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NOVEL ISOFORM OF β1 INTEGRIN EXPRESSED IN SKELETAL AND CARDIAC MUSCLE

Natalia I. Zhidkova[1], Alexey M. Belkin[2], and Richard Mayne[1]*

[1]*Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294*

[2]*Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599*

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We describe a novel isoform of the ß1 integrin subunit called ß1D, which contains a unique cytoplasmic domain, and is expressed specifically in skeletal and cardiac muscle. The ß1D isoform arises from splicing into the final transcript of an additional exon located between exons 6 and 7. The nucleotide sequence of ß1D predicts a cytoplasmic domain of 50 amino acids in which the last 21 amino acids of the ß1A integrin isoform are replaced by a related sequence of 24 amino acids. A ß1D-specific anti-peptide polyclonal antibody was prepared and immunoprecipitation of tissue extracts with subsequent immunoblotting showed expression of ß1D isoform only in striated muscle cells.

Integrins play crucial roles in both cell-matrix and intercellular interactions and are therefore involved in many important biological processes such as cell migration, differentiation, tissue repair and remodeling (1). All integrins are dimers of α and ß subunits and, for most dimers, both of the subunits have large extracellular and short, but highly conserved, intracellular domains. The ß subunit associates with at least 10 different α subunits and in adhesion-dependent cell types members of the ß1 integrin family are usually the most abundant integrins expressed (1). The specificity of ligand binding is largely determined by the α subunit (2), while the ß subunit appears to target the dimer to sites of cell-matrix interaction in a ligand-independent manner (2-4).
Many ß subunits share significant homology in their cytoplasmic tails and the cytoplasmic domains of ß1, ß3, ß5 and ß6 all associate with the actin cytoskeleton and are required for the recruitment of integrins to focal contact sites with the substratum (5). Recent results show that the cytoplasmic domains of the ß1 and ß3 integrins are involved in "inside-out" signaling and therefore may regulate the affinity state and ligand binding properties of the receptor (6-8). Other studies show a role for integrins in "outside-in" signaling with integrin/ligand engagement activating a variety of signaling pathways within the cell (9).

Recently, two novel but minor isoforms of ß1 integrin subunit with altered cytoplasmic domains were described and called ß1B (10) and ß1C (11). In this report, we identify and

*Corresponding author. FAX: (205)934-7029.

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characterize a fourth ß1 integrin isoform called ß1D integrin which is a major ß1 subunit of terminally differentiated skeletal and cardiac muscle cells.

MATERIALS AND METHODS

Cells and Tissues -- All chicken and human cells and tissues were as described previously (12) except adult human skeletal muscle was obtained from a muscle biopsy of a normal thirty-one-year-old black female. L8 rat skeletal muscle myoblasts were obtained from American Type Culture Collection (ATCC CRL-1769) and cultured on collagen in Dulbecco's modified Eagle's medium and medium 199 (4:1 ratio) with 10% horse serum and 1% chicken embryo extract.
RNA Isolation and First Strand cDNA Synthesis -- Total RNA from cells and tissues was obtained by the RNAzol B method (Biotecx Laboratories, Inc., Houston, TX) and poly(A)-rich RNA selected by using the PolyATtract System IV (Promega Corp., Madison, WI). Synthesis of first strand cDNAs was as described previously (12).
RT PCR -- To analyze the cytoplasmic domain of the chicken ß1 integrin, PCR was performed with primers (from reference 13) BD1 (5'-^{2260} GTGGTAGAGACTCCAGAATGCCCT^{2283}-3') and BD2 (5'-^{2493}TTTAGGATTGACCACAGTTGTCAC^{2470}-3') 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. In order to analyze the cytoplasmic domain of human ß1 integrin, PCR was performed with primers (from reference 14)
PE5 (5'-^{2253}TTGTGGAGAATCCAGAGTGTCCCA^{2276}-3') and
PE6 (5'-^{2500}TCATTTTCCCTCATACTTCGGATT^{2477}-3') under the same reaction conditions. For analysis of the cytoplasmic domain of rat ß1 integrin PCR was performed with primer (from reference 15) NZ1 (5'-^{2197}TTGTGGAGACTCCAGACTGTCCTACT^{2222}-3') and PE6 primer.
Genomic Organization of the Cytoplasmic Domain of Human ß1 Integrin and XL PCR -- A YAC clone of 280 kb from the pericentromeric region of chromosome 10 (called yFN10 and containing the ß1 integrin gene) was a gift from Dr. Helen Donis-Keller (16). Yeast cells from the yFN10 clone were grown in YPD-media at 30°C for 18 h. Cells were pelleted and lysed in spheroplasting buffer with subsequent extraction of DNA with phenol/chloroform. DNA was precipitated with ethanol, pelleted and dissolved in TE-buffer before use in all PCR reactions. A map of the genomic structure of the cytoplasmic domain was determined using rTth DNA polymerase and XL PCR reactions (Perkin Elmer). Several sense and antisense primers were designed which span exons 6 and 7 or were located in exons ED and EC (see Fig. 3). All PCR products were separated in TAE-agarose gel, and PCR products were extracted using a QIAEX agarose gel extraction kit (Qiagen Inc., Chatsford, CA). All PCR products after RT-PCR or XL PCR were subcloned into PCR II vector (Invitrogen) for DNA sequencing. All DNA sequencing was performed as described (12).
Northern Hybridization -- A Multiple Tissue Northern Blot (Clontech) of human tissues was hybridized with antisense oligonucleotides. One oligonucleotide was designed to the ß1D isoform: F11 5'-GAGACCAGCTTTACGTCCGTAGTTTGGATTCTTGAAATT-3' and the other ES2 5'-AGTTGTTACGGCACTCTTATAAATAGGATT-3' to a sequence located in exon 7. Oligonucleotides were labelled with [γ-^{32}P]ATP using T4-polynucleotide kinase. Filters were washed once for 15 min in 2 x SSC, 0.5% SDS at room temperature and once for 15 min in 2 x SSC, 0.1% SDS at 44-47°C.
Generation of Antibody against ß1D Integrin Subunit -- Three peptides were synthesized by the Peptide Synthesis Facility, Department of Chemistry, University of North Carolina at Chapel Hill. A 48-mer CKLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK to the cytoplasmic domain of integrin ß1A, a 51-mer
CKLLMIIHDRREFAKFEKEKMNAKWDTQENPIYKSPINNFKNPNYGRKAGL to the cytoplasmic domain of integrin ß1D and a 17-mer CPINNFKNPNYGRKAGL, which contained part of the amino acid sequence unique to ß1D. The 51-mer was conjugated to maleimide-activated keyhole limpet hemacyanin (Imject, Pierce, Rockford, IL) at a ratio of approximately 50-100 moles of peptide to 1 mole of the protein. Rabbits were immunized subcutaneously with 1 mg of this conjugate in complete Freund's adjuvant (Gibco BRL, Grand Island, NY) and boosted 5 weeks later with 0.5 mg of this conjugate in incomplete Freund's adjuvant (Gibco BRL). Boosts were repeated every 4 weeks with 0.5 mg of the conjugate. Both the 48-mer of ß1A and the 17-mer of ß1D were coupled to Thiopropyl/Sepharose 6B (Pharmacia-Biotech, Uppsala). Pooled

antiserum was preadsorbed first to the 48-mer to remove antibodies recognizing epitopes shared by ß1A and ß1D. Unbound material was loaded onto a column of 17-mer to adsorb ß1D-specific antibodies. The column was washed with 50 mM Tris HCl, 1.5 M NaCl, pH 7.5 and bound material eluted with 200 mM Glycine adjusted to pH 2.2 with HCl. The pH of the column eluate was readjusted to pH 8.0 with 1 M Tris and the affinity-purified IgG fraction was dialyzed against 0.1 M Na_3BO_4,150 mM NaCl, pH 8.1 and stored at 4°C in 2% BSA.

Immunoprecipitation , Electrophoresis and Immunoblotting of ß1D Integrin -- Tissues were homogenized and boiled in 1% SDS, spun down and supernatants diluted with 1% Triton X-100 in 50 mM Tris HCl, 150 mM NaCl, pH 7.5. Lysates (1 mg total protein) were immunoprecipitated at 4°C with anti-ß1D integrin specific antibodies (~ 5-10 µg of affinity-purified IgG/sample followed by Protein-Sepharose beads. Immunoblotting was performed as described (10) using goat anti-rabbit IgG conjugated with peroxidase and developed using ECL reagents (Amersham, Arlington Heights, IL).

RESULTS AND DISCUSSION

Initially, RT-PCR reactions were performed using RNA preparations obtained from a variety of 17-day-old chicken embryo tissues and 24-mer oligonucleotides BD1 and BD2 which span the transmembrane and cytoplasmic domains of the ß1 integrin subunit (Fig. 1A). In cardiac (lane 3) and skeletal muscle (lane 5) a band of higher molecular weight was also observed in addition to the band of expected size (234 bp) for ß1A. Similar results were obtained for human tissues using primers PE5 and PE6 (Fig. 1B). Analysis of the appearance of the upper band

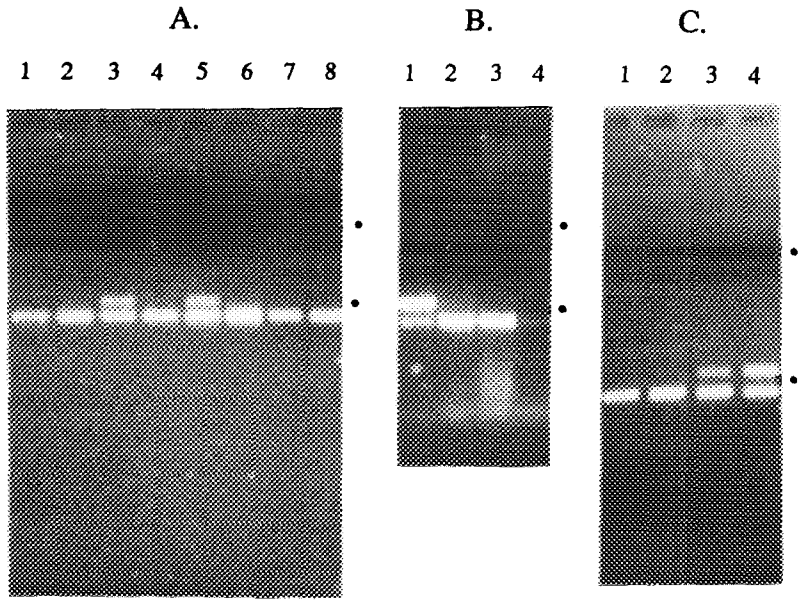

FIG. 1. Identification of the ß1D isoform of integrin by RT-PCR. A. Agarose gel electrophoresis of the products of an RT-PCR reaction performed with RNA from a variety of tissues of 17-day chick embryos using primers BD1 and BD2 which span the cytoplasmic domain of ß1A integrin. Lane: 1, brain; 2, kidney; 3, heart; 4, gizzard; 5, skeletal muscle; 6, liver; 7, skin; 8, chondrocytes. B. RT-PCR reaction performed with RNA from human tissues and cells using primers PE5 and PE6. Lane: 1, adult skeletal muscle; 2, chondrocytes; 3, histiocytic lymphoma cells; 4, fibroblasts. C. RT-PCR reaction performed with RNA from cultured rat L8 cells at different stages of myodifferentiation using primers NZ1 and PE6. Lane: 1, 0 day; 2, 3 day; 3, 12 day; 4, 14 day. Molecular markers: Upper dot is 1 kb; lower dot is 0.3 kb.

during myodifferentiation was performed with rat L8 cells using primers NZ1 and PE6 (Fig. 1C). At initial plating, only the lower band was detected (lane 1) but, after fusion to form myotubes, the proportion of upper to lower band increased until day 14 (lanes 2, 3, and 4). Each band was subcloned into PCR™II vector and subjected to nucleotide sequencing (Fig. 2). The results showed that the lower band is identical to the published sequence of ß1A (data presented only for human). However, the upper band contained an 81 bp insert that was highly conserved between species. This predicts that the C-terminal 21 amino acids of ß1A are replaced by 24 amino acids. The new amino acids differ markedly from the ß1B and ß1C isoforms (10,11) and this variant is called ß1D.

A YAC clone containing the human ß1 integrin gene (16) was mapped using a series of XL PCR reactions with sense and antisense primers located between exons 6 and 7 and within the ß1D sequence (Fig. 3). With primers located in exon 6 and exon 7, it was initially shown that there is a total of 6 kb of intronic sequence. Further XL PCR reactions showed that the ß1D sequence is located within the first 1.5 kb of the intron. Genomic PCR mapping was also performed with primers designed from the published exon sequence of ß1C. The results showed that this exon is located 0.5 kb downstream from ß1D. All PCR products were cloned into the PCR™II vector and all intron-exon boundaries were found to fit canonical sequences for splicing. Fig. 3 shows the origin of the four variants of the cytoplasmic domain of the ß1 subunit. The ß1A isoform arises from splicing together of exons 6 and 7 so that there is an open reading frame of 21

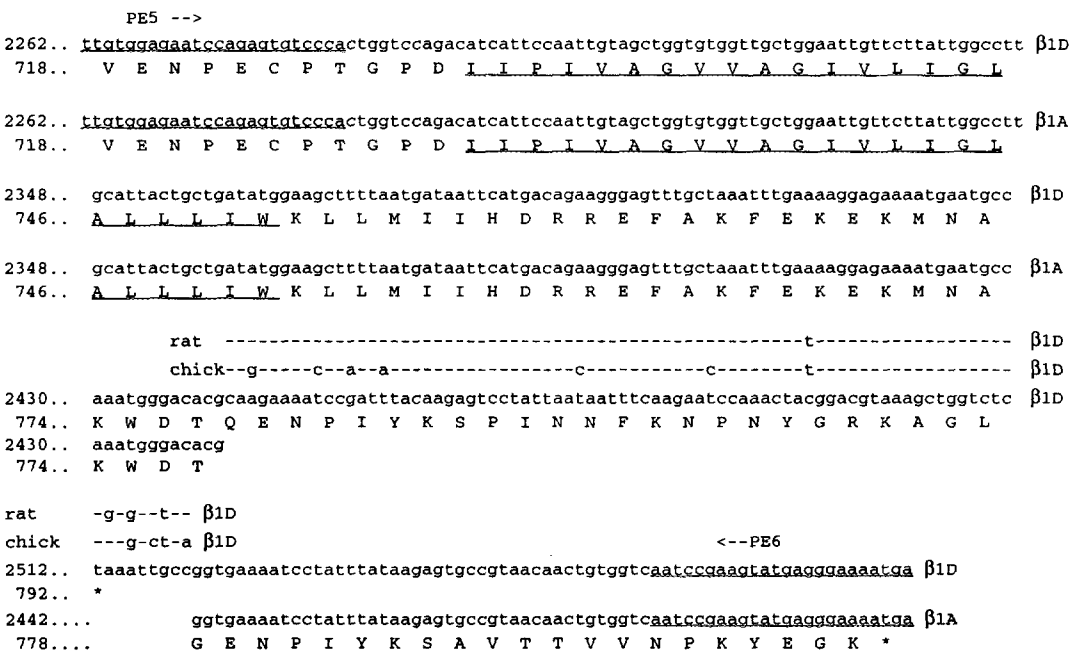

FIG. 2. Comparison of the nucleotide sequence of the β1D isoform with the β1A isoform from human skeletal muscle. RT-PCR was performed using primers PE5 and PE6. Both bands obtained for skeletal muscle (Fig. 1B; Lane 1) were cloned prior to DNA sequencing. Conservative differences were found in the nucleotide sequence of the β1D variant of chicken heart and skeletal muscle (Fig. 1A) and rat skeletal muscle (Fig. 1C), resulting in an identical amino acid sequence.

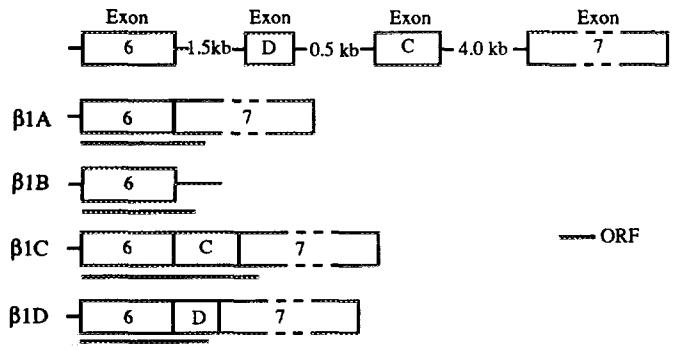

FIG. 3. Exon-intron organization within the cytoplasmic domain of the β1 integrin gene. The β1A isoform arises from splicing together of exons 6 and 7 whereas the β1B variant arises from a failure of this splicing event (10). The β1C and β1D variants arise from alternative splicing of one of the additional exons into the final transcript. The lengths of all open reading frames (ORF) are shown.

amino acids derived from exon 7. The ß1B variant arises from a failure of splicing at the 3' end of exon 6 so that the final transcript contains 12 amino acids derived from the open reading frame of the intronic sequence (10). Both ß1D and ß1C arise from alternative splicing into the final transcript of additional exons (called ED and EC). Exon C has an open reading frame so that translation of ß1C continues into exon 7 for 10 additional amino acids, but in a different reading frame from ß1A (11). For the ß1D isoform, a stop codon is present at the 3' end of exon D (see Fig. 2), which prevents translation continuing into exon 7, and results in 24 new amino acids at the C-terminus.

Northern blots were performed on a variety of tissues to demonstrate that ß1D exists as a full-length transcript of the ß1 integrin gene (Fig. 4). A 30-mer antisense oligonucleotide (called ES2) designed to exon 7 of human ß1, and therefore shared by ß1A, ß1C and ß1D isoforms, gave the expected signal at 4.2 kb in all tissues tested but of varying intensity (Fig. 4A). However, a 40-mer antisense oligonucleotide designed to the unique sequence of ß1D (called F11) gave a signal only for cardiac (lane 1) and skeletal muscle (lane 6) (Fig. 4B).

An anti-peptide isoform-specific antibody against the β1D subunit was used to investigate the expression pattern of this integrin in various tissues of adult chicken by immunoprecipitation and subsequent immunoblotting (Fig. 5). Significant amounts of β1D, represented by a broad band with a M_r around 140,000, were found in two different preparations of skeletal muscle while a slightly slower migrating band of β1D integrin was detected in heart muscle extract. The β1D isoform was not detected in all other tissues analyzed.

The results show the 21 amino acids of the β1A isoform GENPIYKSAVTTVVNPKYEGK are replaced in the β1D isoform by a closely related sequence of 24 amino acids, QENPIYKSPINNFKNPNYGRKAGL. Thus, both the β1A and β1D isoforms share several common structural features with the first 8 amino acids being the same except that the first G is changed to Q. Two NPXY sites are also conserved and it is only at the C-terminus and between the NPXY sites that marked differences are apparent between β1A and β1D. The β1D isoform contains all three sequences required for localization to focal adhesions and, immunofluorescent

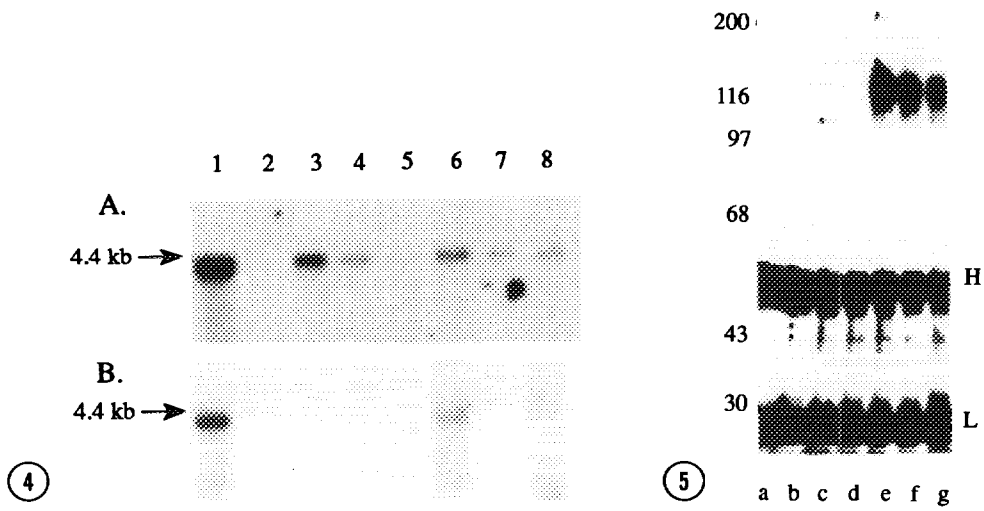

FIG. 4. Northern blot of a variety of adult human tissues with probes specific for β1D and common for β1A, β1C and β1D. In all lanes 2 μg of poly(A)RNA was used. Lane: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas. Panel A was probed with an antisense 30-mer oligonucleotide derived from exon 7 (which is shared by the β1A, β1C and β1D subunits) . Note reactivity in all tissues. Panel B. The blot was stripped and probed with an antisense 40-mer oligonucleotide specific for β1D. Note specific reactivity only with heart and skeletal muscle.

FIG. 5. Expression of β1D integrin isoform in adult chicken muscle tissues. The β1D integrin isoform was immunoprecipitated from different tissues and then immunoblotted with a specific anti-peptide antibody (see Materials and Methods for details). Lane: a, gizzard; b, liver; c, skin; d, brain; e, heart; f, thigh skeletal muscle; g, breast skeletal muscle. Positions of molecular weight markers are indicated to the left of the gel. H and L mark IgG heavy and light chains, respectively.

staining of skeletal and cardiac muscle with the β1D-specific antibody shows localization to costameres and the myotendinous junction. Previous transfection experiments demonstrated a key role for the last 13-16 amino acids of β1A for localization to focal adhesion sites (3,17-19). This sequence includes the region between the two NPXY sites which is the location of major differences in amino acid sequence between β1A and β1D. This sequence includes a site of α-actinin binding (20). The results suggest that β1D, although retaining many of the features of the β1A sequence, is nevertheless likely to differ in its ability to interact with cytoskeletal proteins. It is speculated that β1D plays a key role in the interaction of cytoskeletal proteins with the sarcolemma of skeletal and cardiac muscle, perhaps forming stronger attachment sites required by highly contractile cells.

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