrifuged for 3 min at 12,000 rpm. Radioactivity associated with the cell pellet was determined in a gamma counter.

Flow Cytometry

Cell surface expression of the transfected human $\beta 1A$ or $\beta 1D$ integrins in CHO and GD25 transfectants was assessed with 102DF5 and TS2/16 mAbs, whose binding to the $\beta 1$ subunit is conformation independent. Their expression levels were compared to those of the endogenous hamster $\beta 1$ integrin (examined with 7E2 mAb). 12G10 mAb reacting with activated human $\beta 1$ integrin subunit (Mould et al., 1995) and conformation-specific 9EG7 anti-human $\beta 1$ mAb (Bazzoni et al., 1995) were used either in the absence of Mn^{2+} ions or in the presence of 1 mM Mn^{2+} . Fluorescein-labeled, affinity-purified donkey anti-mouse IgG (Chemicon International, Inc.) was used as secondary antibody.

Fn Matrix Assembly Assays

$\beta 1A$ -CHO, $\beta 1D$ -CHO, as well as $\beta 1A$ -GD25 and $\beta 1D$ -GD25 cells did not assemble Fn matrix well when confluent cell monolayers were cultured in growth medium containing 1% FBS for 2 d. To boost the formation of Fn matrix, exogenous human plasma Fn was added at 200 nM concentration for 2 d to the confluent cell monolayers grown on glass coverslips. Inhibitory mAbs PB1 against hamster $\alpha 5\beta 1$ and H9.2B8 against mouse αv were used to block Fn matrix assembly by the endogenous Fn-binding integrins in CHO and GD25 cells, respectively. Activating TS2/16 and inhibitory P4C10 mAbs were used for the transfected human $\beta 1A$ and $\beta 1D$ integrins. After 2 d, cell monolayers were fixed and stained with anti-Fn antibody. Stained cells were observed using a Zeiss epifluorescence microscope (Carl Zeiss Inc., Thornwood, NY) and representative fields were photographed using equal exposure lengths on Kodak T-Max 400 film (Eastman Kodak, Rochester, NY).

To quantitate Fn incorporation into deoxycholate-insoluble matrix, con-

fluent $\beta 1A$ -CHO, $\beta 1D$ -CHO, $\beta 1A$ -GD25, and $\beta 1D$ -GD25 cultures were incubated for 2 d with 100, 200, or 300 nM of ^{125}I -labeled Fn (sp act 0.08 mCi/nM) in growth medium containing 1% FBS and blocking and activating mAbs as specified above. Deoxycholate-insoluble fraction was obtained from cell monolayers as described (McKeown-Longo and Mosher, 1985; Wu et al., 1993, 1995). ^{125}I -labeled Fn incorporated into the deoxycholate-insoluble extracellular matrix was analyzed by reducing SDS-PAGE (6% running gel) and autoradiography. Iodinated Fn bands were cut out and counted in a gamma counter.

Migration Assays

Migratory properties of $\beta 1A$ -CHO, $\beta 1D$ -CHO, $\beta 1A$ -GD25, and $\beta 1D$ -GD25 cells were examined by a wound closure assay and time lapse videomicroscopy. For the wound closure assay, confluent cell monolayers grown on Fn-coated coverslips were wounded by dragging a sterile 1-mm pipette tip across the monolayer to create cell-free fields (Romer et al., 1994). 2 d later, glass coverslips were fixed with formaldehyde, stained with Coomassie blue, and then photographed.

For time lapse videomicroscopy, $\beta 1A$ - and $\beta 1D$ -transfected CHO and GD25 cells were plated on plastic dishes coated with $10 \mu\text{g/ml}$ of human plasma Fn. Five to six cells were scanned per field in eight different fields, every 20 min for 4 h. The displacement of the cell center as a function of time was calculated for each cell using nonoverlapping time intervals. To block the endogenous Fn receptors, PB1 mAb was used for CHO transfectants and H9.2B8 mAb for GD25 transfectants. TS2/16 was used as the activating mAb and P4C10 as the blocking mAb for the transfected human $\beta 1A$ and $\beta 1D$ integrins.

Analysis of the Association of $\beta 1A$ and $\beta 1D$ Integrins with α Subunits

$\beta 1D$ -CHO cells as well as $\beta 1A$ - and $\beta 1D$ -GD25 transfectants were lysed in buffer containing 1% Triton X-100 in 50 mM TrisCl, 150 mM NaCl, pH 7.5, and protease inhibitors. Each lysate was clarified by centrifugation, divided into four equal parts, and the transfected human $\beta 1$ integrins were immunoprecipitated using TS2/16 mAb, whereas $\alpha 3$, $\alpha 5$, and αv subunits were immunoprecipitated with antibodies against cytoplasmic domains of these integrins. The resulting immunoprecipitates were run on 10% gel and blots were probed with the isoform-specific antibodies against $\beta 1A$ or $\beta 1D$ integrins.

Localization of the Transfected $\beta 1A$ and $\beta 1D$ Integrins and the Endogenous $\beta 1A$ and αv Subunits and Analysis of their Association with the Actin Cytoskeleton

To localize the transfected and the endogenous $\beta 1$ integrins, as well as the endogenous αv integrins in the transfectants, cells cultured on Fn-coated coverslips were fixed with formaldehyde and permeabilized with 0.5% Triton X-100 in PBS. CHO transfectants were costained with fluorescein-labeled A1A5 mAb to human $\beta 1$ and rhodamine-labeled 7E2 mAb to hamster $\beta 1$ integrin. GD25 cells were double stained with mouse fluorescein-labeled A1A5 mAb and rabbit anti- αv antibody followed by rhodamine-labeled donkey anti-rabbit antibody (Chemicon International Inc.). Stained cells were observed using epifluorescence with a Zeiss Axiophot microscope and photographed using Kodak T-Max 400 film.

To study whether the solubility of integrins in digitonin correlates with their cytoskeletal association, ^{35}S -labeled $\beta 1A$ -CHO and $\beta 1D$ -CHO cells, either untreated or treated for 1 h with $10 \mu\text{M}$ of cytochalasin D, were fractionated into soluble and cytoskeleton-associated fractions by sequential extraction at 4°C with 0.1% digitonin in 50 mM Pipes, 1 mM MgCl_2 , 1 mM EGTA, 1 mM EDTA, pH 6.9, and then with radioimmunoprecipitation assay (RIPA) buffer (50 mM TrisCl, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, and 0.1% SDS, pH 7.5). Both buffers contained $10 \mu\text{g/ml}$ leupeptin, $10 \mu\text{g/ml}$ pepstatin, and 0.5 mM PMSF as protease inhibitors. The transfected $\beta 1A$ and $\beta 1D$ integrins were immunoprecipitated from digitonin- and RIPA-soluble fractions using TS2/16 mAb. ^{35}S -labeled $\beta 1$ immunoprecipitates were run on 10% gels and analyzed by autoradiography.

To assess the association of the transfected and the endogenous $\beta 1$ integrin subunits and the endogenous αv integrins with the actin cytoskeleton, ^{35}S -labeled CHO and GD25 transfectants were sequentially extracted with digitonin and RIPA buffers as described above. Immunoprecipitation of the transfected human $\beta 1A$ and $\beta 1D$ integrins from both cellular fractions was performed with TS2/16 mAb. 7E2 mAb antibody was used for the endogenous hamster $\beta 1A$ integrin. Rabbit anti- αv antibody was

used to immunoprecipitate the endogenous $\alpha\text{v}\beta 3/\alpha\text{v}\beta 5$ integrins from GD25 transfectants. ^{35}S -labeled $\beta 1$ and αv immunoprecipitates were analyzed by SDS-PAGE on 10% gels and subsequent autoradiography.

Analysis of the Association of $\beta 1\text{A}$ and $\beta 1\text{D}$ Integrins with Talin and α -Actinin by Coimmunoprecipitation

To compare the association of $\beta 1\text{A}$ and $\beta 1\text{D}$ integrins with talin and α -actinin, 5×10^6 transfected cells were incubated in suspension with 10 μg of either purified TS2/16 mAb, 12G10 mAb, or 7E2 mAb at 4°C for 30 min on a rotator. In the case of 12G10 mAb, cells were either preincubated for 5 min with 1 mM Mn^{2+} or used in the absence of Mn^{2+} . Cells were centrifuged (1,000 rpm, 3 min) and the pellets were extracted for 3 min on ice with buffer containing 0.5% digitonin in 50 mM Pipes, 1 mM MgCl_2 , 1 mM EGTA, 1 mM EDTA, pH 6.9, with 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin, and 0.5 mM PMSF. Under these conditions ~ 80 – 90% of cellular $\beta 1$ integrins was extracted. Cell extracts were centrifuged (12,000 rpm, 30 min, 4°C) and the resulting supernatants incubated at 4°C for 45 min with donkey anti-mouse IgG immobilized on protein A-Sepharose beads. Immunoprecipitates were washed with the same buffer, boiled in SDS sample buffer, and then run on 10% gels. Proteins were transferred onto Immobilon membranes (Millipore Corp., Bedford, MA) and blotted with either rabbit polyclonal antibody to human $\beta 1$ integrin, 8d4 mAb against talin, or 1682 mAb against α -actinin. To verify the equal amount of $\beta 1\text{A}$ and $\beta 1\text{D}$ isoforms in the immunoprecipitates, the blots were stripped and reprobed with the isoform-specific antibodies against $\beta 1\text{A}$ and $\beta 1\text{D}$ (Belkin et al., 1996).

Interaction of Talin and α -Actinin with $\beta 1\text{A}$ and $\beta 1\text{D}$ Cytoplasmic Domain Peptides

Talin was purified from human platelets as described earlier (Collier and Wang, 1982). α -Actinin purification from chicken gizzards was performed as described (Otey et al., 1990). Talin and α -actinin were iodinated using ^{125}I and Iodobeads (Pierce Chemical Co., Rockford, IL). The proteins were labeled to a specific activity of 1.2×10^6 cpm/ μg for talin and 7.5×10^6 cpm/ μg for α -actinin.

Full-length cytoplasmic domain peptides of $\beta 1\text{A}$ and $\beta 1\text{D}$ integrins (Belkin et al., 1996) were iodinated using Iodogen method. Both peptides were initially tested for their binding to the microtiter wells in the range of 1–150 μM concentrations in buffer containing 50 mM TrisCl, 150 mM NaCl, pH 7.5. Since they bound similarly to the wells, saturating 50 μM concentration of $\beta 1\text{A}$ and $\beta 1\text{D}$ peptides was used in subsequent experiments to immobilize them on plastic 96-well microtiter plates for 1 h at 37°C . After blocking with 2% BSA in 50 mM TrisCl, 150 mM NaCl, wells with the bound peptides were incubated with 1 nM of ^{125}I -talin or ^{125}I - α -actinin and 1 nM to 1 μM concentrations of unlabeled talin or α -actinin in the same buffer with 0.1% BSA for 4 h at 37°C . After the incubations, wells were washed three times with the same buffer and bound radioactivity was measured in a gamma counter. Nonspecific background was determined and subtracted for talin and α -actinin binding to BSA-coated wells.

Measurements of Cellular Contractility and Myosin Light Chain Phosphorylation

Silicone rubber substrata for assessing cellular contractility were made as described previously (Harris et al., 1980; Danowski, 1989). The UV glow discharge polymerization was used in combination with gold-palladium coating (Chrzanowska-Wodnicka and Burridge, 1996). 5- and 12-s polymerization was used for CHO and GD25 transfectants, respectively. Cells were plated on the cross-linked rubber substrata in growth medium with 10% FBS and photographed on the next day.

Possible changes in myosin light chain phosphorylation were examined as described (Chrzanowska-Wodnicka and Burridge, 1996). Briefly, subconfluent $\beta 1\text{A}$ -CHO and $\beta 1\text{D}$ -CHO cells cultured on Fn-coated, 35-mm dishes, were labeled for 4 h with 20 $\mu\text{Ci}/\text{ml}$ of ^{35}S -Translabel and 100 $\mu\text{Ci}/\text{ml}$ of [^{32}P]orthophosphate in phosphate-free medium. Cells were washed with PBS and equal amounts of material, as judged by ^{35}S -incorporated radioactivity, were taken for immunoprecipitation with antimyosin antibody, followed by protein A-Sepharose beads. Immunoprecipitates were washed, boiled in SDS sample buffer, and then run on 15% polyacrylamide gel. Phosphorylated myosin light chain bands were visualized by autoradiography using three sheets of aluminum foil to block traces of ^{35}S radiation.

Results

$\beta 1\text{D}$ Integrin Alters Cell Morphology and Inhibits Spreading

The levels of surface expression of the transfected human $\beta 1\text{A}$ and $\beta 1\text{D}$, measured with mAbs 102DF5 and TS2/16, were very close to each other in both CHO and GD25 transfectants (Table I). They were also similar to the levels of the endogenous $\beta 1\text{A}$ in CHO transfectants and αv integrins in GD25 cells (see Fig. 9, *I* and *J*). No difference in association of the transfected $\beta 1\text{A}$ and $\beta 1\text{D}$ with endogenous α subunits was found in the two types of transfectants (Fig. 7).

To determine possible effects of $\beta 1\text{A}$ and $\beta 1\text{D}$ integrin expression on cell morphology, CHO transfectants were grown on Fn for 1 d (Fig. 1, *A* and *B*). $\beta 1\text{D}$ -CHO cells appear more rounded with fewer cytoplasmic extensions at their periphery than their $\beta 1\text{A}$ -transfected counterparts. Statistical analysis of the cell shape was performed for randomly chosen $\beta 1\text{A}$ -CHO and $\beta 1\text{D}$ -CHO cells (Dunn and Brown, 1986). This demonstrated that spread areas were similar for $\beta 1\text{A}$ and $\beta 1\text{D}$ transfectants, but the average cell perimeter was $\sim 20\%$ lower for $\beta 1\text{D}$ -CHO cells (Table II). Two cell shape parameters, dispersion and elongation, representing measures of cell multipolarity and bipolarity, respectively, were significantly lower for $\beta 1\text{D}$ transfectants. These data indicated that pseudopodial activity and cell polarization were reduced in CHO cells expressing $\beta 1\text{D}$ integrin.

To analyze the time course of spreading, $\beta 1\text{A}$ and $\beta 1\text{D}$ transfectants were plated on purified Fn (Fig. 1, *C–F*). Spreading of CHO cells expressing $\beta 1\text{D}$ integrin on Fn was significantly delayed compared with $\beta 1\text{A}$ transfectants. When both $\beta 1\text{A}$ and $\beta 1\text{D}$ transfectants were plated on TS2/16 mAb specific for human $\beta 1$ integrin, $\beta 1\text{D}$ -CHO were less spread than $\beta 1\text{A}$ -CHO cells (Fig. 1, *G* and *H*). Notably, $\beta 1\text{D}$ transfectants also spread more slowly on laminin, vitronectin, and 7E2 mAb against hamster $\beta 1$ integrin (data not shown), indicating that inhibition of spreading was not limited to a certain type of substrate. Likewise, spreading of $\beta 1\text{D}$ -GD25 cells on both Fn and vitronectin was significantly delayed compared with the spreading of

Table I. Expression Levels and Activation States of Transfected Human $\beta 1\text{A}$ and $\beta 1\text{D}$ Integrins in CHO and GD25 Transfectants

| Cell type | mAb 102DF5* | mAb 12G10* | mAb 12G10 | |
|-------------------------|-------------|-------------|------------------------------------|-------------------------------------|
| | | | mAb 12G10 + 1mM Mn^{2+} * | mAb 12G10 + 1 mM Mn^{2+} ‡ |
| $\beta 1\text{A}$ -CHO | 43.1 | 9.2 | 33.6 | 27.4 |
| $\beta 1\text{D}$ -CHO | 34.4 | 21.8 | 28.2 | 77.3 |
| $\beta 1\text{A}$ -GD25 | 42.2 | 12.2 | 32.4 | 37.7 |
| $\beta 1\text{D}$ -GD25 | 38.9 | 29.1 | 36.7 | 79.3 |
| mAb 9EG7 | | | | |
| | | mAb TS2/16* | mAb 9EG7 + 1mM Mn^{2+} * | mAb 9EG7 + 1 mM Mn^{2+} ‡ |
| $\beta 1\text{A}$ -GD25 | | 105 | 39 | 43.8 |
| $\beta 1\text{D}$ -GD25 | | 95 | 69 | 88.4 |

*Values are mean fluorescence intensities from a representative experiment.

‡Values are percentages.

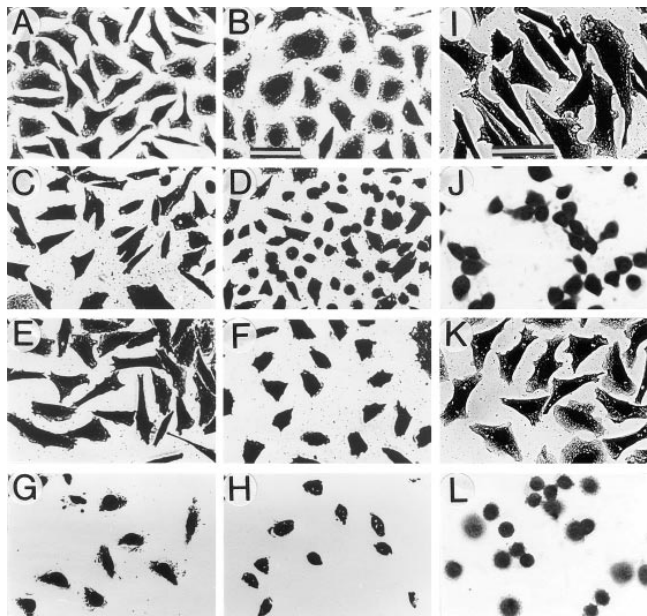


Figure 1. Altered morphology and inhibited spreading of CHO and GD25 cells expressing $\beta 1D$ integrin. $\beta 1A$ -CHO (A) and $\beta 1D$ -CHO (B) cells were plated on Fn and cultured for 1 d. $\beta 1A$ -CHO (C, E, and G) and $\beta 1D$ -CHO (D, F, and H) cells were plated in serum-free medium on Fn for 30 min (C and D), or 1 h (E and F); or on TS2/16 mAb against human $\beta 1$ integrin for 2 h (G and H). $\beta 1A$ -GD25 (I and K) and $\beta 1D$ -GD25 (J and L) cells were plated in serum-free medium on Fn (I and J) or vitronectin (K and L) for 1 h. Cells were fixed with formaldehyde and stained with Coomassie blue. Bar, 50 μ m.

$\beta 1A$ -GD25 cells (Fig. 1, I–L). These observations suggested that retardation of spreading was a general phenomenon of $\beta 1D$ expression.

Constitutive Activation of the $\beta 1D$ Integrin Ectodomain Increases Ligand Binding

To characterize the affinity states of $\beta 1A$ and $\beta 1D$ integrins, the binding of the Fn(III)10 fragment to $\beta 1A$ - and $\beta 1D$ -transfected CHO and GD25 cells in suspension was examined. In the presence of function-blocking mAbs PB1 and H9.2B8, which inhibited endogenous Fn-binding hamster $\alpha 5\beta 1$ and mouse $\alpha v\beta 3$ integrins, respectively, $\beta 1D$ transfectants exhibited a significant increase in binding the soluble ligand compared with $\beta 1A$ -expressing cells (Fig. 2, A and B). TS2/16 mAb, which activates human $\beta 1$ integrin, markedly increased Fn(III)10 fragment binding to both $\beta 1A$ -CHO and $\beta 1A$ -GD25 cells, whereas the ligand binding by $\beta 1D$ transfectants in the presence of TS2/16 mAb remained unaffected (Fig. 2, A and B). Blocking P4C10

Table II. Cell Shape Parameters of $\beta 1A$ -CHO and $\beta 1D$ -CHO Cells

| Cell type | Area, μm^2 [‡] | Perimeter, μm^{\ddagger} | Dispersion [‡] | Elongation [‡] |
|------------------|------------------------------------|-------------------------------------|-------------------------|-------------------------|
| $\beta 1A$ -CHO* | 814 \pm 37 | 147 \pm 4 | 0.237 \pm 0.016 | 1.059 \pm 0.065 |
| $\beta 1D$ -CHO* | 742 \pm 34 | 118 \pm 3 | 0.112 \pm 0.009 | 0.769 \pm 0.045 |

*107 cells were analyzed for $\beta 1A$ -CHO population and 109 cells for $\beta 1D$ -CHO population.

[‡]Given are means and SE.

mAb against human $\beta 1$ integrin completely abolished the binding of Fn(III)10 fragment to all types of transfectants (Fig. 2, A and B), therefore proving its specificity.

To further assess the difference in the conformation of the ectodomains of the transfected $\beta 1A$ and $\beta 1D$, a flow cytometry analysis was used with mAb 12G10. This antibody recognizes preferentially a Mn^{2+} - and ligand-induced conformation of the human $\beta 1$ integrin subunit (Mould et al., 1995; Mould, 1996). The amount of 12G10 mAb bound to the cell surface was significantly higher for $\beta 1D$ -expressing CHO and GD25 cells, than for $\beta 1A$ -expressing cells. Moreover, the binding of 12G10 mAb was almost unchanged for $\beta 1D$ -CHO and $\beta 1D$ -GD25 cells in the presence of Mn^{2+} , whereas the 12G10 mAb binding to both types of $\beta 1A$ transfectants was dramatically increased by Mn^{2+} (Table I). Another mAb, 9EG7, whose binding to human $\beta 1$ integrin is stimulated by ligands and Mn^{2+} , but inhibited by Ca^{2+} (Bazzoni et al., 1995), also reacted more strongly with $\beta 1D$ -GD25 compared with $\beta 1A$ -GD25 cells (Table I). Together, these results showed that in $\beta 1D$ -expressing cells in suspension the majority (~ 77 – 88%) of $\beta 1D$ integrins were constitutively activated on the cell surface, whereas only ~ 27 – 44% of the transfected $\beta 1A$ integrins were present in the activated state.

$\beta 1D$ Enhances Fn Matrix Assembly

Fn biosynthesis and secretion levels in CHO and GD25 cells were not altered by $\beta 1A$ or $\beta 1D$ expression and appeared to be identical for each pair of transfectants (data not shown). Fn matrix assembly by $\beta 1A$ - and $\beta 1D$ -transfected cells was analyzed with the exogenous Fn by both immunofluorescence and measurements of ^{125}I -Fn incorporation into deoxycholate-insoluble matrix (McKeown-Longo and Mosher, 1985; Wu et al., 1993, 1995). Using different concentrations of the exogenous Fn, we consistently observed an enhanced Fn matrix assembly by $\beta 1D$ transfectants compared to their $\beta 1A$ -expressing counterparts (Fig. 3 A). In our subsequent experiments we used 200 nM of exogenous Fn for matrix assembly studies with the transfectants. There was no difference in Fn matrix assembly between $\beta 1A$ and $\beta 1D$ transfectants in the presence of blocking P4C10 mAb against human $\beta 1$ integrin, showing that the observed effects can be ascribed specifically to the expressed $\beta 1D$ (Fig. 3 B).

In the presence of blocking anti-hamster $\alpha 5\beta 1$ integrin mAb PB1 (for CHO cells) or inhibitory anti-mouse αv integrin mAb H9.2B8 (for GD25 cells), $\beta 1D$ transfectants assembled more abundant meshwork of Fn fibrils, than $\beta 1A$ -expressing counterparts (Fig. 4, A, B, E, F, and I, a, b, e, and f). Activating mAb TS2/16 significantly increased Fn matrix assembly by $\beta 1A$ -CHO and $\beta 1A$ -GD25 cells, but did not change the levels of assembly for $\beta 1D$ -transfected CHO and GD25 cells (Fig. 4, C, D, G, H, and I, c, d, g, and h). Quantitation of ^{125}I -Fn incorporated into the extracellular matrix showed a five- to sixfold increase in Fn assembly by $\beta 1D$ compared with $\beta 1A$ integrin in CHO and GD25 cells (Fig. 4, I and J). Interestingly, whereas mAb TS2/16 caused two- to threefold increase in Fn matrix assembly by $\beta 1A$ integrin for both types of transfectants, these levels appeared still much lower than those exhibited by $\beta 1D$ integrin (Fig. 4, I and J).

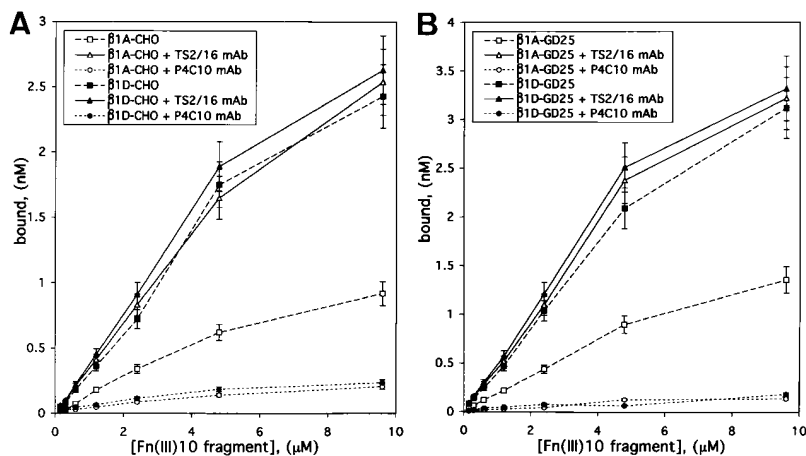


Figure 2. Increased ligand binding by $\beta 1D$ integrin. Ligand-binding properties of $\beta 1A$ - and $\beta 1D$ -transfected CHO (A) and GD25 (B) cells. Binding of ^{125}I -labeled Fn(III)10 fragment to $\beta 1A$ (open marks) and $\beta 1D$ (filled marks) transfectants either in the absence (squares) or presence of the activating anti-human $\beta 1$ integrin TS2/16 mAb (triangles), or the function-blocking anti-human $\beta 1$ integrin P4C10 mAb (circles) was determined as described in the Materials and Methods. The endogenous Fn-binding hamster $\alpha 5\beta 1$ and mouse $\alpha v\beta 3$ integrins were blocked by preincubation of the CHO and GD25 cells with the inhibitory PB1 and H9.2B8 mAbs, respectively. Note that $\beta 1A$ -transfected, but not $\beta 1D$ -transfected CHO and GD25 cells, display a significant increase in Fn(III)10 fragment binding in the presence of activating TS2/16 mAb. Depicted are the means from triplicate measurements.

$\beta 1D$ Integrin Inhibits Cell Migration

Initially, migratory properties of $\beta 1A$ - and $\beta 1D$ -transfected CHO and GD25 cells were analyzed using a monolayer wounding assay. In 2-d wound closure experiments, $\beta 1D$ transfectants exhibited significantly slower migration rates than $\beta 1A$ -expressing cells (Fig. 5, A–D). When migratory behavior of $\beta 1A$ and $\beta 1D$ transfectants was analyzed on Fn substrate by time lapse videomicroscopy, $\beta 1D$ -expressing cells migrated three- to fourfold slower than $\beta 1A$ -transfected cells. Again, blocking P4C10 mAb against human $\beta 1$ integrin completely abolished the effects of $\beta 1D$ on cell migration (Fig. 5 E).

Migration of $\beta 1A$ - and $\beta 1D$ -expressing cells was also examined on Fn by time lapse videomicroscopy in the presence of blocking mAbs PB1 for CHO and H9.2B8 for GD25 transfectants (Fig. 6). In both cases when the endogenous Fn-binding integrins were inhibited, the mean cell speed of

$\beta 1D$ transfectants appeared to be drastically reduced compared to that of $\beta 1A$ -expressing cells. Activating TS2/16 mAb significantly decreased migration mediated by $\beta 1A$ integrin but did not alter the migratory behavior of $\beta 1D$ -expressing cells. The migration rates of $\beta 1A$ -transfected CHO and GD25 cells treated with TS2/16 mAb still exceeded substantially the migration rates of $\beta 1D$ -transfectants.

Interaction of $\beta 1A$ and $\beta 1D$ Integrins with α Subunits in CHO and GD25 Transfectants

Many of the observed properties of $\beta 1D$ including enhanced ligand binding, elevated Fn matrix assembly, and decreased cell motility could be explained by different mode of association of $\beta 1A$ and $\beta 1D$ integrins with α subunits in the transfected cells. Therefore, we examined α subunit association for $\beta 1A$ and $\beta 1D$ in CHO and GD25 transfectants. Among various $\beta 1$ -associated α subunits, $\alpha 3$, $\alpha 5$, and very small amount of αv were detected in both types of transfectants by immunoprecipitation (Fig. 7). Immunoblotting of the corresponding immunoprecipitates from $\beta 1D$ -CHO (Fig. 7, A and B), $\beta 1A$ -GD25 (Fig. 7, C and D), and $\beta 1D$ -GD25 (Fig. 7, E and F) cells showed that equal amounts of $\beta 1D$ and $\beta 1A$ integrins were associated with $\alpha 3$ and $\alpha 5$ subunits in CHO and GD25 transfectants.

Cytoskeletal Association of $\beta 1A$ and $\beta 1D$ Integrins Correlates with Their Insolubility in Digitonin

To determine whether there is a difference in cytoskeletal association between $\beta 1A$ and $\beta 1D$ integrins, we designed a method of sequential extraction using digitonin and RIPA buffers for ^{35}S -labeled cell cultures, followed by integrin immunoprecipitation from both cellular fractions. To test whether integrin insolubility in digitonin is determined by the mode of integrin–cytoskeleton association, we compared $\beta 1$ integrin immunoprecipitates from digitonin and RIPA fractions of untreated and cytochalasin D-treated $\beta 1A$ - and $\beta 1D$ -transfected CHO cells (Fig. 8). Treatment of cultured cells with cytochalasin D shifted almost all $\beta 1A$ and the majority of $\beta 1D$ to the digitonin-soluble fraction. These experiments demonstrated that insolubility of $\beta 1$ integrins in digitonin depends on their association with the actin cytoskeleton.

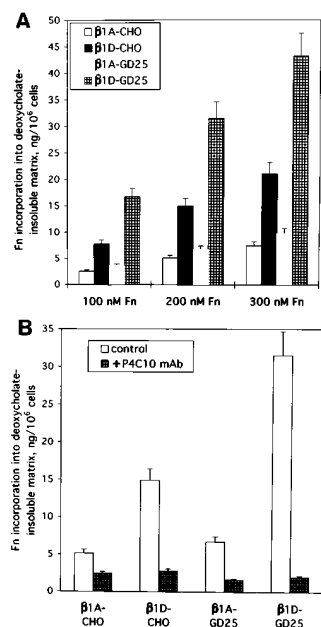


Figure 3. $\beta 1D$ integrin enhances Fn matrix assembly. (A and B) Incorporation of exogenous Fn into deoxycholate-insoluble matrix by $\beta 1A$ - and $\beta 1D$ -transfected CHO and GD25 cells. ^{125}I -Labeled deoxycholate-insoluble Fn was visualized by SDS electrophoresis on 6% gels under reducing conditions, after autoradiography. ^{125}I -Fn bands were cut out and radioactivity was counted in a gamma counter. Bars represent the means of triplicate determinations. (A) Cells were cultured for 2 d with 100, 200, or 300 nM of exogenous Fn. (B) Cells were cultured for 2 d with 200 nM of exogenous Fn in the absence or in the presence of function-blocking P4C10 mAb against the transfected human $\beta 1$ integrins.

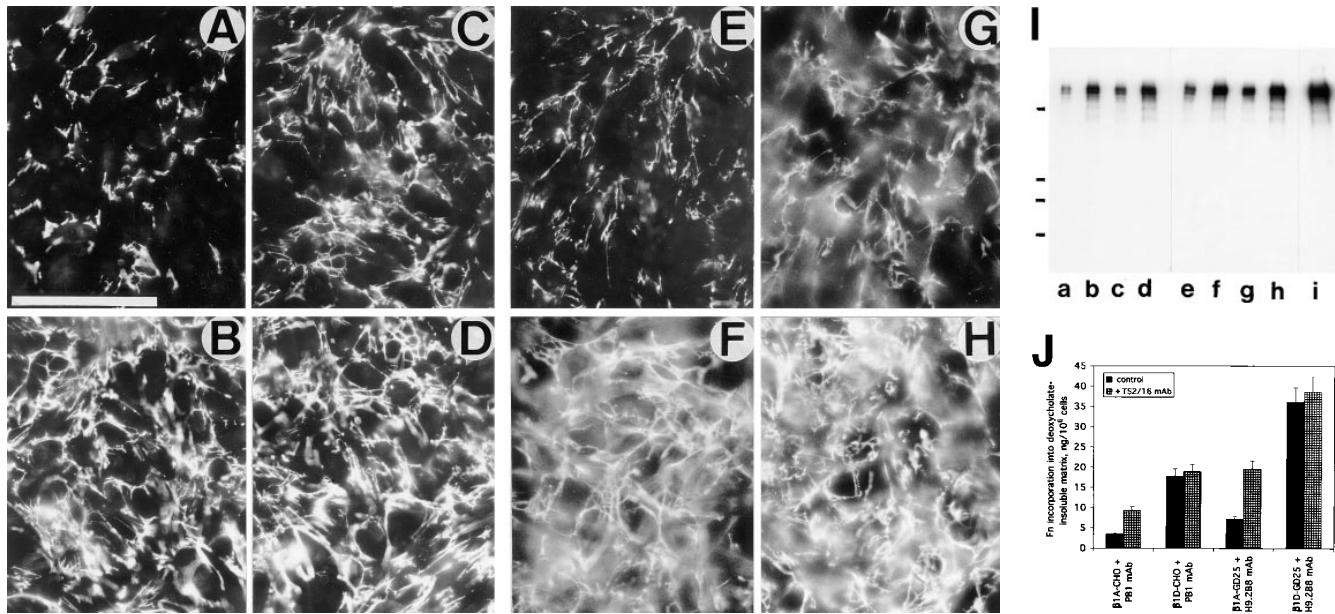


Figure 4. Activation of $\beta 1D$ integrin extracellular domain contributes to the increased $\beta 1D$ -mediated assembly of Fn matrix. (A–H) Immunofluorescent detection of Fn matrix deposition. Confluent monolayers of $\beta 1A$ -CHO (A and C), $\beta 1D$ -CHO (B and D), $\beta 1A$ -GD25 (E and G), and $\beta 1D$ -GD25 (F and H) cells were cultured for 2 d with 200 nM of exogenous human plasma Fn either in the absence (A, B, E, and F) or presence (C, D, G, and H) of activating anti-human $\beta 1$ TS2/16 mAb. Inhibitory PB1 (A–D) and H9.2B8 (E–H) mAbs were used in the growth media to block the endogenous Fn-binding $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins, respectively. Note that more abundant Fn matrix was assembled by $\beta 1D$ -transfected cells (B, D, F, and H). A significant increase in Fn matrix assembly occurred when $\beta 1A$ -transfected cells (C and G), but not $\beta 1D$ -transfected cells (D and H) were incubated in the presence of TS2/16 mAb. Bar, 200 μ m. (I and J) Biochemical evaluation of Fn matrix assembly. Cells were cultured for 2 d with 200 nM of exogenous Fn. (I) ¹²⁵I-labeled Fn was visualized by SDS-PAGE and autoradiography after it had been incorporated into deoxycholate-insoluble matrix of $\beta 1A$ -CHO (a and c), $\beta 1D$ -CHO (b and d), $\beta 1A$ -GD25 (e and g) and $\beta 1D$ -GD25 (f and h) cells either in the absence (I: a, b, e, and f; J, dark bars) or in the presence (I: c, d, g, and h; J, hatched bars) of activating TS2/16 mAb. mAbs PB1 (a–d) and H9.2B8 (e–h) were used as blocking antibodies for the endogenous Fn-binding integrins. ¹²⁵I-Fn was used as a marker for SDS-PAGE (i). Molecular weight markers (from top to bottom) are 200, 116, 97, and 68 kD. They are indicated to the left of the gel. (J) ¹²⁵I-Fn bands for $\beta 1A$ and $\beta 1D$ transfectants from the experiments shown in I were cut out and quantitated in a gamma counter. Bars in J represent the means of triplicate measurements for two independent experiments.

$\beta 1D$ Integrin Displaces the Endogenous $\beta 1A$ and αv Subunits from Focal Adhesions and Associates Strongly with the Digitonin-insoluble Cytoskeleton

Since $\beta 1D$ and $\beta 1A$ integrins have structurally different cytoplasmic domains, and the two types of transfectants displayed dissimilar phenotypes, we next attempted to compare cytoskeletal interactions of the $\beta 1A$ and $\beta 1D$ isoforms. To localize the transfected and the endogenous $\beta 1$ integrins in CHO transfectants, cells grown on Fn were double stained with anti-human $\beta 1$ and anti-hamster $\beta 1$ mAbs. In $\beta 1A$ -CHO cells, both the transfected and the endogenous $\beta 1A$ subunits colocalized at focal adhesions (Fig. 9, A and C). $\beta 1D$ integrin was prominently localized at focal adhesions of $\beta 1D$ -CHO cells. Surprisingly, no endogenous $\beta 1A$ integrin was detected in focal adhesions of $\beta 1D$ transfectants grown on Fn or other extracellular matrix proteins (Fig. 9, B and D; and data not shown). Similarly, both the transfected $\beta 1A$ and $\beta 1D$ integrins were targeted to focal adhesions of GD25 transfectants on Fn (Fig. 9, E and F). The endogenous αv subunit of Fn-binding $\alpha v\beta 3$ integrin in $\beta 1A$ -GD25 cells was at least partially colocalized with $\beta 1A$ at sites of cell–matrix contact (αv does not pair with $\beta 1$ integrins in GD25 cells; Wennerberg et al., 1996). In contrast, $\beta 1D$ displaced the endogenous αv

integrins from focal adhesions (Fig. 9, G and H). Therefore, the displacement of $\beta 1A$ and αv integrins from focal adhesions by expressed $\beta 1D$ was a general phenomenon, suggesting a considerably stronger association of $\beta 1D$ integrin with the actin cytoskeleton.

To define biochemically the modes of $\beta 1A$ and $\beta 1D$ interaction with the cytoskeleton, both the transfected $\beta 1A$ and $\beta 1D$, as well as the endogenous $\beta 1A$ and αv integrins were immunoprecipitated from soluble and cytoskeleton-associated fractions of ³⁵S-labeled transfectants grown on Fn (Fig. 9, I and J). In $\beta 1A$ -CHO cells, the transfected $\beta 1A$ was equally distributed between the soluble and the cytoskeletal fractions, whereas the majority of the endogenous hamster $\beta 1A$ subunit was associated with the cytoskeleton. In contrast, $\beta 1D$ was found exclusively in the cytoskeletal fraction, whereas almost all the endogenous $\beta 1A$ integrin was present in the soluble fraction of $\beta 1D$ -CHO cells, displaced from the cytoskeleton (Fig. 9 I). Cytoskeletal associations of the transfected $\beta 1A$ and $\beta 1D$ integrins in GD25 transfectants were similar to those observed in CHO transfectants. Again, much of the transfected $\beta 1A$ integrin was digitonin soluble. However, the majority of $\beta 1D$ integrin was digitonin insoluble, whereas most of the endogenous $\alpha v\beta 3/\alpha v\beta 5$ integrins were dis-

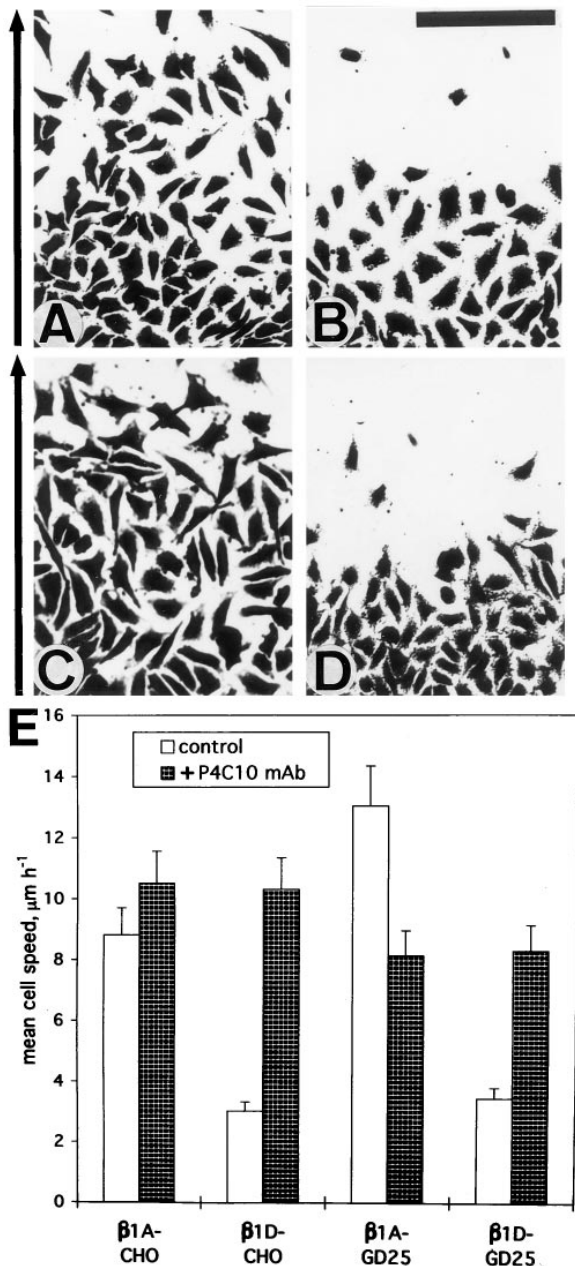


Figure 5. Expression of $\beta 1D$ integrin reduces migration. (A–D) Wounding assays. Confluent monolayers of $\beta 1A$ -CHO (A), $\beta 1D$ -CHO (B), $\beta 1A$ -GD25 (C), and $\beta 1D$ -GD25 (D) cells were scraped to generate 1-mm-wide wounds. After 2 d, cells were fixed, stained and then photographed. The direction of cell migration is shown to the left of the micrographs. (E) Time lapse videomicroscopy analysis of migratory behavior of $\beta 1A$ - and $\beta 1D$ -transfected CHO and GD25 cells. Either untreated cells (light bars), or cells in the presence of blocking P4C10 mAb against human $\beta 1$ integrin (dark bars) were observed. Bar, 200 μm .

placed from their association with the cytoskeleton by the transfected $\beta 1D$ (Fig. 9J).

Differential Interaction of $\beta 1A$ and $\beta 1D$ Integrins with Talin and α -Actinin

At least two cytoskeletal proteins, talin and α -actinin, are known to interact in vitro with the cytoplasmic domain of

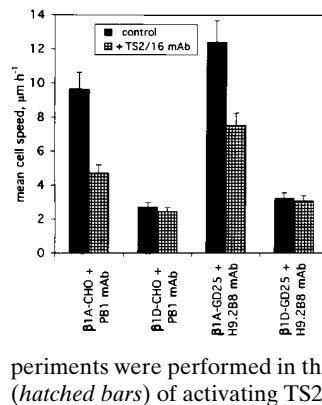


Figure 6. The role of activation of the $\beta 1D$ ectodomain in the decreased migration of $\beta 1D$ transfectants. Cell migration of $\beta 1A$ and $\beta 1D$ transfectants on Fn was analyzed by time lapse videomicroscopy. Inhibitory mAbs PB1 and H9.2B8 were used to block the endogenous Fn-binding $\alpha 5 \beta 1$ integrin in CHO and $\alpha v \beta 3$ integrin in GD25 transfectants. The experiments were performed in the absence (dark bars) or presence (hatched bars) of activating TS2/16 mAb.

$\beta 1A$ integrin (Horwitz et al., 1986; Otey et al., 1990). To identify cytoskeletal proteins, associated preferentially with the $\beta 1D$ integrin subunit in vivo, antibody clustering of the transfected $\beta 1A$ and $\beta 1D$ integrins on the surface of CHO and GD25 transfectants was used in combination with subsequent immunoprecipitation and analysis of the immunoprecipitates (Miyamoto et al., 1995). Several cytoskeletal proteins, including actin, talin, α -actinin, and vinculin coprecipitated with the transfected $\beta 1$ integrins (data not shown). When $\beta 1A$ and $\beta 1D$ immunoprecipitates, obtained with activating TS2/16 anti-human $\beta 1$ mAb, were compared by immunoblotting with mAb 8d4 against talin, a significantly stronger talin band was detected in association with $\beta 1D$ integrin in both CHO and GD25 transfectants (Fig. 10, A, a, a', b, and b'; and D, a, a', b, and b'). This preferential association of talin with $\beta 1D$ compared with $\beta 1A$ integrin did not depend on the nature of anti- $\beta 1$ mAb used for clustering. 12G10 mAb, which recognizes a Mn^{2+} - and ligand-induced conformation of human $\beta 1$ integrin, precipitated more talin from $\beta 1D$ -CHO than from $\beta 1A$ -CHO cells both in the absence or in the presence of 1 mM Mn^{2+} (Fig. 10, A, c, c', d, and d'). In control immunoprecipitations with the 7E2 mAb against the endogenous hamster $\beta 1$ integrin, equal amounts of talin were detected in association with the endogenous $\beta 1A$ in CHO transfectants (Fig. 10, A, e, and e'). Interestingly, when anti-human $\beta 1$ integrin immunoprecipitates from both types of transfectants were probed for α -actinin, more α -actinin was detected in association with $\beta 1A$ than with $\beta 1D$ integrin (Fig. 10, A, f, and f'; and D, c, and c'). To ensure equal amounts of these isoforms in the immunoprecipitates, all the samples used in these experiments were also examined

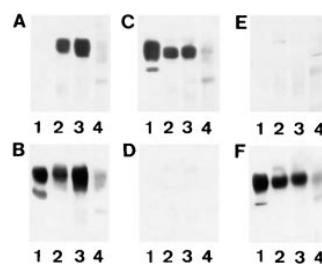


Figure 7. Association of $\beta 1A$ and $\beta 1D$ integrins with α subunits in CHO and GD25 transfectants. Immunoprecipitates containing the transfected human $\beta 1$ integrin subunit (1), $\alpha 3$ subunit (2), $\alpha 5$ subunit (3), or αv subunit (4) from $\beta 1D$ -CHO (A and B), $\beta 1A$ -GD25 (C and D), and $\beta 1D$ -GD25 (E and F) cells were run on 10% gel and subjected to immunoblotting with the isoform-specific antibodies against $\beta 1A$ (A, C, and E) and $\beta 1D$ (B, D, and F) integrins.

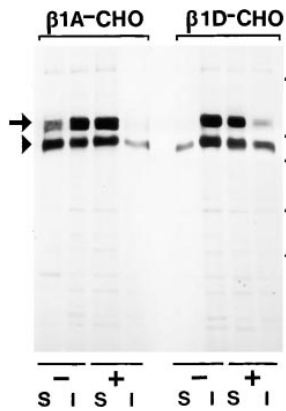


Figure 8. Digitonin insolubility of $\beta 1A$ and $\beta 1D$ integrins is ascribable to cytoskeletal association. ^{35}S -labeled, transfected $\beta 1$ integrins were immunoprecipitated from digitonin-soluble (*S*) and digitonin-insoluble (*I*) fractions of either untreated (–) or cytochalasin D-treated (+) $\beta 1A$ -CHO and $\beta 1D$ -CHO cells. Arrow, the mature $\beta 1$ subunit; and arrowhead, the precursor form. Note a significant increase in solubility of $\beta 1A$ and $\beta 1D$ integrins in 0.1% digitonin after cytochalasin D treatment. Molecular weight markers (from top to bottom) are 200, 116, 97, 68, and 43 kD. They are indicated to the right of the gel.

with the isoform-specific antibodies against $\beta 1A$ (Fig. 10, *B* and *E*) and $\beta 1D$ (Fig. 10, *C* and *F*) integrins (Belkin et al., 1996). Besides the immunoprecipitates from $\beta 1A$ -CHO and $\beta 1D$ -CHO cells with the conformation-specific mAb 12G10 in the absence of Mn^{2+} (Fig. 10, *B*, *c*; and *C*, *c'*), all other samples contained equal amounts of $\beta 1$ integrins (Fig. 10, *B*, *C*, *E*, and *F*).

To compare further the interactions of $\beta 1A$ and $\beta 1D$ integrins with talin and α -actinin, *in vitro* solid phase binding assays were performed with ^{125}I -talin, ^{125}I - α -actinin, and immobilized full-length synthetic peptides, corresponding to the $\beta 1A$ and $\beta 1D$ cytoplasmic domains (Fig. 11). First, we tested the binding of ^{125}I -labeled $\beta 1A$ and $\beta 1D$ cytoplasmic domain peptides to the microtiter wells (Fig. 11 *A*). The amounts of the $\beta 1A$ and $\beta 1D$ peptides adsorbed to the wells were almost indistinguishable within the wide range of concentrations examined.

Then, using the solid phase binding assay we more fully characterized the interactions of ^{125}I -talin and ^{125}I - α -actinin with the $\beta 1A$ and $\beta 1D$ cytoplasmic domain peptides (Fig. 11, *B* and *C*). We found that the binding of ^{125}I -talin to the $\beta 1D$ peptide was severalfold higher than the binding to the $\beta 1A$ peptide. The apparent dissociation constants of 4.2×10^{-8} and 5.9×10^{-9} M were calculated from the competition binding curves for talin interactions with the $\beta 1A$ and $\beta 1D$ cytoplasmic peptides, respectively (Fig. 11 *B*). Conversely, ^{125}I - α -actinin bound more strongly to the $\beta 1A$ than to the $\beta 1D$ cytoplasmic domain peptide (Fig. 11 *C*). In this case the dissociation constants were 1.2×10^{-8} M for α -actinin- $\beta 1A$ binding and 8.8×10^{-8} M for α -actinin- $\beta 1D$ binding. Together, the coimmunoprecipitation analyses and *in vitro* binding data demonstrated that

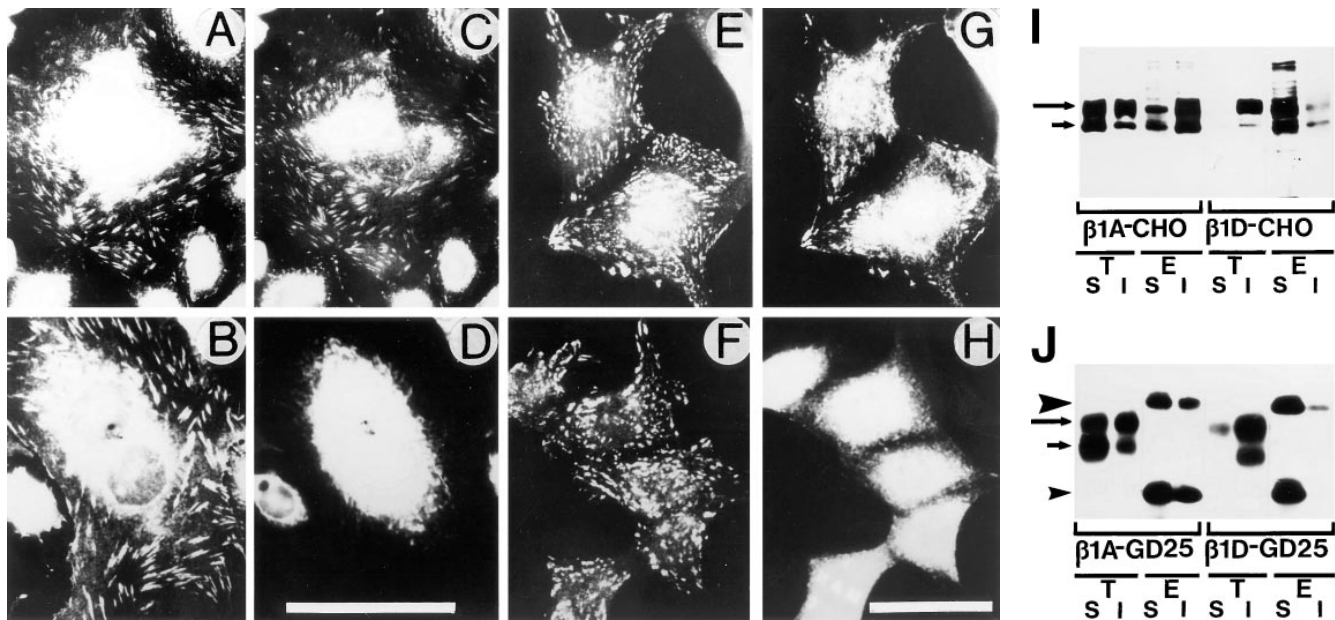


Figure 9. $\beta 1D$ interacts more strongly than $\beta 1A$ with the actin cytoskeleton and displaces the endogenous $\beta 1A$ and αv integrins from focal adhesions. (*A–D*) Localization of the transfected $\beta 1A$ and $\beta 1D$ and the endogenous $\beta 1A$ integrins in CHO transfectants. $\beta 1A$ -CHO (*A* and *C*) and $\beta 1D$ -CHO (*B* and *D*) cells were double stained for human $\beta 1A$ (*A*) and hamster $\beta 1A$ (*C*) integrins, or for human $\beta 1D$ (*B*) and hamster $\beta 1A$ (*D*) integrins. (*E–H*) Localization of the transfected $\beta 1A$ and $\beta 1D$ and the endogenous αv integrins in GD25 transfectants. $\beta 1A$ -GD25 (*E* and *G*) and $\beta 1D$ -GD25 (*F* and *H*) cells were double stained for human $\beta 1A$ (*E*) and mouse αv (*G*) integrins, or for human $\beta 1D$ (*F*) and mouse αv (*H*) integrins. Note colocalization of the transfected $\beta 1A$ with the endogenous $\beta 1A$ and αv integrins, whereas transfected $\beta 1D$ integrin displaces the endogenous $\beta 1A$ from focal adhesions in CHO transfectants and the endogenous αv integrins from focal adhesions in GD25 transfectants. (*I* and *J*) Association of the transfected $\beta 1A$ and $\beta 1D$ and the endogenous $\beta 1A$ and αv integrins with the actin cytoskeleton in CHO and GD25 transfectants. ^{35}S -Labeled integrins were immunoprecipitated from digitonin-soluble (*S*) and digitonin-insoluble (*I*) fractions of $\beta 1A$ - and $\beta 1D$ -transfected CHO (*I*) and GD25 (*J*) cells. (*T*), transfected human $\beta 1A$ or $\beta 1D$ integrin; (*E*), endogenous hamster $\beta 1A$ (*I*) or mouse $\alpha v\beta 3/\alpha v\beta 5$ (*J*) integrins. Long arrows point to the mature $\beta 1$ integrin subunit, short arrows mark the precursor of the $\beta 1$ subunit. The large arrowhead marks the αv subunit and the small arrowhead marks the associated $\beta 3$ and $\beta 5$ subunits, not resolved under conditions of this electrophoresis. Unlike the transfected $\beta 1A$, $\beta 1D$ is present predominantly in the digitonin-insoluble (cytoskeletal) fraction, whereas the endogenous hamster $\beta 1A$ in $\beta 1D$ -CHO and mouse $\alpha v\beta 3/\alpha v\beta 5$ integrins in $\beta 1D$ -GD25 cells are mostly digitonin-soluble. Bars, 50 μm .

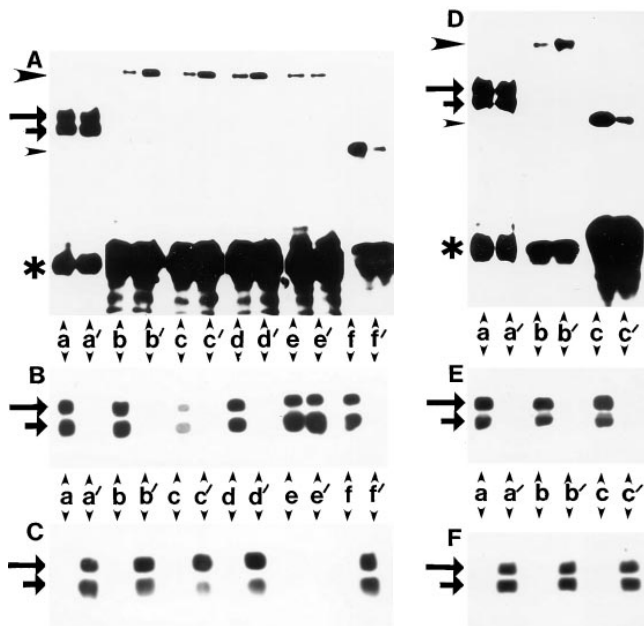


Figure 10. Differential association of β 1A and β 1D integrins with talin and α -actinin. (A and D) Coimmunoprecipitation of talin and α -actinin with β 1D and β 1A integrins. (A) CHO transfectants. Human β 1A (a–d, and f) or β 1D (a'–d', and f') integrins were immunoprecipitated with TS2/16 mAb (a, a', b, b', f, and f') or activation-dependent 12G10 mAb either in the absence of Mn^{2+} (c and c') or in the presence of 1 mM Mn^{2+} (d and d'). Endogenous hamster β 1A was immunoprecipitated from β 1A-CHO (e) or β 1D-CHO (e') cells with 7E2 mAb. Immunoprecipitates were probed for β 1 integrin (a and a'), talin (b, b', c, c', d, d', e, and e'), or α -actinin (f and f'). (D) GD25 transfectants. Human β 1A (a–c) or β 1D (a'–c') integrins were immunoprecipitated with TS2/16 mAb and immunoprecipitates were probed for β 1 integrin (a and a'), talin (b and b'), or α -actinin (c and c'). Long and short arrows mark the β 1 integrin doublet (mature form and precursor, respectively). Large arrowheads point to talin and small arrowheads mark α -actinin. Asterisks indicate IgG heavy chains. (B, C, E, and F) The same immunoprecipitates as in A were probed for β 1A (B) or β 1D (C) integrins with the isoform-specific antibodies. The same immunoprecipitates as in D were blotted for β 1A (E) or β 1D (F).

talin interacts more strongly with the cytoplasmic domain of β 1D than β 1A integrin, whereas α -actinin interacts preferentially with the β 1A integrin isoform.

β 1D Integrin Increases Cellular Contractility

The enhanced association of β 1D integrin with the actin cytoskeleton prompted us to examine whether the β 1D integrin–cytoskeleton interaction increases contractility. When β 1A and β 1D transfectants were plated on flexible silicone rubber substrata for 1 d, β 1D-transfected cells generated prominent wrinkles within the substrata. However, β 1A transfectants were essentially unable to wrinkle these substrata (Fig. 12, A–D). Interestingly, the enhancement of contractility was not accompanied by substantially elevated phosphorylation of myosin regulatory light chains in β 1D-CHO cells (Fig. 12 E). Therefore, the observed increase in cellular contractility of β 1D transfectants appears to be mostly because of enhancement of actin–mem-

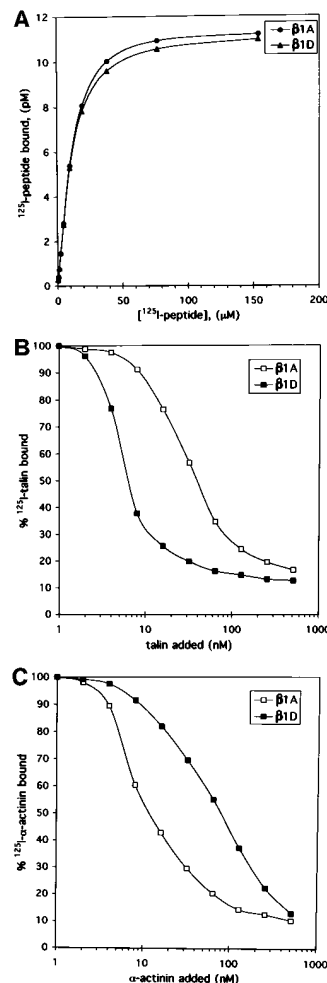


Figure 11. Interaction of talin and α -actinin with β 1A and β 1D cytoplasmic domain peptides. (A) Binding of full-length ^{125}I - β 1A (●) and ^{125}I - β 1D (▲) cytoplasmic domain peptides to the microtiter wells. Shown are the average of triplicate determinations. (B and C) Binding of ^{125}I -talin (B) and ^{125}I - α -actinin (C) to the microtiter wells coated with β 1A (□) or β 1D (■) cytoplasmic domain peptides in the presence of the excess of unlabeled talin (B) or α -actinin (C). All points are the average of quadruplicate determinations.

brane attachment, and did not depend on significant changes of myosin ATPase activity.

Discussion

In this work we have analyzed muscle β 1D integrin, comparing its properties with those of the common β 1A isoform. We found that the unique cytoplasmic sequence of β 1D endows this molecule with the distinctive functional properties. β 1D integrin displays an increased affinity for both the extracellular matrix ligands and the actin cytoskeleton. Expression of β 1D causes a marked phenotypic conversion of both CHO and β 1-deficient GD25 cells. The β 1D phenotype includes altered morphology, retarded spreading, enhanced ligand binding, and extracellular matrix assembly, as well as reduced migration and significantly increased contractility.

Together, the increased integrin–ligand and integrin–cytoskeleton associations mediated by the β 1D isoform, cause a significant reinforcement of the entire cytoskeleton–matrix link. Unlike two other β 1 integrin variants β 1B and β 1C, which are distributed uniformly at the cell surface and are unable to interact with the actin cytoskeleton, β 1D is readily targeted to sites of cell–matrix adhesion upon expression in nonmuscle cells. Furthermore, significantly stronger association of β 1D with the actin cytoskeleton can be mediated by its enhanced interaction with

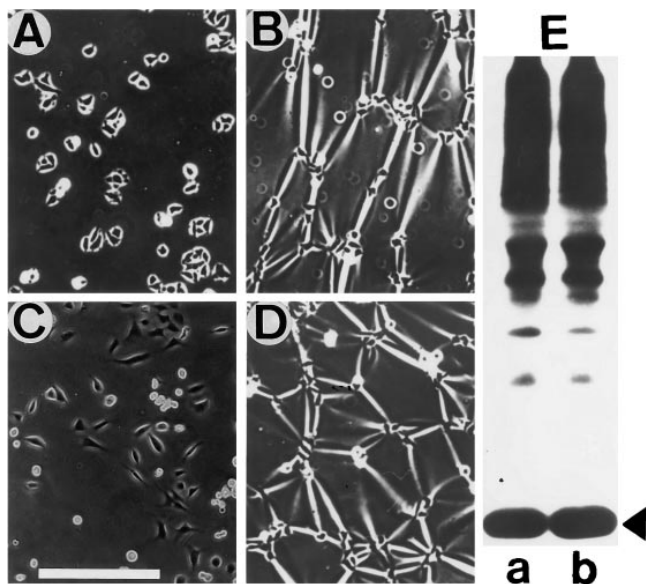


Figure 12. β 1D integrin elevates cellular contractility without affecting phosphorylation of myosin light chains. (A–D) Rubber substrate contractility assay for β 1A and β 1D transfectants. β 1A-CHO (A), β 1D-CHO (B), β 1A-GD25 (C), and β 1D-GD25 (D) cells were plated for 1 d on silicone rubber substrata and photographed. (E) Myosin light chain phosphorylation in CHO transfectants. Myosin was immunoprecipitated from ^{32}P -labeled β 1A-CHO cells (a) and β 1D-CHO cells (b). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Arrowhead points to myosin light chains. Bar, 200 μm .

talins and might change the organization of focal adhesions in β 1D transfectants compared with these structures in β 1A-expressing cells. However, by immunofluorescence with a large panel of antibodies against cytoskeletal and focal adhesion proteins, we did not observe prominent differences in the organization of stress fibers and focal adhesions between β 1D and β 1A transfectants. The intensities of immunostaining for talin, vinculin, α -actinin, paxillin, focal adhesion kinase, and phosphotyrosine at focal adhesions were similar in both types of transfectants (data not shown). Apparently, the differences in the affinities between β 1A/ β 1D and talin/ α -actinin might be insufficient to detect preferential accumulation of these cytoskeletal proteins at focal adhesions of β 1A- and β 1D-transfected cells by immunofluorescence. Nevertheless, β 1D transfectants appeared to be more contractile without changes in myosin light chain phosphorylation and displayed increased resistance of focal adhesions against disassembly by contractility inhibitors BDM and H7 (data not shown). These observations pointed to the increased stability of focal adhesions in cells expressing β 1D integrin that occurs primarily because of enhancement of actin–membrane attachment.

Simultaneously, displacement of the endogenous β 1A subunit from sites of cell–matrix adhesion by β 1D generates a unique “dominant-positive phenotype” of the transfected nonmuscle cells. In this situation, the β 1 integrin-mediated, cytoskeleton–matrix link is built exclusively by the β 1D integrin and lacks the β 1A isoform. Displacement of the major β 1A integrin isoform from cell–matrix adhe-

sion sites appears to be a general phenomenon of β 1D expression, caused by its enhanced association with the actin cytoskeleton. This displacement is observed on various extracellular matrix substrata in a number of nonmuscle cells (Belkin, A.M., unpublished data). Similarly, in GD25 transfectants the major endogenous $\alpha\beta$ 3 integrin appeared to be displaced from the sites of cell–matrix contact. The altered structure of the β 1D integrin cytoplasmic domain also leads to a conformational change in its ectodomain that increases the ligand-binding affinity of β 1D-containing heterodimers. The constitutive activation of β 1D on the cell surface, combined with its stronger interaction with the actin cytoskeleton, enhance Fn matrix assembly by β 1D transfectants (Wu et al., 1995).

Recently, Kucik et al. (1996) demonstrated that nonadhesive inactive state of LFA-1 integrin in lymphocytes is maintained by the cytoskeleton. Both PMA and cytochalasin D were shown to stimulate lymphocyte adhesion, which was accompanied by activation of their major β 2 integrin and its release of cytoskeletal constraints. This points to the opposite mechanisms of cytoskeletal control of integrin activation between β 2 and β 1 integrin subfamilies and might reflect profoundly dissimilar physiology of nonadherent lymphocytes and adhesion-dependent cells, including striated muscles.

Previously, modulation of the ligand-binding affinity of integrin β subunits was demonstrated using either activating antibodies (Arroyo et al., 1992; Faull et al., 1993) or point mutations in the β 1 and β 3 subunit cytoplasmic domains (Ginsberg et al., 1992; Takada et al., 1992; O’Toole et al., 1994, 1995; Schwartz et al., 1995). Here we present the results showing an existence of physiological mechanism reinforcing the cytoskeleton–matrix link in muscle cells based on modulation of integrin adhesive function. This mechanism involves a novel type of inside-out integrin signaling where activation of the extracellular domain of the integrin β subunit is controlled by alternative splicing of its cytodomain. Since the expression of β 1D and β 1A isoforms is developmentally regulated in muscle (Belkin et al., 1996), differentiating muscle cells can control the overall strength of cytoskeleton–matrix attachment via alternative splicing of the β 1 integrin subunit.

The organization of focal adhesions in β 1D-transfected nonmuscle cells appears to be very similar to that found in differentiated muscle cells. In both situations, β 1D is the only β 1 isoform localized at cell–matrix adhesive structures (Belkin et al., 1996). Therefore, the enhanced cytoskeleton–matrix association and stabilization of focal adhesions, mediated by β 1D in the transfectants, might reflect the major role for this integrin in muscle. Since talin, a major structural component of focal adhesions, accumulates at muscle adhesions (Belkin et al., 1986; Tidball et al., 1986), it can also serve as a key cytoskeletal element linking β 1D integrin to actin filaments in muscle. In contrast, we found that α -actinin, a focal adhesion component that interacts with β 1A *in vitro* (Otey et al., 1990), binds β 1D less strongly than β 1A. This correlates with the absence of α -actinin at myotendinous junctions, the major sites of force transmission in muscle (Tidball, 1987). Additionally, certain muscle-specific cytoskeletal proteins, including dystrophin, may contribute to linking β 1D integrin to the subsarcolemmal cytoskeleton.

Many existing models of focal adhesion assembly imply that their formation proceeds from outside the cell inward, starting from integrin clustering by their ligands on the cell surface (Yamada and Miyamoto, 1995; Craig and Johnson, 1996). However, rho-stimulated contractility has been shown to drive the formation of integrin-containing focal adhesion complexes from inside the cell (Hotchin and Hall, 1995; Burridge and Chrzanowska-Wodnicka, 1996; Chrzanowska-Wodnicka and Burridge, 1996). Also, recent data on muscle integrins in *Drosophila melanogaster* point to certain ligand-independent intracellular mechanisms directing localization of β_{ps} integrins (analogous to $\beta 1$ integrins in vertebrates) to sites of their function in embryonic muscles (Martin-Bermudo and Brown, 1996). The altered structure of the $\beta 1D$ cytoplasmic domain and the enhanced interaction of this integrin with the cytoskeleton might determine its ligand-independent targeting to muscle adhesive structures.

Increased Fn matrix assembly by CHO cells expressing $\beta 1D$ can be driven by a combination of higher ligand-binding affinity of this integrin, reinforced actin-membrane association and enhanced contractility of $\beta 1D$ transfectants. Recently, it was shown that the appearance of new Fn matrix assembly sites on the cell surface is stimulated by lysophosphatidic acid, an agent that promotes contractility (Jalink and Moolenaar, 1992; Zhang et al., 1994; Chrzanowska-Wodnicka and Burridge, 1996). Wu and coworkers demonstrated that both integrin activation on the cell surface and integrin-cytoskeleton interactions (postoccupancy events) are essential for the assembly of a Fn matrix (Wu et al., 1995). Our data are in agreement with these findings. The increased Fn matrix assembly mediated by $\beta 1D$ correlates well with the larger proportion of activated integrins on the cell surface, the more stable integrin-cytoskeleton linkage and enhanced contractility in these transfectants. Interestingly, the observed enhancement of Fn matrix assembly by $\beta 1D$ appeared to be greater than the increase in ligand-binding affinity for $\beta 1D$ transfectants. Even though an activation of $\beta 1A$ integrin with TS2/16 mAb significantly increased Fn matrix assembly by $\beta 1A$ transfectants, this still did not convert them entirely to the $\beta 1D$ phenotype. These differences might reflect the enhanced integrin-cytoskeleton association and contractility in $\beta 1D$ transfectants.

Finally, the increased ligand-binding, contractility, and Fn matrix deposition contribute to the reduced cell migration of $\beta 1D$ transfectants. In accordance with recent findings of Palecek et al. (1997), activation of the $\beta 1A$ ectodomain reduced the migration of the transfectants at substrate concentrations and integrin expression levels used in our experiments. Notably, integrin activation in the case of $\beta 1A$ transfectants did not decrease their migration rates to the levels characteristic for $\beta 1D$ -expressing cells, again suggesting an important role for integrin-cytoskeleton interactions in the generation of the $\beta 1D$ phenotype. Together, our data demonstrate that the whole set of alterations displayed by cells expressing $\beta 1D$, is determined by the distinctive structure of the $\beta 1D$ cytoplasmic domain. The alternatively spliced sequence of this $\beta 1$ integrin isoform both enhances its association with the actin cytoskeleton and increases receptor-ligand interaction due to constitutive activation of the $\beta 1D$ ectodomain. Both these

factors contribute to increased Fn matrix assembly and decreased migration of $\beta 1D$ transfectants.

The changes of nonmuscle cells triggered by expression of the $\beta 1D$ isoform are analogous to the transitions that muscle cells undergo during differentiation, accompanied by a gradual increase in the expression of this integrin (Belkin et al., 1996). Thus, growing myotubes possess large, extremely stable adhesions, their spreading is greatly inhibited and even early immature myotubes cease to migrate. Although Fn matrix assembly is strongly decreased in muscle cells, upregulation of synthesis and enhanced deposition of certain laminin isoforms is typical for myodifferentiation both in culture and in vivo. During myodifferentiation, a dramatic increase in cellular contractility has to be counterbalanced by the reinforcement of the cytoskeleton-matrix link. All these phenotypic transitions characteristic for differentiating muscle cells generate a requirement for the novel integrin, strengthening the association between the actin cytoskeleton and the surrounding extracellular matrix. This requirement determines the distinctive properties of $\beta 1D$ and defines a critical role for this $\beta 1$ integrin cytoplasmic domain in the organization and function of adhesive structures in muscle tissues.

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