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**Muscle β1D Integrin Reinforces the Cytoskeleton–Matrix Link: Modulation of Integrin Adhesive Function by Alternative Splicing**

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**Abstract.** Expression of muscle-specific β1D integrin with an alternatively spliced cytoplasmic domain in CHO and GD25, β1 integrin-minus cells leads to their phenotypic conversion. β1D-transfected nonmuscle cells display rounded morphology, lack of pseudopodial activity, retarded spreading, reduced migration, and significantly enhanced contractility compared with their β1A-expressing counterparts. The transfected β1D is targeted to focal adhesions and efficiently displaces the endogenous β1A and αvβ3 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 β1D transfectants. Whereas a significant part of cellular β1A integrin is extractable in digitonin, the majority of the transfected β1D is digitonin-insoluble and is strongly associated with the detergent-insoluble cytoskeleton.

Increased interaction of β1D integrin with the actin cytoskeleton is consistent with and might be mediated by its enhanced binding to talin. In contrast, β1A interacts more strongly with α-actinin, than β1D. Inside-out driven activation of the β1D ectodomain increases ligand binding and fibronectin matrix assembly by β1D transfectants. Phenotypic effects of β1D 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 β1 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 β1D 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, β1 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 β1 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;
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 β1 integrin subunit have been described. Besides the major β1A isoform, characteristic for all known cell types except red blood cells and terminally differentiated striated muscles, two minor cytoplasmic domain isoforms of β1 integrin, β1B and β1C, have been characterized (Altruda et al., 1990; Languino and Ruoslahti, 1992). Although their functions remain uncertain, it has been speculated that β1B can serve as a negative regulator of cell adhesion during development, whereas β1C can strongly inhibit cell growth (Balzac et al., 1993, 1994; Meredith et al., 1995). The alternatively spliced sequences of β1B and β1C have no homology to the major β1A 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, β1B and β1C variants have been found only in humans, whereas the fourth β1 isoform, β1D, 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; van der Flier et al., 1995).

β1 integrin is localized at junctional structures of striated muscles (Boyczcko et al., 1989). Expression of the β1 integrin subunit as well as the ligand occupation of β1-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 β1 integrin in muscles. This function is now attributed primarily to β1D cytoplasmic domain variant, which is a major β1 isoform that completely displaces β1A integrin in differentiated striated muscles (Belkin et al., 1996). Its cytoplasmic domain is highly homologous to that of β1A, including conservation of both NPYX 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). β1D accumulates at all major cell–matrix adhesion sites both in skeletal muscle fibers and cardiomyocytes. α7β1D is a predominant integrin in adult skeletal and heart muscle tissues (Belkin et al., 1996). However, other α subunits, including α5 and α6A, can pair with β1D in developing heart muscle (Branaccio et al., 1997). The data obtained so far lead to the suggestion that β1D 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, β1D is targeted to focal adhesions, proving that the muscle-specific isoform of β1 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 β1D and β1A cytoplasmic domain isoforms in CHO cells and the mouse GD25 cell line. GD25 cells lack endogenous β1 integrin as a consequence of gene inactivation (Wennerberg et al., 1996). Here we report that the expression of β1D 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 β1D integrin with both the actin cytoskeleton and extracellular matrix ligands. β1D-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 β1D 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 β1D 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 β1 integrin were used in this study: TS2/16 mAb to human β1 subunit, which activates ligand binding by β1-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 β1 integrin (Carter et al., 1990) was from GIBCO BRL (Gaithersburg, MD); 102DF5 mAb against human β1 integrin (Ylanne and Virtanen, 1989); 12G10 mAb, which reacts with activated (high affinity conformation for ligand binding) human β1 integrin (Hemler et al., 1984); conjugated with fluorescein and rabbit polyclonal antibody against human β1 integrin (Belkin et al., 1990). 7E2 mAb against hamster β1 integrin and inhibitory PBI mAb against intact hamster α5β1 integrin were generous gifts from Dr. R. Juliano (University of North Carolina, Chapel Hill, NC) (Brown and Juliano, 1983, 1988). Integrin-specific antibodies against β1A and β1D 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, α3, and α5 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 dissolution in water, filtering, and lyophilization before use. 32P-Translabel and methionine- and cysteine-free medium were from ICN Biomedicals Inc. (Costa Mesa, CA). Na232P and 32POrthophosphate were from Du Pont-NEN (Boston, MA).

Expression Constructs, Transfection, and Cell Culture

Human full-length cDNAs encoding β1A integrin or β1D integrin in SV40-based expression vector PECE (Ellis et al., 1986), were transfected into CHO cells or β1-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 β1 integrin subunit; the expression level of the transfected β1A and β1D integrins were very similar and comparable to the level of the endogenous hamster β1 integrin subunit in CHO cells and close to the levels of the endogenous αv and β3 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 β1A-CHO and β1D-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 V.1.0 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, β1A- and β1D-transfected CHO and GD25 cells were plated in serum-free medium on Fn, laminin, vitronectin, or on immobilized mAb TS2/16 to human β1 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 125I-labeled Fn(III)10 fragment to β1A-CHO, β1D-CHO, β1A-GD25, and β1D-GD25 cells in suspension was quantified as described (O’Toole et al., 1990; Wu et al., 1995). Since CHO cells express the endogenous α5β1, and β1-minus GD25 cells express αvβ3 as a major Fn-binding integrin (Wennerberg et al., 1996), inhibitory mAbs PB1 against hamster α5β1 or αvβ3 were used with CHO and GD25 cells, respectively. In some experiments, blocking P4C10 mAb against human β1 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 x 10⁵ cells/ml) in Tyrode’s buffer were incubated with specified concentrations of 125I-labeled Fn(III)10 fragment (sp act 0.12 mCi/mg) for 30 min at 37°C either alone or in the presence of 10 μg/ml of purified TS2/16 mAb, which activates human β1 integrins. Coincubation with an excess of unlabelled Fn(III)10 fragment (0.5 mg/ml) was used to determine and subtract the nonspecific background binding, 50-μl 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 β1A or β1D integrins in CHO and GD25 transfectants was assessed with 102DF5 and TS2/16 mAbs, whose binding to the β1 subunit is conformation independent. Their expression levels were compared to those of the endogenous hamster β1 integrin (examined with 7E2 mAb). 12G10 mAb reacting with activated human β1 integrin subunit (Mould et al., 1995) and conformation-specific 9EG7 anti-human β1 mAb (Bazzoni et al., 1995) were used either in the absence of Mn²⁺ ions or in the presence of 1 mM Mn²⁺. Fluorescein-labeled, affinity-purified donkey anti-mouse IgG (Chemicon International, Inc.) was used as secondary antibody.

**Fn Matrix Assembly Assays**

β1A-CHO, β1D-CHO, as well as β1A-GD25 and β1D-GD25 cells did not assemble Fn matrix well when confluent cell monolayers were cultured in growth medium containing 1% PBS 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 α5β1 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 β1A and β1D 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 quantify Fn incorporation into deoxycholate-insoluble matrix, con-fluent β1A-CHO, β1D-CHO, β1A-GD25, and β1D-GD25 cultures were incubated for 2 d with 100, 200, or 300 nM of 125I-labeled Fn (sp act 0.08 mCi/mg) 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). 125I-labeled Fn incorporated into the deoxycholate-insoluble extracellular matrix was examined 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 β1A-CHO, β1D-CHO, β1A-GD25, and β1D-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, β1A- and β1D-transfected CHO and GD25 cells were plated on plastic dishes coated with 10 μg/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 β1A and β1D integrins.

**Analysis of the Association of β1A and β1D Integrins with α Subunits**

β1D-CHO cells as well as β1A- and β1D-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 β1 integrins were immunoprecipitated using TS2/16 mAb, whereas α3, α5, 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 isofrom-specific antibodies against β1A or β1D integrins.

**Localization of the Transfected β1A and β1D Integrins and the Endogenous β1A and αv Subunits and Analysis of their Association with the Actin Cytoskeleton**

To localize the transfected and the endogenous β1 integrins, as well as the endogenous αv integrins in the transfectedants, 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 β1 and rhodamine-labeled 7E2 mAb to hamster β1 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 Axioshot microscope and photographed using Kodak T-Max 400 film.

To study whether the solubility of integrins in digitonin correlates with their cytoskeletal association, 35S-labeled β1A-CHO and β1D-CHO cells, either untreated or treated for 1 h with 10 μg 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 MgCl₂, 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 μg/mg leupeptin, 10 μg/ml pepstatin, and 0.5 mM PMSF as protease inhibitors. The transfected β1A and β1D integrins were immunoprecipitated from digitonin- and RIPA-soluble fractions using TS2/16 mAb. 35S-labeled β1 integrin immunoprecipitates were run on 10% gels and analyzed by autoradiography.

To assess the association of the transfected and the endogenous β1 integrin subunits and the endogenous αv integrins with the actin cytoskeleton, 35S-labeled CHO and GD25 transfectants were sequentially extracted with digitonin and RIPA buffers as described above. Immunoprecipitation of the transfected human β1A and β1D integrins from both cellular fractions was performed with TS2/16 mAb. 7E2 mAb antibody was used for the endogenous hamster β1A integrin. Rabbit anti-αv antibody was used...
used to immunoprecipitate the endogenous αβ3/αvβ5 integrins from GD25 transfectants. 35S-labeled β1 and αv immunoprecipitates were analyzed by SDS-PAGE on 10% gels and subsequent autoradiography.

**Analysis of the Association of β1A and β1D Integrins with Talin and α-Actinin by Com Immunoprecipitation**

To compare the association of β1A and β1D integrins with talin and α-actinin, 5 × 10⁶ transfected cells were incubated in suspension with 1 μM 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 Mn²⁺ or used in the absence of Mn²⁺. Cells were centrifuged (1000 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 MgCl₂, 1 mM EGTA, 1 mM EDTA, pH 6.9, with 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 0.5 mM PMSF. Under these conditions ~80–90% of cellular β1 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 β1 integrin, 844 mAb against α-actinin, or 1682 mAb against talin. To verify the equal amount of β1A and β1D isoforms in the immunoprecipitates, the blots were stripped and reprobed with the isoform-specific antibodies against β1A and β1D (Belkin et al., 1996).

**Interaction of Talin and α-Actinin with β1A and β1D 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 125I Iodobeads (Pierce Chemical Co., Rockford, IL). The proteins were labeled to a specific activity of 1.2 × 10⁶ cpm/μg for talin and 7.5 × 10⁵ cpm/μg for α-actinin.

Full-length cytoplasmic domain peptides of β1A and β1D integrins (Belkin et al., 1996) were iodinated using iodogen method. Both peptides were labeled at their binding sites for the microtrophin wells in the 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 concentrations of β1A and β1D peptides was used in subsequent experiments to immobilize them on plastic 96-well microtrophin 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 μM of 125I-talin or 125I-α-actinin and 1 mM 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., 1989; Danowski, 1989). The UV glow discharge polymerization was used in combination with gold-palladium discharge polymerization was used in combination with gold-palladium deposition. In the case of 12G10 mAb, cells were either preincubated for 5 min with 1 mM Mn²⁺ or used in the absence of Mn²⁺. Cells were centrifuged (1000 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 MgCl₂, 1 mM EGTA, 1 mM EDTA, pH 6.9, with 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 0.5 mM PMSF. Under these conditions ~80–90% of cellular β1 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 β1 integrin, 844 mAb against α-actinin, or 1682 mAb against talin. To verify the equal amount of β1A and β1D isoforms in the immunoprecipitates, the blots were stripped and reprobed with the isoform-specific antibodies against β1A and β1D (Belkin et al., 1996).

**Results**

β1D Integrin Alters Cell Morphology and Inhibits Spreading

The levels of surface expression of the transfected human β1A and β1D, 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 β1A in CHO transfectants and αv integrins in GD25 cells (see Fig. 9, I and J). No difference in association of the transfected β1A and β1D with endogenous α subunits was found in the two types of transfectants (Fig. 7).

To determine possible effects of β1A and β1D integrin expression on cell morphology, CHO transfectants were grown on Fn for 1 d (Fig. 1, A and B). β1D-CHO cells appeared more rounded with fewer cytoplasmic extensions at their periphery than their β1A-transfected counterparts. Statistical analysis of the cell shape was performed for randomly chosen β1A-CHO and β1D-CHO cells (Dunn and Brown, 1986). This demonstrated that spread areas were similar for β1A and β1D transfectants, but the average cell perimeter was ~20% lower for β1D-CHO cells (Table II).

Two cell shape parameters, dispersion and elongation, representing measures of cell multipolarity and bipolarity, respectively, were significantly lower for β1D transfectants. These data indicated that pseudopodial activity and cell polarization were reduced in CHO cells expressing β1D integrin.

**Table I. Expression Levels and Activation States of Transfected Human β1A and β1D Integrins in CHO and GD25 Transfectants**

| Cell type | mAb 102DF5 | mAb 12G10 | mAb 12G10 + 1 mM Mn²⁺ | mAb 12G10 + 1 mM Mn²⁺ |
|-----------|------------|-----------|----------------------|----------------------|
| β1A-CHO   | 43.1       | 9.2       | 33.6                 | 27.4                 |
| β1D-CHO   | 34.4       | 21.8      | 28.2                 | 77.3                 |
| β1A-GD25  | 42.2       | 12.2      | 32.4                 | 37.7                 |
| β1D-GD25  | 38.9       | 29.1      | 36.7                 | 79.3                 |

| β1A-GD25  | 38.9       | 29.1      | 36.7                 | 79.3                 |

| mAb 9EG7  | mAb 12G10 | 1 mM Mn²⁺ | mAb 12G10 | 1 mM Mn²⁺ |
|-----------|------------|-----------|-----------|-----------|
| mAb 9EG7  | 105        | 39        | 89        | 43.8      |
| β1D-GD25  | 95         | 69        | 78        | 88.4      |

*Values are mean fluorescence intensities from a representative experiment.

1Values are percentages.
remained unaffected (Fig. 2, B), but markedly increased Fn(III) fragment binding to both CHO (C, E, and G) and 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 β1 integrin for 2 h (G and H). 1A-GD25 (J and K) and 1D-GD25 (J and L) cells were plated in serum-free medium on Fn (J and L) or vitronectin (K and L) for 1 h. Cells were fixed with formaldehyde and stained with Coomassie blue. Bar, 50 μm.

β1A-GD25 cells (Fig. 1, I–L). These observations suggested that retardation of spreading was a general phenomenon of β1D expression.

Constitutive Activation of the β1D Integrin Ectodomain Increases Ligand Binding

To characterize the affinity states of β1A and β1D integrins, the binding of the Fn(III)10 fragment to β1A- and β1D-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 αβ1 and mouse αβ3 integrins, respectively, β1D transfecteds exhibited a significant increase in binding to the soluble ligand compared with β1A-expressing cells (Fig. 2, A and B). TS2/16 mAb, which activates human β1 integrin, markedly increased Fn(III)10 fragment binding to both β1A-CHO and β1A-GD25 cells, whereas the ligand binding by β1D transfecteds in the presence of TS2/16 mAb remained unaffected (Fig. 2, A and B). Blocking P4C10 mAb against human β1 integrin completely abolished the binding of Fn(III)10 fragment to all types of transfecteds (Fig. 2, A and B), therefore proving its specificity.

To further assess the difference in the conformation of the ectodomains of the transfected β1A and β1D, a flow cytometry analysis was used with mAb 12G10. This antibody recognizes preferentially a Mn2+ and ligand-induced conformation of the human β1 integrin subunit (Mould et al., 1995; Mould, 1996). The amount of 12G10 mAb bound to the cell surface was significantly higher for β1D-expressing CHO and GD25 cells, than for β1A-expressing cells. Moreover, the binding of 12G10 mAb was almost unchanged for β1D-CHO and β1D-GD25 cells in the presence of Mn2+, whereas the 12G10 mAb binding to both types of β1A transfecteds was dramatically increased by Mn2+ (Table I). Another mAb, 9EG7, whose binding to human β1 integrin is stimulated by ligands and Mn2+, but inhibited by Ca2+ (Bazzoni et al., 1995), also reacted more strongly with β1D-GD25 compared with β1A-GD25 cells (Table I). Together, these results showed that in β1D-expressing cells in suspension the majority (~77–88%) of β1D integrins were constitutively activated on the cell surface, whereas only ~27–44% of the transfected β1A integrins were present in the activated state.

β1D Enhances Fn Matrix Assembly

Fn biosynthesis and secretion levels in CHO and GD25 cells were not altered by β1A or β1D expression and appeared to be identical for each pair of transfecteds (data not shown). Fn matrix assembly by β1A- and β1D-transfected cells was analyzed with the exogenous Fn by both immunofluorescence and measurements of 125I-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 β1D transfecteds compared to their β1A-expressing counterparts (Fig. 3 A). In our subsequent experiments we used 200 nM of exogenous Fn for matrix assembly studies with the transfecteds. There was no difference in Fn matrix assembly between β1A and β1D transfecteds in the presence of blocking P4C10 mAb against human β1 integrin, showing that the observed effects can be ascribed specifically to the expressed β1D (Fig. 3 B).

In the presence of blocking anti-hamster αβ1 integrin mAb PB1 (for CHO cells) or inhibitory anti-mouse αβ1 integrin mAb H9.2B8 (for GD25 cells), β1D transfecteds assembled more abundant meshwork of Fn fibrils, than β1A-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 β1A-CHO and β1A-GD25 cells, but did not change the levels of assembly for β1D-transfected CHO and GD25 cells (Fig. 4, C, D, G, H, and I, c, d, g, and h). Quantitation of 125I-Fn incorporated into the extracellular matrix showed a five- to sixfold increase in Fn assembly by β1D compared with β1A 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 β1A integrin for both types of transfecteds, these levels appeared still much lower than those exhibited by β1D integrin (Fig. 4, I and J).

Table II. Cell Shape Parameters of β1A-CHO and β1D-CHO Cells

| Cell type     | Area, μm² | Perimeter, μm | Dispersion | Elongation |
|---------------|-----------|---------------|------------|------------|
| β1A-CHO*      | 814 ± 37  | 147 ± 4       | 0.237 ± 0.016 | 1.059 ± 0.065 |
| β1D-CHO*      | 742 ± 34  | 118 ± 3       | 0.112 ± 0.009 | 0.769 ± 0.045 |

*107 cells were analyzed for β1A-CHO population and 109 cells for β1D-CHO population.

Given are means and SE.
\(\beta 1D\) 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 were a display significant increase in Fn(III)10 fragment binding in the presence of activating TS2/16 mAb. Depicted are the means from triplicate measurements.

Interaction of \(\beta 1A\) and \(\beta 1D\) Integrins with \(\alpha\) 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 \(\alpha\) subunits in the transfected cells. Therefore, we examined \(\alpha\) subunit association for \(\beta 1A\) and \(\beta 1D\) in CHO and GD25 transfectants. Among various \(\beta 1\)-associated \(\alpha\) subunits, \(\alpha 3\), \(\alpha 5\), and very small amount of \(\alpha 6\) 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) 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 \(^{35}\text{S}\)-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 cytchalasin D–treated \(\beta 1A\)- and \(\beta 1D\)-transfected CHO cells (Fig. 8). Treatment of cultured cells with cytchalasin 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.
β1D Integrin Displaces the Endogenous β1A and αv Subunits from Focal Adhesions and Associates Strongly with the Digitonin-insoluble Cytoskeleton

Since β1D and β1A integrins have structurally different cytoplasmic domains, and the two types of transfectants displayed dissimilar phenotypes, we next attempted to compare cytoskeletal interactions of the β1A and β1D isoforms. To localize the transfected and the endogenous β1 integrins in CHO transfectants, cells grown on Fn were double stained with anti–human β1 and anti–hamster β1 mAbs. In β1A-CHO cells, both the transfected and the endogenous β1A subunits colocalized at focal adhesions (Fig. 9, A and C). β1D integrin was prominently localized at focal adhesions of β1D-CHO cells. Surprisingly, no endogenous β1A integrin was detected in focal adhesions of β1D transfectants grown on Fn or other extracellular matrix proteins (Fig. 9, B and D; and data not shown). Similarly, both the transfected β1A and β1D integrins were targeted to focal adhesions of GD25 transfectants on Fn (Fig. 9, E and F). The endogenous αv subunit of Fn-binding αvβ3 integrin in β1A-GD25 cells was at least partially colocalized with β1A at sites of cell–matrix contact (αv does not pair with β1 integrins in GD25 cells; Wennerberg et al., 1996). In contrast, β1D displaced the endogenous αv integrins from focal adhesions (Fig. 9, G and H). Therefore, the displacement of β1A and αv integrins from focal adhesions by expressed β1D was a general phenomenon, suggesting a considerably stronger association of β1D integrin with the actin cytoskeleton.

To define biochemically the modes of β1A and β1D interaction with the cytoskeleton, both the transfected β1A and β1D, as well as the endogenous β1A and αv integrins were immunoprecipitated from soluble and cytoskeleton-associated fractions of 35S-labeled transfectants grown on Fn (Fig. 9, I and J). In β1A-CHO cells, the transfected β1A was equally distributed between the soluble and the cytoskeletal fractions, whereas the majority of the endogenous hamster β1A subunit was associated with the cytoskeleton. In contrast, β1D was found exclusively in the cytoskeletal fraction, whereas almost all the endogenous β1A integrin was present in the soluble fraction of β1D-CHO cells, displaced from the cytoskeleton (Fig. 9 I). Cytoskeletal associations of the transfected β1A and β1D integrins in GD25 transfectants were similar to those observed in CHO transfectants. Again, much of the transfected β1A integrin was digitonin soluble. However, the majority of β1D integrin was digitonin insoluble, whereas most of the endogenous αvβ3/αvβ5 integrins were dis-
or cells in the presence of blocking P4C10 mAb against human integrin (known to interact in vitro with the cytoplasmic domain of

Differential Interaction of β1A and β1D 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 β1A integrin (Horwitz et al., 1986; Otey et al., 1990). To identify cytoskeletal proteins, associated preferentially with the β1D integrin subunit in vivo, antibody clustering of the transfected β1A and β1D 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 β1 integrins (data not shown). When β1A and β1D immunoprecipitates, obtained with activating TS2/16 anti–human β1 mAb, were compared by immunoblotting with mAb 8d4 against talin, a significantly stronger talin band was detected in association with β1D 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 β1D compared with β1A integrin did not depend on the nature of anti-β1 mAb used for clustering. 12G10 mAb, which recognizes a Mn$^{2+}$- and ligand-induced conformation of human β1 integrin, precipitated more talin from β1D-CHO than from β1A-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 β1 integrin, equal amounts of talin were detected in association with the endogenous β1A in CHO transfectants (Fig. 10, A, e, and e'). Interestingly, when anti–human β1 integrin immunoprecipitates from both types of transfectants were probed for α-actinin, more α-actinin was detected in association with β1A than with β1D 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...
To compare further the interactions of β1A and β1D integrins with talin and α-actinin, in vitro solid phase binding assays were performed with 125I-talin, 125I-α-actinin, and immobilized full-length synthetic peptides, corresponding to the β1A and β1D cytoplasmic domains (Fig. 11). First, we tested the binding of 125I-labeled β1A and β1D cytoplasmic domain peptides to the microtiter wells (Fig. 11 A). The amounts of the β1A and β1D 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 125I-talin and 125I-α-actinin with the β1A and β1D cytoplasmic domain peptides (Fig. 11, B and C). We found that the binding of 125I-talin to the β1D peptide was several fold higher than the binding to the β1A peptide. The apparent dissociation constants of $4.2 \times 10^{-8}$ and $5.9 \times 10^{-9}$ M were calculated from the competition binding curves for talin interactions with the β1A and β1D cytoplasmic peptides, respectively (Fig. 11 B). Conversely, 125I-α-actinin bound more strongly to the β1A than to the β1D cytoplasmic domain peptide (Fig. 11 C). In this case the dissociation constants were $1.2 \times 10^{-8}$ M for α-actinin–β1A binding and $8.8 \times 10^{-8}$ M for α-actinin–β1D binding. Together, the coimmunoprecipitation analyses and in vitro binding data demonstrated that

with the isoform-specific antibodies against β1A (Fig. 10, B and E) and β1D (Fig. 10, C and F) integrins (Belkin et al., 1996). Besides the immunoprecipitates from β1A-CHO and β1D-CHO cells with the conformation-specific mAb 12G10 in the absence of Mn2+ (Fig. 10, B, C, and C'), all other samples contained equal amounts of β1 integrins (Fig. 10, B, C, E, and F).

To compare further the interactions of β1A and β1D integrins with talin and α-actinin, in vitro solid phase binding assays were performed with 125I-talin, 125I-α-actinin, and immobilized full-length synthetic peptides, corresponding to the β1A and β1D cytoplasmic domains (Fig. 11). First, we tested the binding of 125I-labeled β1A and β1D cytoplasmic domain peptides to the microtiter wells (Fig. 11 A). The amounts of the β1A and β1D 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 125I-talin and 125I-α-actinin with the β1A and β1D cytoplasmic domain peptides (Fig. 11, B and C). We found that the binding of 125I-talin to the β1D peptide was several fold higher than the binding to the β1A peptide. The apparent dissociation constants of $4.2 \times 10^{-8}$ and $5.9 \times 10^{-9}$ M were calculated from the competition binding curves for talin interactions with the β1A and β1D cytoplasmic peptides, respectively (Fig. 11 B). Conversely, 125I-α-actinin bound more strongly to the β1A than to the β1D cytoplasmic domain peptide (Fig. 11 C). In this case the dissociation constants were $1.2 \times 10^{-8}$ M for α-actinin–β1A binding and $8.8 \times 10^{-8}$ M for α-actinin–β1D binding. Together, the coimmunoprecipitation analyses and in vitro binding data demonstrated that
talin interacts more strongly with the cytoplasmic domain of β1D than β1A integrin, whereas α-actinin interacts preferentially with the β1A integrin.

**β1D Integrin Increases Cellular Contractility**

The enhanced association of β1D integrin with the actin cytoskeleton prompted us to examine whether the β1D integrin–cytoskeleton interaction increases contractility. When β1A and β1D transfectants were plated on flexible silicone rubber substrata for 1 d, β1D-transfected cells generated prominent wrinkles within the substrata. However, β1A 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 β1D-CHO cells (Fig. 12 E). Therefore, the observed increase in cellular contractility of β1D transfectants appears to be mostly because of enhancement of actin–membrane attachment, and did not depend on significant changes of myosin ATPase activity.

**Discussion**

In this work we have analyzed muscle β1D integrin, comparing its properties with those of the common β1A isoform. We found that the unique cytoplasmic sequence of β1D endows this molecule with the distinctive functional properties. β1D integrin displays an increased affinity for both the extracellular matrix ligands and the actin cytoskeleton. Expression of β1D causes a marked phenotypic conversion of both CHO and β1-deficient GD25 cells. The β1D 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 β1D isoform, cause a significant reinforcement of the entire cytoskeleton–matrix link. Unlike two other β integrin variants β1B and β1C, which are distributed uniformly at the cell surface and are unable to interact with the actin cytoskeleton, β1D is readily targeted to sites of cell–matrix adhesion upon expression in nonmuscle cells. Furthermore, significantly stronger association of β1D with the actin cytoskeleton can be mediated by its enhanced interaction with...
The mediated, cytoskeleton–matrix link is built exclusively by infected nonmuscle cells. In this situation, the 

subunit from sites of cell–matrix adhesion by 

attachment. 

ates a unique “dominant-positive phenotype” of the trans-

increased resistance of focal adhesions against disassembly 

focal adhesions were similar in both types of transfectants (data not 

proteins at focal adhesions of 

showed). Apparently, the differences in the affinities be-

ences between 

b 

1D and 

b 

1A-transfected 

subunit cytoplasmic do-

2 and 

3 subunit cytoplasmic do-

1 transfectants. The intensities of immunostaining for talin, vinculin, 

-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 

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1A/ 

b 

1D talin and -actinin might be insufficient to detect preferential accumulation of these cytoskeletal proteins at focal adhesions of 

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1A- and 

b 

1D-transfected 

cells by immunofluorescence. Nevertheless, 

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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 

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1D integrin that occurs primarily because of enhancement of actin–membrane at-

PMA and cytochalasin D were shown to stimulate lymphocyte adhesion, which was accompanied by activation of their major 

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2 integrin and its release of cytoskeletal constraints. This points to the opposite mechanisms of cytoskeletal control of integri-

activation between 

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2 and 

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1 integrin subfamilies and might reflect profoundly dissimilar physiology of nonad-

herent lymphocytes and adhesion-dependent cells, including striated muscles. 

Previously, modulation of the ligand-binding affinity of integrin 

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subunits was demonstrated using either activating antibodies (Arroyo et al., 1992; Faull et al., 1993) or point mutations in the 

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1 and 

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3 subunit cytoplasmic do-

mains (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 mecha-

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 sig-

aling where activation of the extracellular domain of the integrin 

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subunit is controlled by alternative splicing of its cytodomain. Since the expression of 

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1D and 

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1A iso-

forms 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 

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1 integrin subunit. 

The organization of focal adhesions in 

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1D-transfected nonmuscle cells appears to be very similar to that found in differentiated muscle cells. In both situations, 

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1D is the only 

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1 isoform localized at cell–matrix adhesive 

structures (Belkin et al., 1996). Therefore, the enhanced cyto-

skeleton–matrix association and stabilization of focal adhe-

sions, mediated by 

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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 

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1D integrin to actin filaments in muscle. In con-

trast, we found that -actinin, a focal adhesion component that interacts with 

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1A in vitro (Otey et al., 1990), binds 

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1D less strongly than 

b 

1A. This correlates with the absence of -actinin at myotendinous junctions, the major 
sites of force transmission in muscle (Tidball, 1987). Addi-

tionally, certain muscle-specific cytoskeletal proteins, in-

cluding dystrophin, may contribute to linking 

b 

1D integrin to the subsarcolemmal cytoskeleton.

Figure 12. 

β1D integrin elevates cellular contractility without affecting phosphatase activity for 

β1A and 

β1D transfectants. 

β1A-

CHO (A), 

β1D-CHO (B), 

β1A-GD25 (C), and 

β1D-GD25 (D) cells were plated for 1 d on silicon rubber substrata and photographed. (E) Myosin light chain phosphorylation in CHO transfectants. Myosin was immunoprecipitated from 32P-labeled 

β1A-

CHO cells (a) and 

β1D-CHO cells (b). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Arrowhead points to myosin light chains. Bar, 200 μm.
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 β₁₉ integrins (analogous to β₁ integrins in vertebrates) to sites of their function in embryonic muscles (Martin-Bermudo and Brown, 1996). The altered structure of the β₁D 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 β₁D can be driven by a combination of higher ligand-binding affinity of this integrin, reinforced actin-membrane association and enhanced contractility of β₁D 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 β₁D 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 β₁D appeared to be greater than the increase in ligand-binding affinity for β₁D transfectants. Even though an activation of β₁A integrin with TS2/16 mAb significantly increased Fn matrix assembly by β₁A transfectants, this still did not convert them entirely to the β₁D phenotype. These differences might reflect the enhanced integrin–cytoskeleton association and contractility in β₁D transfectants.

Finally, the increased ligand-binding, contractility, and Fn matrix deposition contribute to the reduced cell migration of β₁D transfectants. In accordance with recent findings of Palecek et al. (1997), activation of the β₁A 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 β₁A transfectants did not decrease their migration rates to the levels characteristic for β₁D-expressing cells, again suggesting an important role for integrin–cytoskeleton interactions in the generation of the β₁D phenotype. Together, our data demonstrate that the whole set of alterations displayed by cells expressing β₁D, is determined by the distinctive structure of the β₁D cytoplasmic domain. The alternatively spliced sequence of this β₁ integrin isoform both enhances its association with the actin cytoskeleton and increases receptor–ligand interaction due to constitutive activation of the β₁D ectodomain. Both these factors contribute to increased Fn matrix assembly and decreased migration of β₁D transfectants.

The changes of nonmuscle cells triggered by expression of the β₁D 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 β₁D and defines a critical role for this β₁ integrin cytoplasmic domain in the organization and function of adhesive structures in muscle tissues.

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