β1D Integrin Displaces the β1A Isoform in Striated Muscles: Localization at Junctional Structures and Signaling Potential in Nonmuscle Cells

Alexey M. Belkin,* Natalia I. Zhidkova,‡ Fiorella Balzac,§ Fiorella Altruda,§ Daniela Tomatis,§ Alfred Maier,‡ Guido Tarone,§ Victor E. Koteliansky,‖ and Keith Burridge*

*Department of Cell Biology and Anatomy, University of North Carolina, Chapel Hill, North Carolina 27599;‡Department of Cell Biology, University of Alabama, Birmingham, Alabama 35294;§Department of Genetics, Biology and Medical Chemistry, University of Torino, Italy; and ‖CNRS-Institut Curie, Paris, France 75232

Abstract. The cytoplasmic domains of integrins provide attachment of these extracellular matrix receptors to the cytoskeleton and play a critical role in integrin-mediated signal transduction. In this report we describe the identification, expression, localization, and initial functional characterization of a novel form of β1 integrin, termed β1D. This isoform contains a unique alternatively spliced cytoplasmic domain of 50 amino acids, with the last 24 amino acids encoded by an additional exon. Of these 24 amino acids, 11 are conserved when compared to the β1A isoform, but 13 are unique (Zhidkova, N. I., A. M. Belkin, and R. Mayne. 1995. *Biochem. Biophys. Res. Commun.* 214:279–285; van der Flier, A., I. Kuikman, C. Baudoin, R. van der Neuf, and A. Sonnenberg. 1995. *FEBS Lett.* 369:340–344). Using an anti-peptide antibody against the β1D integrin subunit, we demonstrated that the β1D isoform is synthesized only in skeletal and cardiac muscles, while very low amounts of β1A were detected by immunoblot in striated muscles. Whereas β1A could not be detected in adult skeletal muscle fibers and cardiomyocytes by immunofluorescence, β1D was localized to the sarcolemma of both cell types. In skeletal muscle, β1D was concentrated in costameres, myotendinous, and neuromuscular junctions. In cardiac muscle this β1 isoform was found in costameres and intercalated discs. β1D was associated with α7A and α7B in adult skeletal muscle. In cardiomyocytes of adult heart, α7B was the major partner for the β1D isoform. β1D could not be detected in proliferating C2C12 myoblasts, but it appeared immediately after myoblast fusion and its amount continued to rise during myotube growth and maturation. In contrast, expression of the β1A isoform was downregulated during myodifferentiation in culture and it was completely displaced by β1D in mature differentiated myotubes.

We also analyzed some functional properties of the β1D integrin subunit. Expression of human β1D in CHO cells led to its localization at focal adhesions. Clustering of this integrin isoform on the cell surface stimulated tyrosine phosphorylation of pp125^{FAK} (focal adhesion kinase) and caused transient activation of mitogen-activated protein (MAP) kinases. These data indicate that β1D and β1A integrin isoforms are functionally similar with regard to integrin-mediated signaling.

INTEGRINS are a large family of transmembrane heterodimeric receptors mediating association between the extracellular matrix (ECM)[1] and cytoskeletal elements (Hynes, 1992; Schwartz, 1992; Juliano and Haskill, 1993; Clark and Brugge, 1995). Integrins play a crucial role in cell adhesion including cell–matrix and intercellular interactions and therefore are involved in various biological phenomena, including cell migration, differentiation, tissue repair and remodeling, programmed cell death, etc. Both α and β subunits are composed of large extracellular and short intracellular domains with the exception of β4 integrin subunit that possesses an extremely long cytoplasmic tail. Experiments with chimeric integrins demonstrated that β subunits are necessary and sufficient for targeting integrins to sites of cell–matrix adhesion (focal adhesions, focal contacts) in a ligand-independent manner, while α subunits mostly determine the specificity of the ligand binding (Hayashi et al., 1992; LaFlamme et al., 1992; Ylanne et al., 1993). More than 20 different integrin heterodimeric receptors have been described, whereas 8 integrin β subunits and 15 α subunits have been identified so far. Among different β subunits, the β1 integrin subunit is usually the most abundant integrin expressed by adhesion-dependent cell types (Hynes, 1992). Beta 1 integrin associates with at least 10 α subunits to form distinct inte-

Address all correspondence to Dr. Alexey M. Belkin, Dept. of Cell Biology and Anatomy, Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7090. Tel.: (919) 966-5783. Fax: (919) 966-1856.

1. *Abbreviations used in this paper*: ECM, extracellular matrix; MAP, mitogen-activated protein; MBP, myelin basic protein; pp125^{FAK}, focal adhesion kinase; RT-PCR, reverse transcription polymerase chain reaction.

grin dimers, capable of interacting with various extracellular matrix molecules as well as some cell adhesion molecules (Hynes, 1992).

In contrast to the α subunit cytoplasmic domains, which are quite divergent, most β subunits, particularly β1 and β3 subunits, share significant homology within their cytoplasmic tails (Tamkun et al., 1986; Argraves et al., 1987; Fitzgerald et al., 1987). Cytoplasmic domains of several integrin β subunits, including β1, β3, β5, and β6 associate with the actin cytoskeleton and are required for the recruitment of integrins to focal adhesions of cultured cells (Sastry and Horwitz, 1993; Hemler et al., 1994). Results of in vitro experiments showed that at least two major actin-associated proteins present at focal adhesions, talin and α-actinin, are capable of interacting directly with β1 integrin intracellular domain peptides (Horwitz et al., 1986; Tapley et al., 1989; Otey et al., 1990, 1993). Integrin-mediated cell-matrix interaction during the adhesion process induces tyrosine phosphorylation of $pp125^{FAK}$ and paxillin (Guan et al., 1991; Kornberg et al., 1991, 1992; Burridge et al., 1992; Guan and Shalloway, 1992; Schaller et al., 1992). Clustering of integrins in the plasma membrane with extracellular matrix ligands or anti-integrin antibodies triggers association of some cytoskeletal proteins, including talin, vinculin, α-actinin, tensin, paxillin, and zyxin with integrins and leads to nucleation of assembly of actin filament bundles at sites of cell–matrix adhesion (Lewis and Schwartz, 1995; Miyamoto et al., 1995). Concomitantly, a number of signaling molecules like $pp125^{FAK}$, PKCγ, PI-3K, and G protein γ subunit accumulate at focal adhesions after integrin engagement and activate a variety of signaling pathways within the cell (Clark and Brugge, 1995). Mutants of the β1 integrin cytoplasmic domain are unable to localize to focal adhesion sites (Marcantonio and Hynes, 1990; Reszka et al., 1992; Akiyama et al., 1994). The cytoplasmic domain mutants as well as autonomously expressed cytoplasmic domain of β1 subunit were shown to have a dominant-negative effect on cell adhesion and inhibit outside-in signal transduction of integrins (LaFlamme et al., 1994; Lukashev et al., 1994; Smilenov et al., 1994). Growing data on β3 integrin subunit cytoplasmic domain mutants also indicate a role for both the entire cytoplasmic domain and some particular amino acid motifs within it for inside-out integrin signaling and regulation of the affinity state and ligand-binding properties of the receptor (Chen et al., 1994; O'Toole et al., 1994, 1995).

Recently, two novel isoforms of the β1 integrin subunit were described with altered cytoplasmic domains. One of them, β1B, is an isoform in which the 26-membrane–proximal amino acids of the cytoplasmic domain, encoded by exon 6 of the β1 integrin gene, are retained and then followed by 12 amino acids derived from the intron sequence located adjacent to exon 6 (Altruda et al., 1990). This is a rather minor isoform and is coexpressed with β1A in some tissues and cells (Balzac et al., 1993). Upon transfection into cultured cells, β1B serves as a dominant negative integrin variant, inhibiting cell adhesion and motility apparently due to its inability to interact with the actin cytoskeleton (Balzac et al., 1993, 1994). Based on this evidence, it was suggested that the β1B isoform may function as a "de-adhesion" integrin receptor during embryogenesis and in some physiological situations in adult tissues (Balzac et al., 1994). A similar isoform of the cytoplasmic domain was also described earlier for the β3 integrin subunit (van Kuppevelt et al., 1989). Another β1 integrin isoform with an alternatively spliced cytoplasmic domain, β1C, was identified in megakaryocytes, platelets and some other blood cells (Languino and Ruoslahti, 1992). Its cytoplasmic domain consists of 26 amino acids encoded by exon 6 and 48 novel amino acids derived from an additional exon of the β1 integrin gene. Upon transient transfection into mouse 10T1/2 fibroblasts, β1C integrin isoform does not localize to focal adhesions and at moderate concentrations has no effect on actin stress fibers and focal adhesions. However, β1C expression markedly inhibits DNA synthesis and causes growth arrest at the late G_1 phase of the cell cycle (Meredith et al., 1995).

In this report, we describe intracellular localization, expression during myodifferentiation, interaction with α subunits, and some signaling properties of a β1 integrin isoform, termed β1D, which has an alternatively spliced cytoplasmic domain, where the 24 most COOH-terminal amino acids are encoded by a novel exon of the β1 integrin gene (Zhidkova et al., 1995; van der Flier et al., 1995). This fourth variant of β1 integrin appears to be a muscle-specific isoform, replacing the common β1A isoform in terminally differentiated striated muscles. The β1D isoform localizes at various adhesive structures of muscle cells which were thought to contain β1A and is associated primarily with α7 subunit variants. We also show in CHO cells transfected with full-length β1D cDNA that the exogenous muscle-specific β1D subunit is localized to focal adhesions of nonmuscle cells and that antibody-induced clustering of β1D integrin causes an increase in tyrosine phosphorylation of $pp125^{FAK}$ and leads to a transient activation of MAP kinases.

Materials and Methods

Sources of Cells and Tissues

C2C12 mouse skeletal myoblasts were obtained from American Type Culture Collection (ATCC CRL-1772, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. To switch cells to myodifferentiation, fetal bovine serum was replaced with 3% horse serum in the growth medium. Cells were cultured on plastic dishes coated with 0.1% gelatin. CHO cells were obtained from ATCC and cultured in HAM's F12 medium plus 10% fetal bovine serum. All culture media and supplements were from GIBCO BRL (Gaithersburg, MD). Mouse tissues were dissected without dissociation into individual cells. Mouse and chicken skeletal muscle samples were taken from adult animals.

Isolation of RNA and First Strand cDNA Synthesis

Total RNA from C2C12 cells was obtained by the RNAzol™B method (Biotecx Laboratories, Inc., Houston, TX) and poly(A)-rich RNA selected by using the polyATract System IV (Promega Corp., Madison, WI) or FastTrack mRNA isolation kit (Invitrogen Corp., San Diego, CA). Synthesis of first strand cDNAs was based on a kit (Reverse Transcription System; Promega Corp.) using 2–5 μg of poly(A)-rich RNA in 50 μl of buffer. A mixture of random primers (10 μl) was added and the mixture incubated at 65°C and rapidly cooled on ice (1 min). To the RNA/primer mixture, 5 μl of 100 mM methylmercuric hydroxide (Alpha/Johnson Matthey, Ward Hill, MA) was added with incubation for 10 min at room temperature followed by 5 μl of 700 mM β-mercaptoethanol and incubation for 5 min at room temperature. For first strand cDNA synthesis, avian myeloblastosis virus reverse transcriptase (Promega Corp.) was employed with incubation at 45°C for 45 min followed by 55°C for 15 min. The cDNAs were precipitated with ethanol and resuspended in 50 μl of water.

Polymerase Chain Reaction (PCR)

Synthesized cDNAs were used as a template in the amplification by PCR in a Programmable Thermal Controller (MJ Research Inc., Watertown, MA). In order to analyze the cytoplasmic domain of mouse β1 integrin, PCR was performed with primers NZ1 (5′-^{2197}TTGTGGAGACTC-CAGACTGTCCTACT^{2222}-3′) and PE6 (5′-^{2500}TCATTTTCCCTCATA-CTTCGGATT^{2477}-3′), designed from Argraves et al., 1987; Holers et al., 1989; using PCR buffers and Taq 1 polymerase (Perkin Elmer, Norwalk, CT). The cycle parameters were: denaturation at 94°C for 2 min, annealing at 58°C for 1.5 min, extension at 72°C for 3 min for 33 cycles with 5 min final elongation at 75°C.

DNA Sequencing

All sequencing was performed on both strands with Sequenase™ version 2.0 DNA sequencing kit. (U.S. Biochem. Corp., Cleveland, OH). All plasmid templates for sequencing were isolated using the Wizard™ miniprep DNA purification system (Promega Corp.).

Antibodies

Rabbit polyclonal anti-peptide antibody against the last 12 amino acids (CTTVVNPKYEGK) of β1A integrin, which is isoform-specific and does not crossreact with either β1D or other known β1 subunit variants, was described and characterized earlier (Balzac et al., 1993, 1994). Rabbit polyclonal antibodies against synthetic peptides derived from the COOH-terminal sequences of α1, α2, α3, α4, α5, and αV integrin subunits were generated as described previously (Defilippi et al., 1992). These anti-peptide antibodies were purified by affinity chromatography on the relevant peptides coupled to Sepharose resin. They showed a broad cross-species reactivity. Antibodies to the COOH-terminal sequences of α7A and α7B were a kind gift from Dr. Ginetta Collo (Glaxo, Geneva, Switzerland). 7F9 monoclonal antibody against vinculin was described earlier (Glukhova et al., 1990). Anti-phosphotyrosine mAb PY20 was purchased from Transduction Laboratories (Lexington, KY). Anti-MAP kinase rabbit polyclonal antibodies *sc-93* and *sc-94* were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody N681 against human platelet talin was characterized earlier (Fath et al., 1989). Rat mAb M1.2 against an extracellular epitope of mouse β1 integrin was a kind gift of Dr. B. Chen (University of Western Ontario, London, Ontario, Canada). mAb TS2/16 against human integrin β1 subunit was a gift from Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). Rabbit antiserum 5158 against COOH-terminal portion of pp125^{FAK} was kindly provided by Dr. L. Romer (University of North Carolina, Chapel Hill).

Generation of Antibody against β1D Integrin Subunit

Three integrin cytoplasmic domain peptides were synthesized at the Peptide Synthesis Facility of the Department of Chemistry, University of North Carolina, Chapel Hill. A 48-mer CKLLMIIHDRREFAKFEKEK-MNAKWDTGENPIYKSAVTTVVNPKYEGK corresponded to the amino acid sequence of integrin β1A whole cytoplasmic domain. A second peptide was a 51-mer CKLLMIIHDRREFAKFEKEKMNAKWDTQENPI-YKSPINNFKNPNYGRKAGL, which represented the entire cytoplasmic domain sequence of the novel β1D integrin subunit. The third peptide was a 17-mer CPINNFKNPNYGRKAGL, which contained an amino acid sequence present only in the β1D isoform, and not in β1A isoform. An NH_2-terminal cysteine, was added to all peptides to orient peptide coupling to either carrier protein keyhole limpet hemocyanin or to the activated resin thiopropyl Sepharose 6B (Pharmacia-Biotech., Uppsala, Sweden). The 51-mer corresponding to the whole cytoplasmic sequence of β1D was conjugated to Imject™ maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL) at a ratio of ~50–100 mol of peptide to 1 mol of the protein. Rabbits were immunized subcutaneously with 1 mg of this conjugate in complete Freund's adjuvant (GIBCO BRL) and boosted 5 wk later with 0.5 mg of this conjugate in incomplete Freund's adjuvant (GIBCO BRL). Boosts were repeated every 4 wk with 0.5 mg of the conjugate. Serum was collected which gave some positive reaction in immunoblotting of extracts of muscle tissues. Both a 48-mer representing the whole cytoplasmic domain of β1A subunit and a 17-mer, containing the new amino acid sequence specific for β1D subunit, were coupled to thiopropyl Sepharose 6B according to the manufacturer's instructions. Pooled antiserum was preadsorbed first with immobilized β1A integrin cytoplasmic domain peptide and the unbound material was loaded onto a column containing immobilized 17-mer, representing the new sequence of β1D integrin isoform. The column was extensively washed with 50 mM Tris-HCl, 1.5 M NaCl, pH 7.5, and bound material was eluted with 200 mM Glycine adjusted to pH 2.2 with HCl. The pH of the column eluate was immediately readjusted to pH 8.0 with 1 M Tris, and the affinity-purified IgG fraction was extensively dialyzed against 0.1 M Na_3BO_4, 150 mM NaCl, pH 8.1, and stored at 4°C in the presence of 2% BSA.

Immunoprecipitation, Electrophoresis, and Immunoblotting of β1A and β1D Integrins

For immunoprecipitation, various adult mouse tissues were homogenized and boiled in 1% SDS, tissue extracts were spun down and supernatants were diluted with 1% Triton X-100 in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, to give a final SDS concentration of 0.1%. Protein concentration was determined in tissue extracts using Micro BCA Protein Assay Reagent Kit (Pierce) and samples containing 1 mg of total protein were taken for subsequent immunoprecipitation with antibodies. Cell and tissue lysates were subjected to immunoprecipitation with either anti-β1A or anti-β1D integrin antibodies (~5–10 μg of affinity-purified anti-β1A or anti-β1D IgG were used per one sample), followed by protein A–Sepharose beads. Both incubations were done at 4°C on the rotator. Beads were extensively washed with 1% Triton X-100 in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, and immunoprecipitates were boiled in electrophoresis SDS sample buffer. For electrophoresis, 10% polyacrylamide gels, containing 0.13% bisacrylamide, were used (Laemmli, 1970). Proteins were transferred onto Immobilon Membranes (Millipore, Bedford, MA) as described by Towbin et al. (1979). Blots were blocked with 2% BSA, 2% cold water fish gelatin, 0.1% Tween 20 in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h. Then blots were incubated with 0.5 μg/ml of anti-β1A or anti-β1D IgG. Goat anti–rabbit affinity-purified IgG, conjugated with peroxidase (Jackson Immunoresearch, West Grove, PA), diluted 1:10,000, was used as the secondary antibody. Blots were extensively washed with TBS plus 0.1% Tween 20, then with TBS and finally developed using ECL reagents (Amersham, Arlington Heights, IL).

To study the expression patterns of β1D and β1A during myodifferentiation in culture, C2C12 cells, taken on day 1 to day 9 after plating, were lysed in 1% SDS. 0.5 mg of total cellular protein was taken for immunoprecipitation using the method described above. To separate myotubes from the remaining myoblasts in culture for the analysis of β1D and β1A content in both cell populations, C2C12 mixed myoblast/myotube cultures were treated first with 0.01% trypsin, 0.004% EDTA for 15 min. Under these conditions myotubes slowly detached from the substrate, whereas 0.05% trypsin and 0.02% EDTA was needed to deadhere remaining myoblasts.

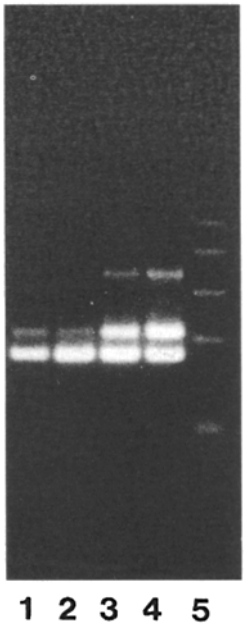
Constructs and Transfections

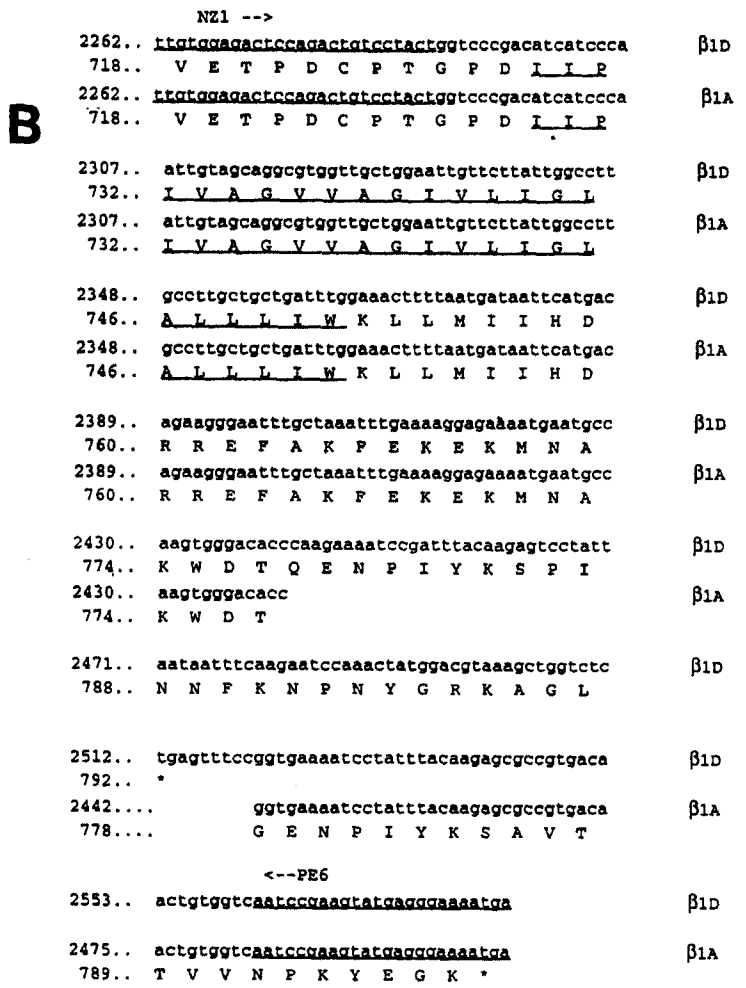
The cDNA for the β1D isoform was prepared from the human β1B cDNA in the pECE vector (Balzac et al., 1994). The Hind III site in the polylinker of the vector was removed and the sequence encoding the cytoplasmic region of β1B between the two Hind III sites (positions 2250 and 2742, respectively, starting from the ATG) was then replaced with the new sequence specific for the β1D variant. The DNA fragment corresponding to the β1D specific sequence was prepared by PCR amplification using mouse cDNA as a template. The PCR primers included Hind III sites for cloning into the vector.

Stable transfectants of CHO cells expressing the human integrin β1A or β1D were obtained as described previously (Balzac et al., 1993, 1994). Briefly, a 3.5-kb EcoRI fragment of the β1D integrin containing the entire coding sequence was inserted into the EcoRI-cloning site of the SV40-based expression vector pECE (Ellis et al., 1986). The full-length cDNA for the human β1A integrin cloned in the pECE vector was a kind gift of Dr. Filippo Giancotti (Giancotti and Ruoslahti, 1990). CHO cells were cotransfected with 20 μg of the plasmid containing either β1A or β1D cDNA and 2 mg of pSV2-neo (Southern and Berg, 1982), and neomycin-resistant clones were selected in Ham's F12 medium with 10% FCS and 800 μg/ml of G418 (GIBCO BRL). Cells expressing the transfected protein were selected by plating on a dish coated with 50 μg/ml of mAb BV7 reacting with an extracellular epitope of the human β1 integrin (Martin-Padura et al., 1994). Adherent cells were grown and subjected to repeated cycles of selection on the antibody until the appropriate expression level was achieved. Flow cytometry analysis of the transfectants was performed as described earlier (Balzac et al., 1994).

Adhesion Experiments with β1A- and β1D-Transfectants

CHO transfectants were kept in serum-free Ham's F12 medium for ~3 h before their detachment. Confluent cells were de-adhered from culture flasks with 0.05% trypsin, 0.53 mM EDTA and turkey egg trypsin inhibitor (Sigma) was immediately added to the cell suspension up to a final concentration of 0.5 mg/ml. The suspended cells were washed twice with Ham's F12 medium plus 2% BSA and cell suspensions were incubated for 1 h at 37°C on the rotator. For plating experiments, T25 tissue culture flasks were precoated with mAb TS2/16 against human β1 integrin, at a concentration of 10 μg/ml, as described earlier (Balzac et al., 1994).





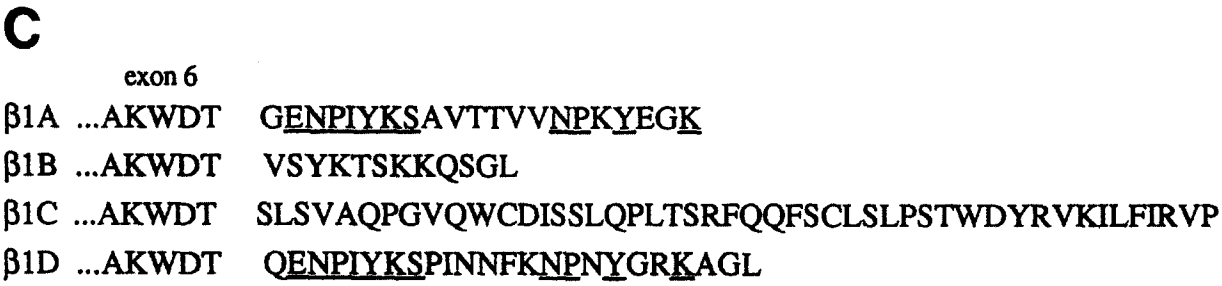


Figure 1. Identification of the β1D integrin isoform by RT-PCR, sequence of β1D integrin and its comparison to other β1 integrin cytoplasmic domain variants. (*A*) Agarose gel electrophoresis of the products of an RT-PCR reaction performed with RNA from cultured C2C12 cells taken at different stages of myodifferentiation using primers NZ1 and PE6. Lane *1*, 1 day; lane *2*, 3 days; lane *3*, 9 days; lane *4*, 12 days; lane *5*, molecular markers, from top to bottom are: 1 kb; 0.7 kb; 0.5 kb; 0.3 kb; 150 bp; 50 bp. (*B*) Sequence of mouse β1D isoform and its comparison to β1A. RNA was isolated from differentiated C2C12 cells (day 9, lane *3*) and both PCR bands were cloned before DNA sequencing. Note that the β1D isoform contains an insert of 81 nucleotides of which 72 nucleotides are in an open reading frame. The amino acid sequence of the transmembrane domain of β1D and β1A is underlined. NZ1 and PE6 are antisense oligonucleotides used for PCR amplification. These sequence data are available from GenBank/EMBL/DDBJ under accession number U37029. (*C*) Comparison of the amino acid sequences of the β1 integrin cytoplasmic domain isoforms. Shown are the predicted amino acid sequences of the COOH-terminal part of the cytoplasmic tails of β1A, β1B, β1C and β1D isoforms as the result of differential splicing events. Note that β1A and β1D, but not β1B and β1C isoforms, share a significant homology including two conserved NPXY motifs (identical amino acids in β1A and β1D are underlined).

To examine tyrosine phosphorylation of pp125^{FAK} upon adhesion of CHO transfectants on TS2/16 mAb, plated cells, taken at various timepoints (15, 30, 60, and 150 min) and cells kept in suspension, were rinsed twice with PBS and lysed with buffer, containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 1 mM Na orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. 1 ml of lysis buffer was used for each T25 tissue culture flasks. Cell lysates were precleared by centrifugation (10,000 rpm, 15 min, 4°C). Protein content in cell lysates was determined using Coomassie Protein Assay Reagent (Pierce) and 1 mg of total cellular protein was taken for immunoprecipitation. Rabbit antiserum 5158 against pp125^{FAK} (5 μl per sample) and protein A–Sepharose were used to precipitate pp125^{FAK} from cell lysates. The resulting immunoprecipitates were washed five times with lysis buffer, once with PBS and finally boiled in SDS sample buffer for electrophoresis. Samples were loaded on 10% gels and blots were probed with PY20 mAb (1 μg/ml) followed by goat anti–mouse affinity-purified IgG, conjugated to peroxidase (Jackson) taken at 1:10,000 dilution.

To study activation of MAP kinases by gel mobility shift assay in response to CHO cell attachment and spreading via either β1A or β1D integrins, CHO transfectants were plated on TS2/16 mAb as described above. Cells kept in suspension or plated on the antibody-coated surfaces for 30, 90, or 200 min, were rinsed with PBS and cells lysed with 2% SDS plus 1 mM Na orthovanadate (200 μl of lysis buffer per each T25 flask or pellet of suspended cells) and cell lysates were immediately boiled for 5 min. Protein content in different samples was equalized and 50 μg of total cellular protein was loaded onto each gel lane. Electrophoresis on 15% polyacrylamide gel was used to improve the resolution of MAP kinase bands. Polyclonal antibody *sc-94*, reacting with both MAPK1 and MAPK2 kinases (1:1,000 dilution) was used in combination with secondary goat anti–rabbit affinity-purified IgG (Jackson, diluted 1:10,000) for visualization of MAP kinase bands on the blot.

To analyze activation of MAP kinases by immune complex kinase activity assay in CHO cells transfected with β1A and β1D integrins, cells were kept in suspension or plated on TS2/16 mAb-coated dishes for 15, 30, 45, or 150 min. Cells were lysed with 1 ml of buffer, containing 150 mM NaCl, 1% Nonidet P-40, 0.5% Na-deoxycholate, 50 mM Hepes, pH 7.5, 1 mM Na orthovanadate, 50 mM NaF, 1 mM *p*-nitrophenyl phosphate, 20 nM calyculin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein content in various samples was equalized using Coomassie Protein Assay Reagent (Pierce) and 0.5 mg of total cellular protein was taken for immunoprecipitation. MAP kinases were immunoprecipitated using *sc-93* rabbit antibody (1 μg per sample) followed by protein A–Sepharose. Immune complexes were washed four times with the lysis buffer, twice with 0.25 M Tris-HCl, pH 7.6, and once with 0.1 M NaCl, 50 mM Hepes, pH 8.0. The immunoprecipitated MAP kinases were incubated with 100 ml of a mixture, containing 1 μCi of [γ-^{32}P]ATP, 50 μM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM benzamidine, 0.3 mg/ml myelin basic protein (MBP), and 25 mM Hepes, pH 8.0, at 30°C for 20 min. The reaction was stopped by boiling supernatants in SDS sample buffer. 10 μg of MBP was loaded on a 15% polyacrylamide gel. Proteins were fixed, stained with Coomassie Brilliant Blue and the gel was dried and exposed to Kodak x-ray Film for visualization of phosphorylated MBP bands.

Immunofluorescence

For immunofluorescent staining of skeletal and cardiac muscle sections, 5–7 μm cryosections of mouse or chicken adult muscle tissues were fixed for 10 min with ice-cold acetone. Cryosections were blocked with 2% BSA in PBS and then incubated with 10 μg/ml of either anti-β1A or anti-β1D IgG. Affinity-purified goat anti–rabbit IgG, coupled with Texas Red (Chemicon, Temecula, CA), taken at 1:40 dilution, was used as secondary antibody. Localization of β1D and β1A in mouse adult skeletal and cardiac muscle tissues was also compared with the distribution of vinculin, visualized with 7F9 mAb. In this case, a mixture of donkey anti–rabbit IgG, conjugated with Texas Red (Chemicon, 1:40 dilution), and donkey anti–mouse IgG, conjugated with fluorescein (Chemicon, 1:40 dilution), was used as both secondary antibodies for simultaneous visualization of antigens. To visualize β1D or β1A integrins at neuromuscular junctions, chicken skeletal muscle tissue sections were double stained using fluorescein-conjugated goat anti–rabbit IgG in combination with rhodamine-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR). Adult chicken skeletal muscle tissue sections, containing myotendinous junctions were double-stained with anti-β1D or anti-β1A variant-specific antibodies followed by fluorescein-conjugated goat anti–rabbit IgG (1:40 dilution) and 7F9 mAb against vinculin followed by rhodamine-conjugated donkey anti–mouse IgG (1:40 dilution).

To study localization of β1D in differentiating C2C12 myocytes by immunofluorescence, C2C12 cells, growing on laminin-coated coverslips, were taken at various timepoints of myodifferentiation, fixed with ice-cold methanol and stained with 10 μg/ml of anti-β1D antibody, followed by goat anti-rabbit IgG, conjugated with Texas Red (1:40 dilution).

For immunofluorescent staining of β1A- and β1D-expressing CHO transfectants, cells cultured in Ham's F12 medium with 10% FCS, were detached with trypsin and then replated on fibronectin-precoated glass coverslips for 3 h. Cells were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 2 min. For double-staining of β1A- and β1D-CHO transfectants, TS2/16 mAb against human β1 integrin was used in combination with rabbit polyclonal anti-talin antibody. A mixture of donkey anti–mouse IgG conjugated with rhodamine (1:40 dilution) and donkey anti–rabbit IgG coupled with fluorescein (1:40 dilution), was used for both secondary antibodies.

Analysis of β1D Association with α Subunits

Frozen mouse skeletal and cardiac muscle tissues were triturated in liquid nitrogen, extracted and sonicated at 4°C in 0.5% Triton X-100 in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml aprotinin. After centrifugation at 10,000 *g* for 10 min, extracts were preincubated with nonimmune rabbit IgG, coupled to Sepharose for removing the material that nonspecifically binds Sepharose. Integrin complexes were immunoprecipitated by incubating with the specific antibodies to alpha subunits for 15 h at 4°C with gentle agitation, followed by 1 h incubation with protein A–Sepharose

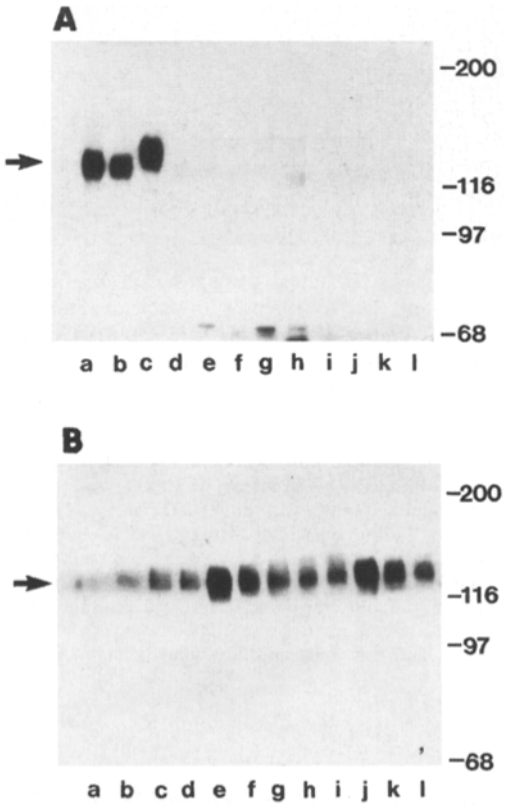


Figure 2. β1D integrin subunit is expressed only in skeletal and cardiac muscles of adult mouse. Immunoblotting of corresponding immunoprecipitates (see Materials and Methods for details) from thigh skeletal muscle (*a*), diaphragm skeletal muscle (*b*), heart (*c*), aorta (*d*), placenta (*e*), lung (*f*), spleen (*g*), skin (*h*), liver (*i*), gut (*j*), kidney (*k*), or brain (*l*) of adult mouse was performed with anti-peptide isoform-specific antibodies against β1D (*A*) and β1A (*B*). Positions of molecular weight markers in kilodaltons are indicated to the right of each blot. Arrows show β1D bands in *A* and β1A bands in *B*.

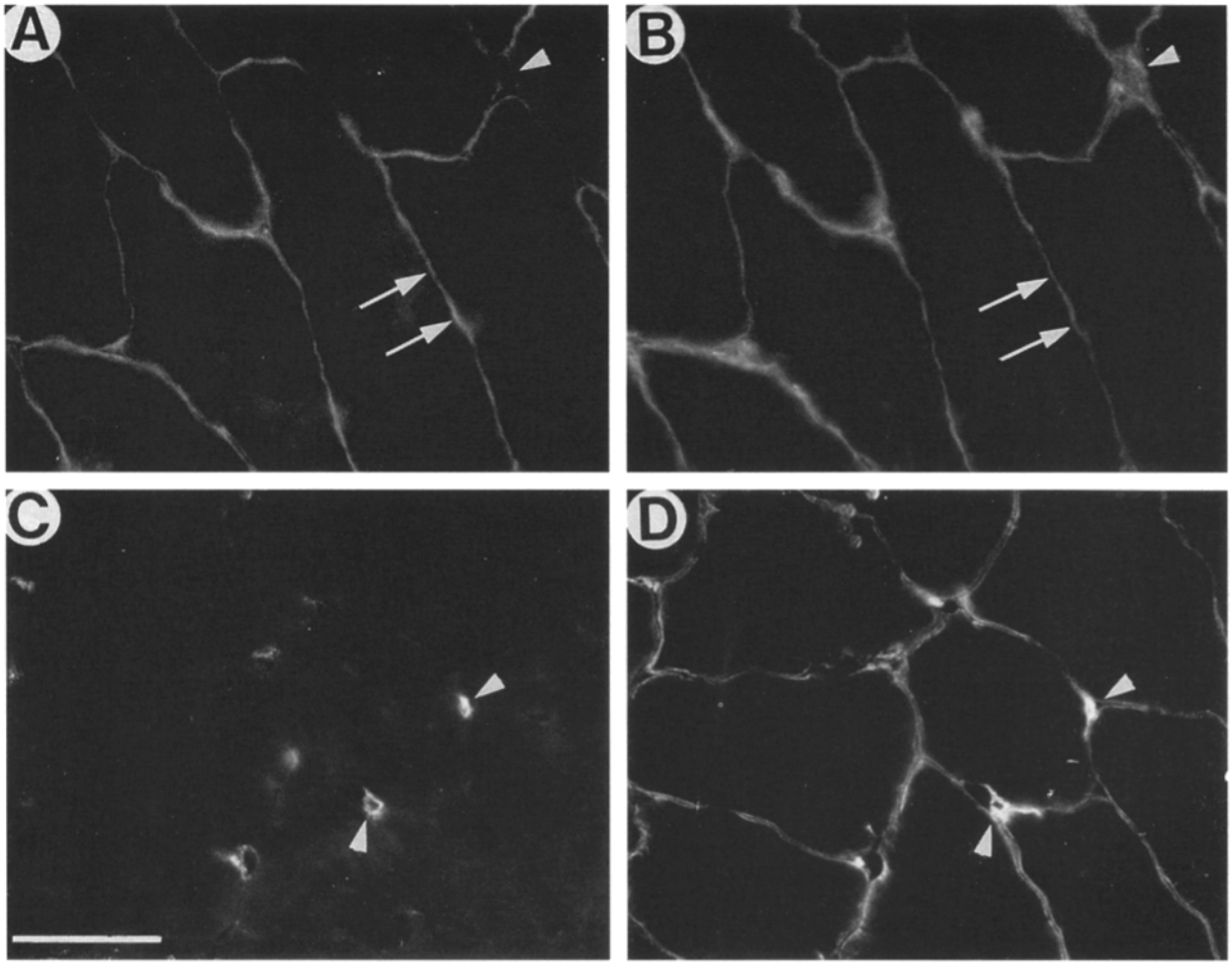


Figure 3. Localization of β1D and β1A integrin isoforms in adult skeletal muscle. Transverse cryostat sections of skeletal muscle from adult mouse (*5-μm*) were double-stained with polyclonal antibody against β1D (*A*) and anti-vinculin mAb 7F9 (*B*) or polyclonal antibody against β1A (*C*) and anti-vinculin mAb 7F9 (*D*). Arrows point to the sarcolemma of skeletal muscle fibers, arrowheads indicate nonmuscle β1A integrin-positive cells, not expressing β1D integrin isoform. Bar, 50 μm.

beads (Pharmacia). After extensive washing, bound material was eluted by boiling the beads in SDS sample buffer with or without β-mercaptoethanol respectively for immunoblotting with either β1D antibody or rat M1.2 mAb against mouse β1 integrin subunit. Proteins were run on a 6% SDS–polyacrylamide gel, transferred to nitrocellulose and probed with either anti-β1D antibody (0.5 μg/ml) or with 0.2 μg/ml of the rat mAb M1.2 to the mouse β1 integrin subunit. Peroxidase-conjugated anti-rabbit IgG (Sigma, Italy) or peroxidase-conjugated anti-rat IgG (Boehringer Mannheim Corp., Mannheim, Germany) were used as secondary antibodies.

Results

Identification and Amino Acid Sequence of Mouse β1D Integrin Cytoplasmic Domain Isoform

In preliminary experiments, it was observed that a polyclonal anti-peptide antibody to the cytoplasmic domain of β1 integrin (Marcantonio and Hynes, 1988) failed to give immunofluorescent staining of the sarcolemma of adult skeletal and cardiac muscles (A. M. Belkin, N. I. Zhidkova and V. E. Koteliansky, unpublished observations). However, polyclonal or monoclonal antibodies to the extracellular domain of β1 integrin always gave strong immunofluorescent staining of the surface of adult striated muscle cells (Bozyczko et al., 1989; Swasdison and Mayne, 1989). These observations first suggested the possibility that an isoform of the cytoplasmic domain of β1 integrin might displace the ubiquitously expressed β1A isoform in muscle cells. To examine this possibility, reverse transcription polymerase chain reaction (RT-PCR) reactions were performed using RNA preparations obtained from C2C12 cells taken at various timepoints of myodifferentiation and 24-mer antisense oligonucleotides NZ1 and PE6 that span the portion of β1 integrin subunit mRNA encoding transmembrane and cytoplasmic domains. At early timepoints preceeding cell fusion in myogenic culture, only one band most likely corresponding to the β1A isoform was detected (Fig. 1 *A*, lanes *1* and *2*). However, after cell fusion occurs in C2C12 culture and myotubes grow and differentiate, a prominent upper band was detected together with the original lower band (Fig. 1 *A*, lanes *3* and *4*). Each band was subcloned into the PCR™II vector and subjected to nucleotide sequencing (Fig. 1 *B*). The results showed that the lower band had an identical nucleotide sequence to the published sequence for the mouse β1A iso-

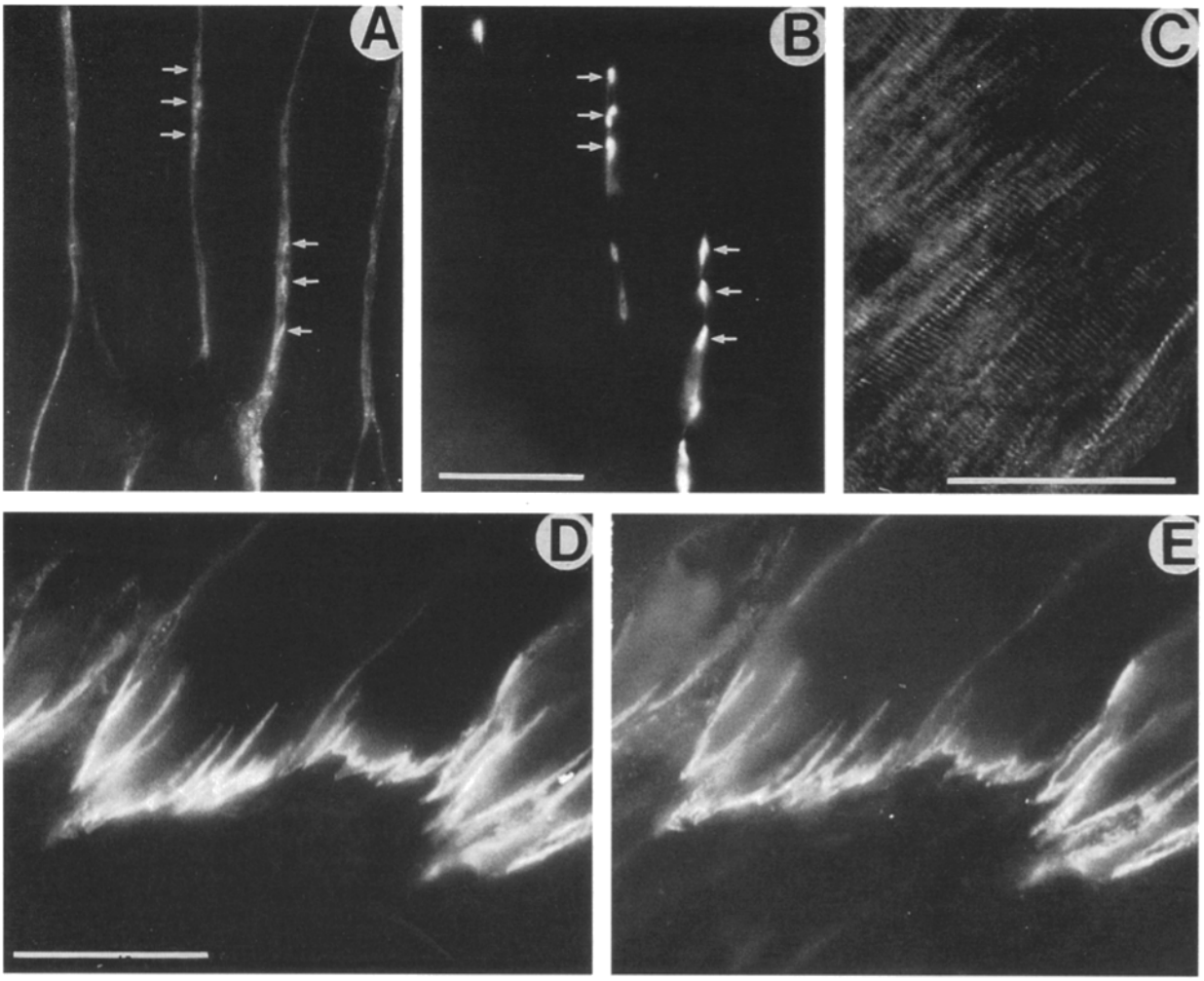


Figure 4. Localization of β1D integrin at various adherens-type junctions of adult skeletal muscle. 5-μm longitudinal cryostat sections of *tibialis anterior* skeletal muscle from 8-wk-old chicken were prepared. Sections were double stained with anti-b1D antibody (*A*) and α-bungarotoxin (*B*). Small arrows in *A* and *B* depict acetylcholine receptor clusters, colocalized with β1D integrin. (*C*) β1D integrin is localized at skeletal muscle costameres. Micrograph represents a field where the section plane is close to the surface of myofibers. Muscle-tendon interface was costained with antibodies against β1D integrin (*D*) and vinculin (*E*) to show accumulation of β1D integrin at myotendinous junctions. Bar, 50 μm.

form (Holers et al., 1989). However, the upper PCR-generated band contained an additional 81-bp insert with an open reading frame for 72 bp after the stop codon. The position of the 81-bp insert, encoded by a novel exon of the β1 integrin gene coincided with the boundary between exons 6 and 7 of the gene (Lanza et al., 1990) where all the diversity of previous β1 integrin cytoplasmic domain isoforms was shown to be generated by differential splicing (Altruda et al., 1990; Languino and Ruoslahti, 1992). The deduced amino acid sequence differed markedly from the previously described β1B and β1C isoforms and this newly identified β1 integrin variant was therefore assigned β1D. Fig. 1 *C* shows the comparison of β1D integrin with all other known cytoplasmic domain isoforms. Notably, the COOH-terminal parts of β1A and β1D cytoplasmic domain variants, unlike the alternatively spliced amino acid sequences of β1B and β1C, share 11 amino acids in common, including two conserved NPXY motifs at the same positions (Fig. 1 *C*, *underlined*). The amino acid sequence for mouse β1D was identical to the recently reported chicken and human β1D sequences (Zhidkova et al., 1995).

The β1D Integrin Isoform Is the Predominant β1 Integrin Isoform Expressed in Skeletal and Cardiac Muscles

Using an anti-peptide antibody specific for the β1D sequence, we examined the expression pattern of this integrin in various tissues of adult mouse by immunoprecipitation and subsequent immunoblotting and compared it to that of β1A isoform. Significant amount of β1D integrin, represented by a broad band with a M_r around 140 kD was found in two different types of skeletal muscle (Fig. 2 *A*, *a* and *b*). In heart muscle extracts a slightly slower migrating band of β1D was detected (Fig. 2 *A*, *c*). The β1D isoform was not detected in any other tissues analyzed except for a very weak reaction in skin. This may be explained by a trace contamination of this sample with skeletal muscle cells. In contrast, the β1A isoform was detected in all the

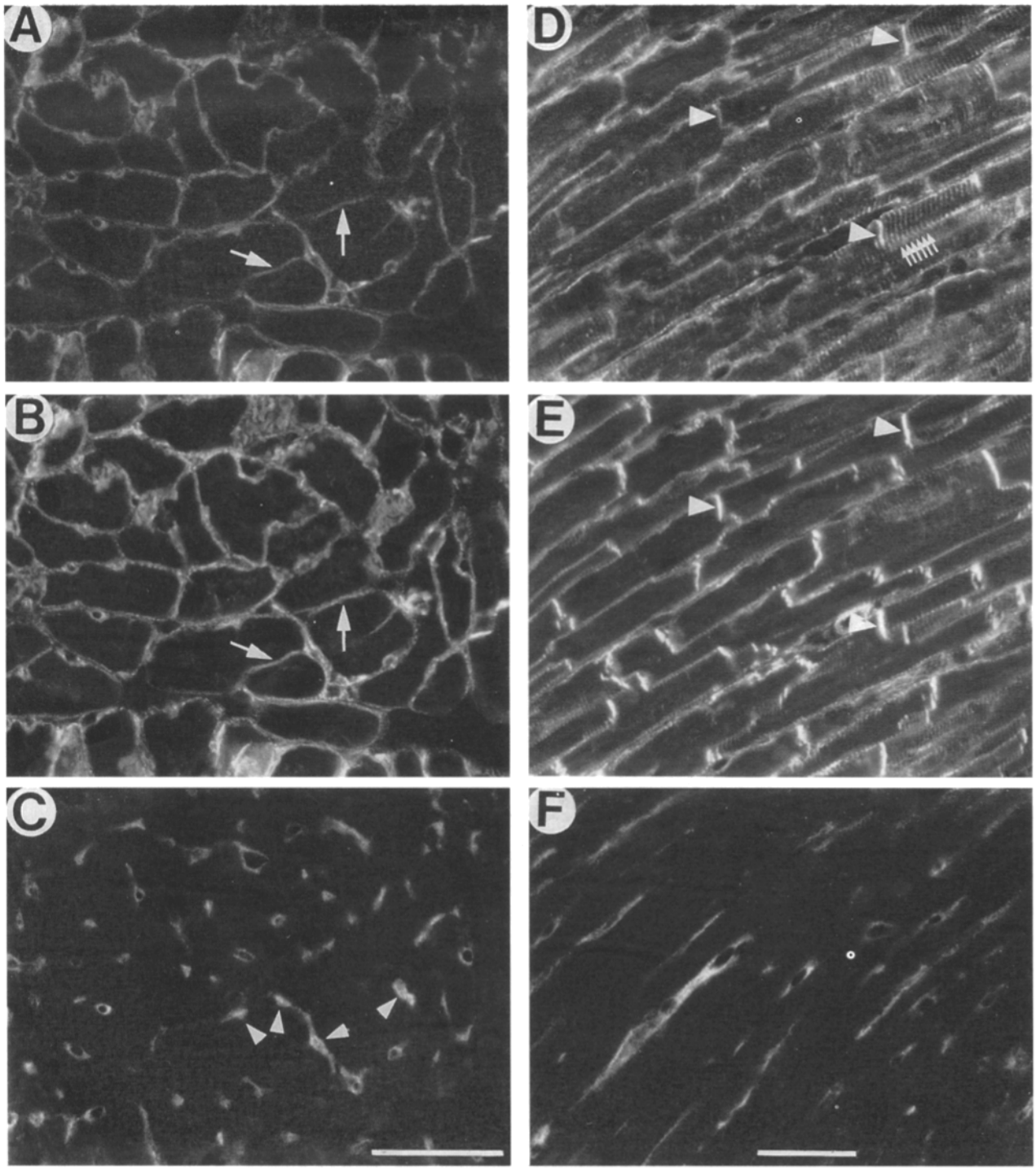


Figure 5. Localization of β1D and β1A integrin isoforms in adult cardiac muscle. (*A–C*) Serial transverse cryostat sections of mouse adult cardiac muscle (*5-μm*) were stained with antibody against β1D (*A*), anti-vinculin mAb 7F9 (*B*), or antibody against β1A (*C*). Arrows mark the cardiomyocyte sarcolemma, arrowheads indicate nonmuscle cells, expressing β1A but lacking β1D integrin isoform. (*D–F*) Longitudinal cryostat sections of mouse adult cardiac muscle (*5-μm*) were either double-stained with antibody against β1D (*D*) and anti-vinculin mAb 7F9 (*E*) or stained with antibody against β1A (*F*). Small arrows mark cardiomyocyte costameres, arrowheads point to intercalated discs. Bar, 20 μm.

tissue samples analyzed, but with slightly weaker reaction in cardiac muscle and notably fainter bands in the two skeletal muscle samples analyzed (Fig. 2 *B*). These data indicated that β1D is the predominant β1 integrin variant in adult striated muscles.

Localization of β1D and β1A Integrin Isoforms in Adult Skeletal and Cardiac Muscles

Double immunostaining of transverse sections of skeletal muscle with antibodies against the β1D integrin variant

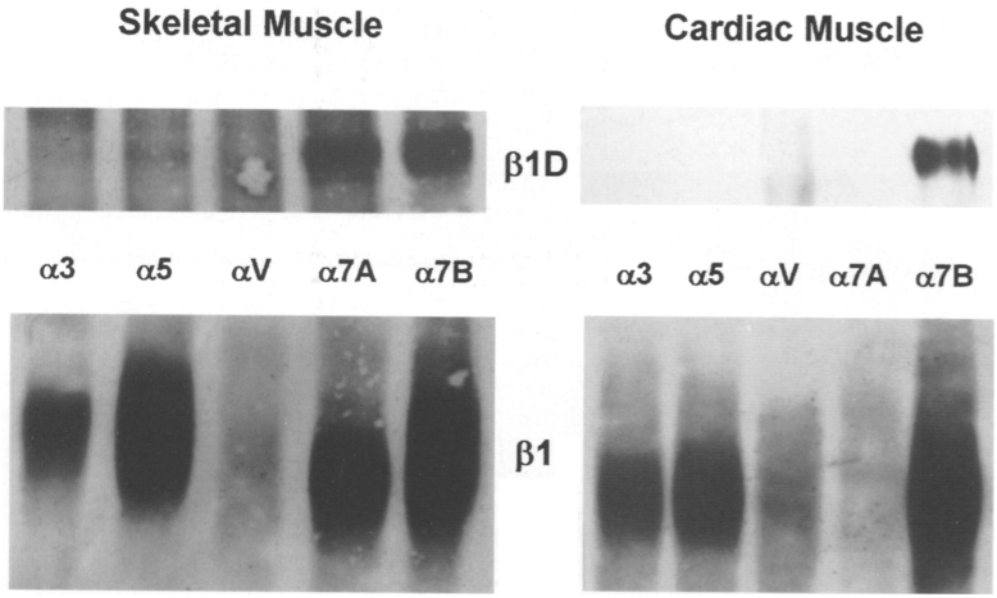


Figure 6. Association of β1D integrin isoform with integrin α subunits in adult mouse skeletal and cardiac muscles. Mouse muscle tissues were extracted under conditions favoring association of α and β subunits and integrin complexes were immunoprecipitated with antibodies specific for α3, α5, αV, α7A, and α7B integrin subunits. After electrophoresis and electrotransfer, co-precipitated β1 integrin isoforms were visualized on immunoblots by reacting with antibodies specific to the β1D isoform (*top*) or antibody to an extracellular epitope, common to all four known β1 integrin isoforms. Note that β1D was detected in association only with α7B subunit in heart muscle and in skeletal muscle β1D is associated with α7A and α7B.

and vinculin showed that β1D was localized at the sarcolemma of skeletal muscle fibers (Fig. 3 *A*, *arrows*). Some nonmuscle cells, present in the skeletal muscle tissue, were positive for vinculin but did not express any β1D (Fig. 3, *A* and *B*, *arrowheads*). In contrast, the β1A integrin isoform was virtually undetectable at the sarcolemma, but was clearly visualized in some connective tissue and capillary cells of the tissue (Fig. 3, *C* and *D*, *arrows*, *arrowheads*).

Detailed analysis of the intracellular localization of the β1D isoform in skeletal muscle showed that this integrin subunit is accumulated at all major adherens-type junctions of skeletal muscle fibers (Fig. 4). Double-labeling with α-bungarotoxin revealed the presence of β1D integrin at neuromuscular junctions (Fig. 4, *A* and *B*, *arrows*). β1D was also localized at skeletal muscle fiber costameres (Fig. 4 *C*). Finally, a very intense staining of the myotendinous junction was obtained with anti-β1D antibody, showing that β1D is particularly enriched at these cell–matrix junctional sites (Fig. 4, *D* and *E*). The β1A integrin isoform was not identified at any of these junctional structures (data not presented).

In cardiac muscle, the β1D integrin isoform was prominent at the sarcolemma of cardiomyocytes and codistributed with vinculin (Fig. 5, *A* and *B*, *arrows*). Although abundantly expressed in nonmuscle cells present in this tissue, the β1A integrin subunit was not detected at the sarcolemma (Fig. 5 *C*, *arrowheads*). With longitudinal sections of cardiac muscle, we could detect β1D integrin staining at both costameres and intercalated discs, two major adherens-type junctions of cardiomyocytes (Fig. 5 *D*) which also contain vinculin (Fig. 5 *E*). Again, the β1A integrin variant was not localized at these two types of junctional structures in cardiac muscle (Fig. 5 *F*).

Association of β1D with α7 Subunit Isoforms In Vivo

Western blot analysis with a panel of antibodies specific for α subunits indicated that α3, α5, αV, α7A, and α7B were the major integrin α subunits expressed in adult skeletal muscle (data not shown). In adult heart muscle a similar pattern of α subunits was detected except that α7A was absent (Fig. 6, *bottom*), confirming previous report (Collo et al., 1993). To assess which α subunit was associated with β1D integrin, tissue extracts were immunoprecipitated with various antibodies against α subunits and immunoprecipitates were probed by immunoblotting with anti-β1D antibody or an anti-β1 mAb reacting with all known β1 isoforms. As shown in Fig. 6, β1D integrin appeared to be associated exclusively with α7A and α7B subunit variants in skeletal muscle, whereas in cardiac muscle α7B was the major pairing partner for the β1D. α3 and α5 subunits which are known to be expressed in the connective tissue and blood vessels present in muscle tissues, are associated with a different β1 isoform, probably β1A, since there was a prominent reaction in immunoblot of anti-α3 and anti-α5 immunoprecipitates with anti-β1 antibody (Fig. 6). Only a very minor portion of β1 was found associated with αV in adult muscle tissues.

Expression and Localization of β1D Integrin during Myodifferentiation

During myodifferentiation of mouse C2C12 cells in culture, β1D could not be detected in replicating myoblasts (Fig. 7 *A*, *a* and *b*). Immediately after cell fusion occurred, a β1D band was detectable (Fig. 7 *A*, *c*). Thereafter, the amount of β1D continued to increase reaching its highest level at day 9 through day 10, when mature differentiated myotubes appear in the cell culture (Fig. 7 *A*, *d* and *e*). In contrast, large amounts of β1A isoform were found in prefusion myoblasts (Fig. 7 *B*, *a* and *b*). A significant decrease in the content of β1A acccompanied subsequent stages of myodifferentiation after cell fusion, and only a weak β1A band was seen at late timepoints in C2C12 culture (Fig. 7

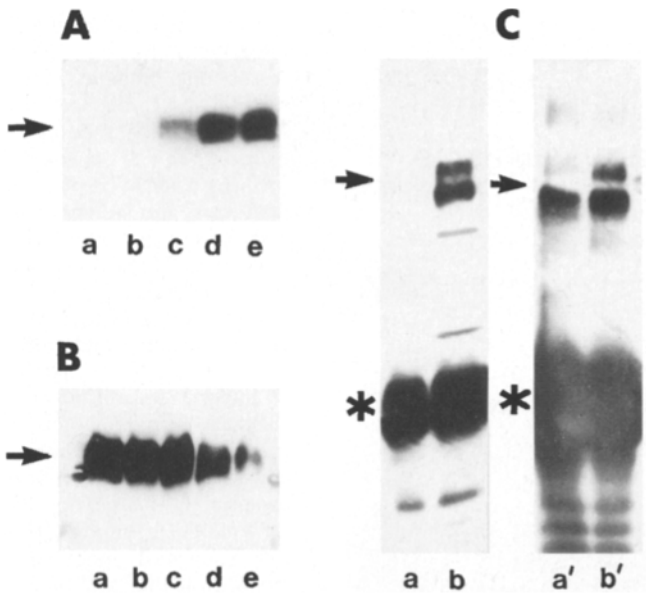


Figure 7. Expression of β1D and β1A integrin isoforms during myodifferentiation of C2C12 cells. (*A* and *B*) Cells, taken on day 1 (*a*), day 3 (*b*), day 5 (*c*), day 7 (*d*), and day 9 (*e*) after plating, were lysed and cell lysates were subjected to immunoprecipitation and subsequent immunoblotting with anti-β1D (*A*) or anti-β1A (*B*) antibody. (*C*) Myotubes (*b* and *b'*) were separated from the remaining nonfused myoblasts (*a* and *a'*) on day 6 of culture, both cell populations were lysed and the corresponding cell lysates were immunoprecipitated and immunoblotted with antibodies against β1D (*a* and *b*) or β1A (*a'* and *b'*). Arrows point to β1D and β1A bands, asterisks in *C* indicate immunoglobulin heavy chains.

B, *c–e*). Upon the separation of myotubes and myoblasts in culture on day 6 after plating, we found that β1D was abundantly expressed in growing myotubes but was absent from postmitotic myoblasts (Fig. 7 *C*, *a* and *b*). In contrast, comparable levels of β1A expression in myoblasts and differentiating myotubes were found at this timepoint of myodifferentiation (Fig. 7 *C*, *a'b'*).

By immunofluorescence, we also did not see any β1D in C2C12 myoblasts except for some distinct perinuclear staining. This most likely corresponded to β1D accumulation in the Golgi apparatus (Fig. 8 *A*, *arrows*). As cell fusion occurred in culture, early myotubes having 2–4 nuclei displayed bright regions of β1D immunostaining, with clear accumulation of the protein at focal adhesions in myotube tips (Fig. 8 *B*, *long arrow*, *arrowheads*). In larger, more mature myotubes, β1D appeared to accumulate at focal adhesions located throughout the entire ventral surface of the myotubes (Fig. 8 *C*, *arrowheads*). A fibrillar pattern of β1D on the dorsal surface of branching myotubes suggested codistribution with ECM fibrils (Fig. 8 *D*). Finally, β1D integrin was found redistributed to costameres of terminally differentiated contractile myotubes (Fig. 8 *E*, *arrows*).

β1D is Localized to Focal Adhesions in Transfected CHO Cells

CHO cells were transfected with plasmids encoding full-length human β1A and β1D isoforms of the β1 integrin subunit. As determined by FACS analysis of β1A integrin- and β1D integrin-transfected CHO cells, these cell lines expressed comparable amounts of transfected integrin subunits on their surface (Table I). To analyze the intracellular localization of β1D in transfected CHO cells, we performed double immunofluorescent staining of these cells with TS2/16 mAb, which is specific for the extracellular domain of human integrin β1 subunits, and polyclonal anti-talin antibodies. Immunofluorescent staining showed that β1D integrin is specifically accumulated at focal adhesions of CHO cells plated on fibronectin and is codistributed with talin at these sites (Fig. 9, *C* and *D*). The pattern for transfected β1D integrin subunit in these cells was largely indistinguishable from the distribution of transfected β1A (Fig. 9, *A* and *B*). Both β1A- and β1D-CHO transfectants adhered and spread similarly on surfaces coated with anti-human integrin TS2/16 mAb. However, in this case both types of CHO transfectants remained less spread than on a fibronectin substrate, and a somewhat less distinct pattern of β1D and β1A localization in focal adhesions was observed (data not shown).

Adhesion via β1D Integrin Causes Tyrosine Phosphorylation of pp125^{FAK}

We also used β1D-transfected CHO cells in adhesion experiments with TS2/16 mAb-coated culture flasks to determine whether clustering β1D integrin leads to increased tyrosine phosphorylation of pp125^{FAK}. After plating of the β1D-transfected CHO cells on the antibody substrate, we consistently observed an increase in tyrosine phosphorylation of ~130-kD protein band corresponding to pp125^{FAK} (Fig. 10 *B*, *arrow*). This was detected as early as 15 min after plating of β1D-CHO transfectants on TS2/16 mAb (Fig. 10 *B*, *b*). The level of tyrosine phosphorylation of pp125^{FAK} in β1D-CHO cells significantly increased by 30 min and reached a plateau thereafter (Fig. 10 *B*, *c–e*). A similar time course of pp125^{FAK} tyrosine phosphorylation was observed with the β1A-CHO transfectants adhering to surfaces coated with the same antibody, except a slightly higher level of pp125^{FAK} phosphorylation was detected in β1A-CHO cells (Fig. 10 *A*, *a–d*). It should be noted that lower levels of tyrosine phosphorylation of pp125^{FAK} in β1D-CHO cells at early timepoints of adhesion corresponded to a slower and less efficient spreading of β1D-CHO transfectants compared with the β1A-CHO counterparts (data not shown).

β1D Integrin-Mediated Adhesion Leads to Activation of MAP Kinases

Activation of MAP kinases upon adhesion of β1A- and β1D-CHO transfectants on TS2/16 mAb was examined first by mobility shift assay as described previously (Chen et al., 1994; Zhu and Assoian, 1995). Plating of the β1D-CHO transfectants on anti-integrin antibody caused a distinct electrophoretic mobility shift of both the 42- and 44-kD MAP kinase bands (Fig. 11 *B*, *a* and *b*, *arrows*, *arrowheads*). The observed activation of MAP kinase was transient, peaking at 30 min and had dropped by ~90 min after plating (Fig. 11 *B*, *c* and *d*). As detected by mobility shift kinase assay, the observed pattern of MAP kinase activation in the case of β1D-mediated adhesion looked identi-

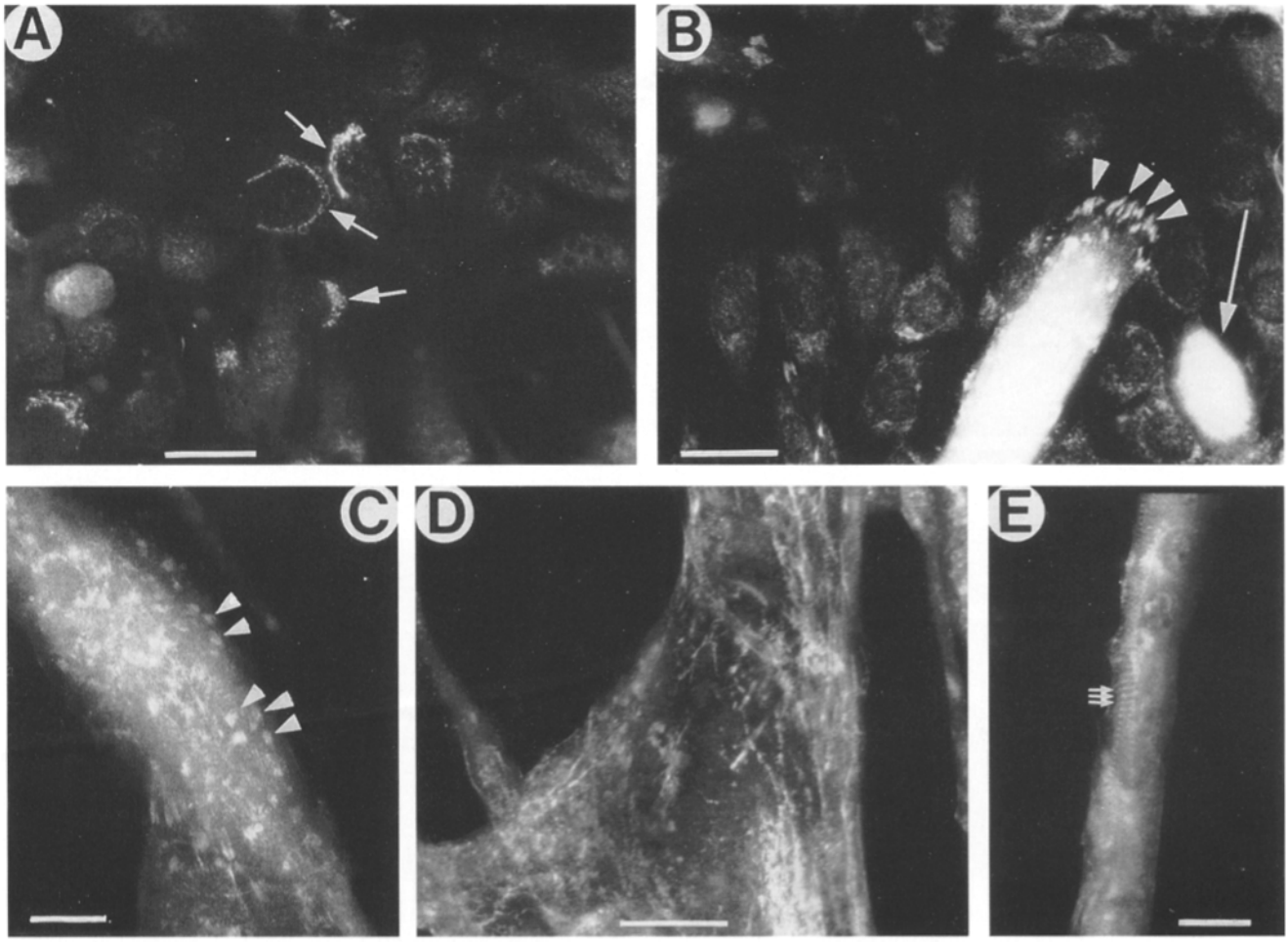


Figure 8. Localization of β1D integrin isoform in differerentiating cultured C2C12 myocytes. C2C12 myocytes were taken on day 3 (*A*), day 5 (*B*), day 7 (*C*), day 9 (*D*) and day 11 (*E*) of culture and stained with antibody specific for β1D integrin isoform. Arrows in *A* mark β1D localization in Golgi apparatus of growth-arrested myoblasts. Long arrow in *B* points to accumulation of β1D isoform in early myotubes. Arrowheads in *B* and *C* indicate localization of β1D at myotube's focal adhesions. Small arrows in *E* mark presence of β1D integrin at costameres of differentiated contractile myotubes. Bar, 10 μm.

cal to that seen for β1A-CHO transfectants (Fig. 11 *A*, *arrows*, *arrowheads*).

To study adhesion-dependent activation of MAP kinase in β1D-CHO and β1A-CHO cells in more detail, we immunoprecipitated MAP kinases and examined their enzymatic activity by immune complex kinase assay using MBP as an exogenous substrate (Fig. 11, *C* and *D*). Again, we demonstrated that MAP kinases underwent transient activation in CHO cells adhered via the β1D integrin isoform. The overall level and time course of adhesion-dependent MAP kinase activation in β1D-CHO transfectants were largely indistinguishable from those observed for β1A-CHO cells.

Discussion

Identification of the β1D Isoform and its Comparison with Other β1 Integrin Cytoplasmic Domain Variants

It is commonly accepted that all adhesion-dependent cell types express one or more β1 integrin heterodimers (Hynes, 1992). Of the three previously identified cytoplasmic domain variants of β1 integrin, two (β1B and β1C) are minor isoforms (Languino and Ruoslahti, 1992; Balzac et al., 1993), leading to the idea that β1A is the predominant variant in most cells. It was surprising, therefore, that in our preliminary experiments an anti-peptide antibody against the β1A cytoplasmic domain failed to recognize the antigen in either adult skeletal or cardiac muscles, even though antibodies to the extracellular domain of β1

Table I. Expression Level of the Human β1A and β1D Integrin Subunits in CHO Cells

Cell type	% positive cells	Mean fluorescence intensity
CHO-β1A	98.6	330
CHO-β1D	95.4	250
CHO	7.0	81

CHO cells transfected with full-length human β1D or β1A cDNA and control untransfected CHO cells were reacted with saturating amounts of mAb BV7 specific for human β1 integrin subunit, followed by fluorescein-labeled anti-mouse IgG and cell populations were analyzed by FACS.

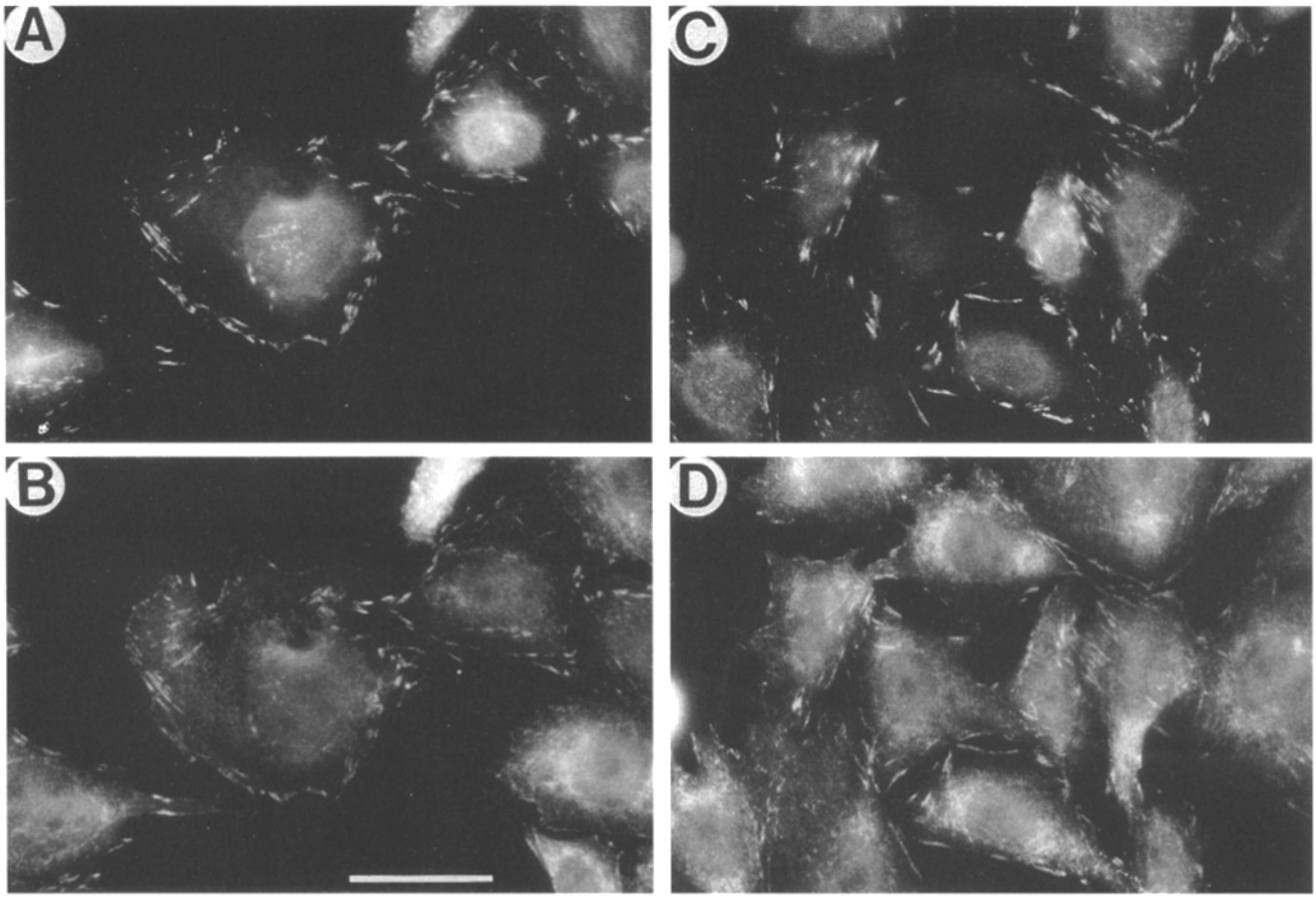


Figure 9. Localization of β1D integrin isoform at focal adhesions of transfected CHO cells. β1A- (*A* and *B*) and β1D- (*C* and *D*) transfected CHO cells were plated on fibronectin substrate for 3 h and then double-stained with mAb TS2/16 specific for human β1 integrin subunit (*A* and *C*) and polyclonal antibody against talin (*B* and *D*). Bar, 50 μm.

integrin had been reported to stain the sarcolemma brightly (Bozyczko et al., 1989; Swasdison and Mayne, 1989). This observation has led us to this identification of a fourth cytoplasmic domain variant, β1D, which is specifically expressed in muscle cells. This variant arises through differential splicing of β1 integrin pre-mRNA. Analysis of the exon-intron organization of the human β1 integrin gene shows that the novel amino acid sequence of β1D is encoded by a single additional exon D, located between exons 6 and 7 and upstream from exon C which encodes part of the β1C cytoplasmic domain variant (N. I. Zhidkova et al., 1995). Recently, similar data were obtained for mouse β1 integrin gene and β1D isoform (A. van der Flier et al., 1995).

Comparing the four known cytoplasmic domain variants of the β1 integrin subunit (Fig. 1C) indicates that β1D is closest to the widely distributed β1A. In contrast, β1B and β1C are distinct and in the alternatively spliced regions of the cytoplasmic domain they show no homology with β1A (Tamkun et al., 1986; Argraves et al., 1987; Altruda et al., 1990; Languino and Ruoslahti, 1992). Of the 24 amino acids encoded by the newly identified exon D, 13 are unique, whereas 11 are conserved between β1D and β1A. Notably, both NPXY motifs (780-783 and 792-795) are conserved between these two cytoplasmic domain variants.

β1D Integrin Is the Predominant β1 Integrin Isoform Expressed in Striated Muscles and Is Localized at Various Adherens-Type Junctions

Analysis of the tissue distribution of the novel β1D integrin isoform showed that on the protein level its expression is strictly limited to skeletal and cardiac muscle tissues. During myodifferentiation of C2C12 myocytes, β1A becomes progressively replaced by β1D. Moreover, the expression and localization of β1D and β1A in adult skeletal and cardiac muscle tissues demonstrate that β1D displaces the common β1A isoform in terminally differentiated muscle cells. Although we cannot say that mature striated muscle cells have no β1A, the level appears to be very low and possibly negligible. Differentiated muscle cells appear, therefore, to be one of the few adherent vertebrate cells that have little or no β1A integrin. β1C is not expressed in adult striated muscles (G. Tarone, personal communication), whereas β1B was only detected in trace amounts in muscle tissues (Balzac et al., 1993). Together these results suggest that β1D is the predominant β1 integrin variant in striated muscles.

Immunolocalization of β1D integrin in muscle cells revealed its presence in all those junctional structures, including myotendinous junctions and neuromuscular junc-

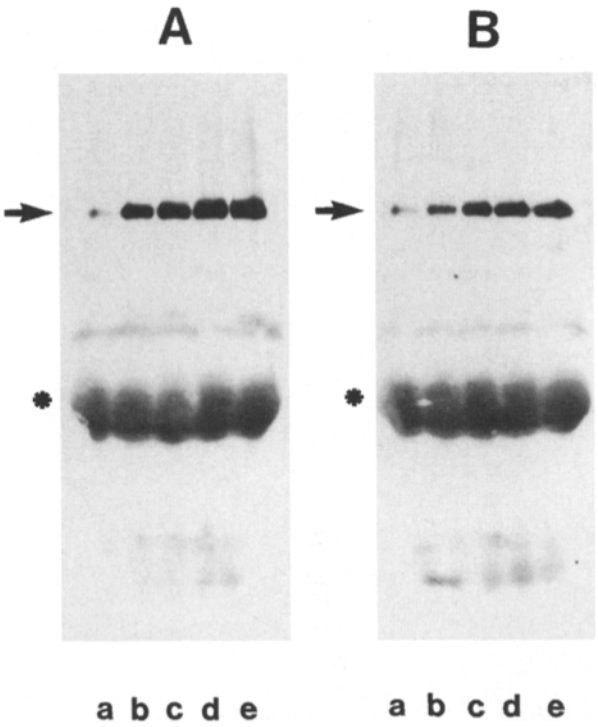


Figure 10. β1D integrin isoform mediates adhesion-induced tyrosine phosphorylation of pp125^{FAK}. β1A- (*A*) and β1D-transfected CHO cells (*B*) were either kept in suspension (*a*) or plated on TS2/16-covered tissue culture flasks for 15 min (*b*), 30 min (*c*), 60 min (*d*), or 150 min (*e*). pp125^{FAK} was immunoprecipitated using rabbit antiserum 5158, immunoprecipitates were run on a 10% gel, proteins were transferred to the membrane and blots were probed using PY20 anti-phosphotyrosine mAb, followed by goat anti–mouse IgG, conjugated with peroxidase. Arrows point to pp125^{FAK} bands. Asterisks indicate immunoglobulin heavy chains.

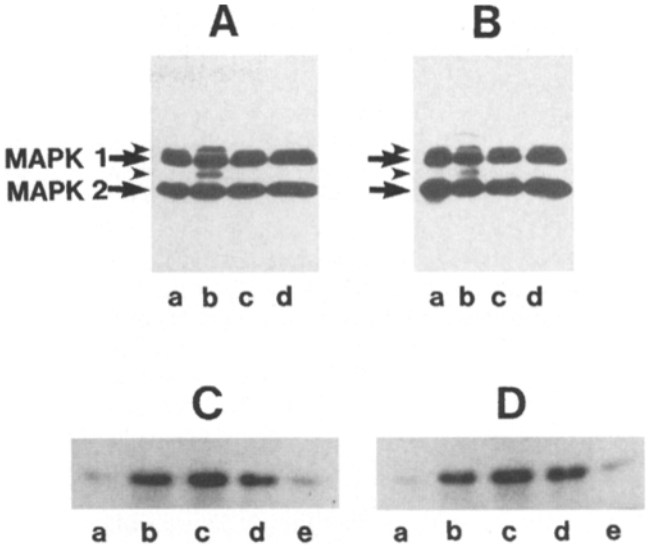


Figure 11. Cell adhesion mediated by β1D integrin causes transient activation of MAP kinases. (*A* and *B*) Mobility shift assay for β1D- and β1A-mediated MAP kinase activation. β1A- (*A*) and β1D-transfected CHO cells (*B*) were either kept in suspension (*a*) or plated on TS2/16-covered tissue culture flasks for 30 min (*b*), 90 min (*c*) or 200 min (*d*). Cell lysates were run on a 15% gel, proteins were transferred to the membrane and blots were probed with polyclonal antibody *sc-94* against MAP kinases. Positions of MAPK1 at 44 kD and MAPK2 at 42 kD are indicated by arrows. Small arrowheads point to the bands of activated MAP kinases. (*C* and *D*) Immune complex kinase assay for β1D- and β1A-mediated MAP kinase activation. β1A- (*C*) and β1D-transfected CHO cells (*D*) were either kept in suspension (*a*) or plated on TS2/16 mAb for 15 min (*b*), 30 min (*c*), 45 min (*d*) or 150 min (*e*). MAP kinases were immunoprecipitated using *sc-93* polyclonal antibody and immune complex kinase reaction was performed with the exogenous substrate MBP, as described in Materials and Methods. Shown are phosphorylated MBP bands.

tions of skeletal muscle and costameres of both skeletal and cardiac muscle, that were previously described to contain the β1 integrin subunit (Bozyczko et al., 1989; Swasdison and Mayne, 1989). Since we were unable to detect any β1A isoform in terminally differentiated muscle cells by immunostaining, we conclude that all these cell–matrix junctions contain only the β1D integrin variant. Here we also report localization of the β1D integrin isoform at intercalated discs, major cell–cell contact structures of cardiomyocytes. To our knowledge, this is the first demonstration of β1 integrins in intercalated discs. It will be interesting to identify the extracellular ligand for β1D-containing integrins at these sites.

Analysis of the association of β1D integrin with α subunits in vivo showed that among the α subunits analyzed in our experiments, α7B is the only α subunit complexed with β1D in cardiac muscle, whereas both α7A and α7B variants interact with β1D in skeletal muscle. Cytoplasmic domain structure does not contribute to the specificity of α/β subunit association (Hemler et al., 1994), but the restricted tissue expression for both β1D and α7 integrin subunits and their colocalization in skeletal muscle fibers are striking (Song et al., 1992; Bao et al., 1993). α7Aβ1D and α7Bβ1D appear to be the major integrin receptors involved in cytoskeletal-matrix interactions of differentiated striated muscles.

Possible Functions of the β1D Integrin Isoform in Muscle Cells

When β1D was expressed in CHO cells, it concentrated in focal adhesions. This distinguishes it from the β1B isoform, which neither targets to focal adhesions nor is detected in adherens-type junctions (Balzac et al., 1993). The recruitment of β1D to focal adhesions is consistent with the similarity of its cytoplasmic domain to that of β1A and suggests that β1D may interact with some of the same cytoskeletal proteins as β1A. Together, these observations reflect constraints for the overall structure of the β1 integrin cytoplasmic domain with regard to its interaction with the actin cytoskeleton (Marcantonio et al., 1990; LaFlamme et al., 1992, 1994; Reszka et al., 1992; Pasqualini and Hemler, 1994; Lewis and Schwartz, 1995). Integrins of the β1A subfamily have been shown not only to have a structural role linking the cytoskeleton to the ECM, but also to be involved in signaling. Transfection of human β1D into CHO cells enabled us to ask whether this integrin is also capable of signaling. Clustering of β1D integrins in these trans-

fected CHO cells stimulated tyrosine phosphorylation of pp125^{FAK}. The response was similar to the tyrosine phosphorylation of pp125^{FAK} generated as a result of clustering transfected β1A, but less effective, presumably due to slower spreading of β1D-CHO transfectants on anti-β1 integrin antibody. pp125^{FAK} has been identified at myotendinous junctions (Baker et al., 1994). Although the function of pp125^{FAK} at these sites has not been determined, it seems likely that it is associated with β1D. The interaction of β1A integrins with ECM also activates the MAP kinase pathway, possibly as a downstream consequence of pp125^{FAK} activation (Chen et al., 1994; Schlaepfer et al., 1994; Zhu and Assoian, 1995). Again, using the transfected CHO cells, we have shown that β1D is capable of activating MAP kinases and the overall level and time course of this activation were similar for β1D and β1A isoforms. The relevance of this pathway, however, to mature skeletal muscle cells is uncertain, since these cells are withdrawn irreversibly from the cell cycle after myoblast fusion. Also, it should be mentioned, that even though the β1D- and β1A-mediated response looked similar with the high concentration of aggregating antibody, used in these experiments, at lower concentrations of the same antibody, we observed much slower spreading of the β1D-CHO transfectants compared with the β1A-CHO cells. This was accompanied by decreased tyrosine phosphorylation of pp125^{FAK} and delayed activation of MAP kinases in the case of β1D transfectants (data not shown). These observations suggest that under conditions of nonmuscle environment, β1D may be less effective than β1A with regard to integrin-mediated signaling. In muscle cells, however, one cannot exclude that β1D transmits different or substantially modified signals compared with β1A. This might occur if muscle-specific signaling molecules are expressed that interact with β1D.

Expression of the β1 integrin subunit, as well as the occupation of β1-containing integrin heterodimers is necessary for myodifferentiation and the formation of sarcomeric cytoarchitecture (Menko and Boettiger, 1987; Volk et al., 1990). Since the majority of β1 integrins are represented by the β1D isoform in differentiated skeletal muscle, the function of β1 integrin during myodifferentiation should be mostly attributed to this novel cytoplasmic domain isoform. A major function for integrins is to transmit tension generated by the actin cytoskeleton across the plasma membrane to the ECM. The forces generated by striated muscles are considerably greater than those of most other cells. It is easy to envisage how there may be a need for an integrin with a unique cytoplasmic domain to transmit this higher tension across the muscle membrane. The restricted distribution of β1D and its enrichment at myotendinous junctions and costameres, which represent major force transmission sites in skeletal muscle, are consistent with such a function. Like β1D, several components of the peripheral membrane cytoskeleton are expressed only in muscle tissues. These include dystrophin, syntrophin and aciculin (Froehner et al., 1987; Hoffman et al., 1987; Watkins et al., 1988; Ervasti and Campbell, 1991, 1993; Sealock et al., 1991; Porter et al., 1992; Straub et al., 1992; Adams et al., 1993; Yang et al., 1994; Belkin and Burridge, 1994, 1995). One mode of attachment of dystrophin to the sarcolemma involves a non-integrin ECM receptor dystroglycan (Ervasti and Campbell, 1991, 1993; Ibraghimov-Beskrovnaya et al., 1992). However, additional linkages may also be involved and it will be important to explore whether the β1D cytoplasmic domain binds to dystrophin or other unique components of the subsarcolemmal cytoskeleton. With most integrins, the interactions with ECM ligands, as well as with cytoskeletal proteins, involves relatively low affinities (Horwitz et al., 1986; Otey et al., 1990; Hynes, 1992). Presumably these are sufficient for the necessary anchorage and traction of most cells, without precluding the remodeling that must occur during cell migration. With striated muscles the requirements are different. Mature muscle cells do not migrate and, as mentioned above, the tensile forces that have to be transmitted are high. Consequently, high affinity interactions involving integrins may be both tolerated and desirable. One possibility is that the unique cytoplasmic sequence of the β1D integrin subunit increases the affinity of β1D integrin heterodimers for cytoskeletal or both cytoskeletal and extracellular ligands. We hope to test this possibility in our future work.

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