H36-α7 Is a Novel Integrin Alpha Chain That Is Developmentally Regulated During Skeletal Myogenesis

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Abstract. H36 is a 120,000-D membrane glycoprotein that is expressed during the differentiation of skeletal muscle. H36 cDNA clones were isolated from a lambda UniZapXR rat myotube cDNA library and sequenced. The deduced amino acid sequence demonstrates that H36 is a novel integrin alpha chain that shares extensive homology with other alpha integrins that includes: (a) the GFFKR sequence found in all alpha integrins; (b) a single membrane spanning region; (c) conservation of 18 of 22 cysteines; and (d) a protease cleavage site found in the non-I region integrin alpha chains. The cytoplasmic domain of H36 is unique and additional regions of nonhomology further indicate H36 is distinct from all other alpha chains. In keeping with current nomenclature we designate this alpha chain α7. Northern blots demonstrate that expression of H36-α7 mRNA is regulated both early in the development of the myogenic lineage and later, during terminal differentiation. Detection of H36-α7 mRNA coincides with conversion of H36- myogenic precursor cells to H36+ cells. H36-α7 mRNA is present in replicating myoblasts: expression increases upon terminal differentiation and is markedly reduced in developmentally defective myoblasts. In addition, H36-α7 mRNA is not detected in C3H10T1/2 cells. It is in myotubes derived from myoblasts obtained by treatment of 10T1/2 cells with azacytidine or transfection with MRF4. Immunoblots and immunofluorescence demonstrate that the H36-α7 chain is associated with integrin β1. Affinity chromatography demonstrates that H36-α7β1 selectively binds to laminin. The expression of H36-α7 on secondary myoblasts during the development of the limb in vivo corresponds with the appearance of laminin in the limb, with the responsiveness of secondary myoblast proliferation to laminin, and with the onset of increased muscle mass, suggesting that H36-α7 modulates this stage in limb development. We conclude that H36-α7 is a novel alpha integrin laminin binding protein whose expression is developmentally regulated during skeletal myogenesis.

A continuum of cell interactions takes place throughout the differentiation of skeletal muscle: distinct cell and molecular interactions underlie the development and function of each stage. In the early stages of myogenesis cells replicate, migrate, and maintain an autonomy from one another that is typical of most other cells. During this stage the primary interactions of cells are with their molecular environment that includes nutrients, growth factors, and extracellular matrix proteins. This serves to increase cell mass and localize the sites of future development. Subsequently, upon termination of the proliferative stage, the cells interact and fuse to form elongate fibers. At this same stage of development the genes that encode the myofibrillar proteins and ATP-generating enzymes are expressed. The transition between these stages of myogenic development is regulated by the interactions of heterodimeric complexes of helix-loop-helix proteins with regulatory sites in the genome (Murre et al., 1989; Davis et al., 1990; for review see Olson, 1990) and by the interactions of cells with growth factors (Lim and Hauschka, 1984; Ewton and Florini, 1990; Massague et al., 1986; Jin et al., 1991) and extracellular matrix proteins (Foster et al., 1987; Ocalan et al., 1988). The assembly of the contractile proteins into sarcomeres and interactions between fibers and nerve cells results in the formation of functional muscle, responsive to neuronal input. Extracellular matrix proteins may promote sarcomere assembly (Volk et al., 1990) as well as localize acetylcholine receptors (Axelrod et al., 1976; Dymtrenko et al., 1990). Neuromuscular junctions are formed and stabilized by further molecular interactions, each cell type providing specific proteins (Fullon et al., 1985; Bloch and Froehner, 1987; Hunter et al., 1989; Carr et al., 1989). Specific ion channels and transport proteins in the muscle membrane produce the ionic discontinuities between muscle and the extracellular environment that are essential to muscle activation. Formation of myotendinous junctions and additional anchoring of the skeletal fibers further stabilize the muscle and direct movement. Dystrophin, the protein defective or absent in patients with Duchenne muscular dystrophy (Bonilla et al., 1988), and integrin, the extracellular matrix receptor (Bozyczko et al., 1989), may be of particular importance to this latter stage of development. Thus as cells develop from precursors to myoblasts to functional contractile fibers diverse interactions occur on the plasma membrane. A remodeling of the membrane that
presumably reflects these diverse events accompanies each stage of development (Kaufman and Foster, 1985). The specific molecules in the membrane and mechanisms that underlie each of these stages of myogenic development are known to differing degrees.

One membrane protein, H36, has proven useful as a cell surface marker to identify and isolate myogenic cells. H36 is a developmentally regulated integral membrane glycoprotein on skeletal muscle (Kaufman et al., 1985). It was originally identified using immunoaffinity chromatography and isolated by immunoaffinity chromatography with a mAb. H36 is expressed on replicating myoblasts and this expression is increased before fusion. This upregulation is dependent on new transcription and is inhibited by a variety of conditions which inhibit differentiation (Kaufman et al., 1985; Kaufman, 1990). Cells selected from heterogeneous populations by flow cytometry based on their expression of H36 differentiate into skeletal muscle (Schweitzer et al., 1987; Kaufman and Foster, 1988). Mutants isolated from the L8/E63 myogenic line based on their lack of expression of H36 do not differentiate. H36 has also served as a marker for identifying cells in the myogenic lineage (Kaufman and Foster, 1988). Precursor cells in the embryonic limb bud do not initially express H36, but do so upon subsequent development (Kaufman et al., 1991). Immunocytochemistry of cryosections confirms the expression of H36 on fetal skeletal muscle in vivo and has contributed to demonstrating that H36 is expressed at different stages during the formation of primary and secondary fibers (Kaufman et al., 1991; George-Weinstein, M., manuscript submitted for publication). During primary myogenesis, H36 is first expressed upon terminal differentiation whereas during secondary myogenesis it is present on replicating myoblasts and its expression is subsequently upregulated upon terminal differentiation.

Because of the developmental specificity and regulation of H36 during the embryonic and fetal development of skeletal muscle we have cloned the H36 gene to determine the structure and function of this protein. In these experiments we report the cloning and sequencing of H36 cDNA and its identification as a novel integrin alpha chain.

**Materials and Methods**

**Cells**

L8/E63 cells, a myogenic clone of L8 rat skeletal myoblasts and f1 cells, a developmentally defective clone of L8, that have lost normal growth control, were grown in DME supplemented with 10% horse serum (Gibco Laboratories, Grand Island, NY) as described (Kaufman and Parks, 1977). C2A2 myoblasts, subcloned from the C2 line of mouse satellite cells (Yaffe et al., 1977), and C2A2A myoblasts, subcloned from the C2A2 myogenic cells derived by treatment of C3H10T1/2 cells with 5-azacytidine (Konieczny et al., 1984), were grown in 15% colostrum-free bovine serum (Irvine Scientific, Santa Ana, CA) in DME. MRF4/7 mouse myogenic cells, derived by transfection of C3H10T1/2 cells with MRF4 cDNA (Rhodes and Koni-eczny, 1989), and BC3H1 non-fusing mouse myoblasts (Schubert et al., 1974) were grown in 20% FBS (Sigma Chemical Co., St. Louis, MO) in DME; RMO rat myoblasts (Merrill, 1989) were grown in 15% FBS. The 23A2 and MRF4 myogenic lines were provided by Drs. C. Emerson, Jr. (Fox Chase Institute, Philadelphia, PA) and S. Konieczny (Purdue University, Lafayette, IN), respectively. C2 cells and BC3H1 cells were provided by Dr. E. Olson (M. D. Anderson Cancer Center, Houston, TX) and RMO myoblasts were provided by Dr. G. Merrill (Oregon State University, Corvallis, OR). P1C1 human satellite cells were obtained from Dr. H. Blau (Stanford University, Stanford, CA) and grown in F10 medium containing 20% FBS, and 0.5% chick embryo extract as described (Blau and Webster, 1981).

Differentiation was promoted in these cell lines by growth in 2% horse serum. The qual myogenic line, QM7, provided by Drs. P. Antin and C. Or- dol (University of California, San Francisco, CA), was grown in medium 199 containing 10% FBS and 1% trypsin phosphate and allowed to differen- tiate in 0.5% FBS in medium 199 (Antin and Ordahl, 1991). C3H10T1/2 cells (Reznikoff et al., 1973) were provided by Dr. P. Jones (Uni- versity of Southern California, Los Angeles, CA) and grown in 10% horse serum. BHK cells were grown in DME containing 10% FBS and 10% tryp- tone phosphate. Primary cultures of Sprague/Dawley newborn rat thigh muscle (Holtzmann) were prepared as described by Foster et al. (1987). Em- bryonic day 12 cultures of hindlimb buds were prepared from rats in timed pregnancy and serially passaged as described (Kaufman et al., 1991). Chick embryo muscle cultures were prepared from day 21 White Leghorn em- bryos (O'Neil and Stockdale, 1972). Media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml kanamycin and all cultures were grown at 37°C, in 10% CO₂.

**Antibodies**

H36 protein was isolated by immunoaffinity chromatography. The hind- limbs of 120 5-d-old rat pups were skinned, feet removed, teased in 40 ml buffer A (250 mM KCl, 1 mM CaCl₂, 3 mM MgCl₂, 200 mM sucrose, 1% aprotinin, 100 μg PMPSF, 50 μg Tris-maleate, pH 5.5), and homogenized in an equal volume of buffer A in a Polytron at 10,000 rpm for 30 s. The supernatant was added to 10 ml glass wool columns (Bio-Rad Laboratories, Cambridge, MA) coupled with 10 mg purified anti-H36 antibody (Kaufman et al., 1985). The column was washed with 500 mM NaCl, 1 mM EDTA, 10 mM CHAPS, 0.02% NΑ₂, 50 mM Hepes, pH 8.0 and then with 150 mM NaCl, 10 mM CHAPS, 0.02% NΑ₂, 50 mM Hepes, pH 8.0, until A₂₈₀ = 0. Bound H36 was eluted in 0.5 M acetic acid, pH 2.0.

BALB/c mice, 8-wk old, were immunized four times, at 3-wk intervals, with 100 μl containing 35 μg antigen in RIBI adjuvant (Ribi Immunocore Research, Inc., Hamilton, MT). Hybridomas were prepared by fusion of immune spleen cells with SP2/0 cells in the University of Illinois Cell Science Center (Urbana, IL). Supernatants were screened by immunofluorescence and immunoautoradiography. All positive clones were subcloned and restested for reactivity and specificity. The 05 antibody reacts with purified H36 in immunoblots and was used to screen the cDNA library. The 04-1 antibody reacts with integrin β1 chain in the affinity purified H36 complex and myo- tubes lysates. 026 antibody reacts with purified H36 in immunoblots and on cells. Rabbit antisera specific for the cytoplasmic domains of integrin β1 (two sera provided by Drs. C. Buck, Wistar Institute, Philadelphia, PA and Dr. F. Horwitz, University of Illinois, Urbana, IL), β3 (Dr. T. F. Wangberg, Scripps Institute, La Jolla, CA), β4 and β6 (Dr. V. Quaranta, Scripps Institute, La Jolla, CA) and β5 (Dr. L. Reichardt, University of California, San Francisco, CA), and for extracellular determinants on β1 (two sera provided by Drs. C. Buck and K. Yamada, NIH, Bethesda, MD) were gener- ously provided as indicated.

**Immunofluorescence**

Cells cultured on No. 1 glass coverslips (Belco Glass, Inc., Vineland, NJ) coated with 0.1% gelatin, and cryosections prepared from adult Sprague-Dawley thigh muscle were processed for immunofluorescence as reported (Kaufman et al., 1991). H36 anti-alpha chain antibody, purified from ascites fluid, was used at a dilution of 5 μg/ml. 04-1 anti-β1 antibody in hybridoma culture fluid was used undiluted, and rabbit antibody reactive with the cytoplasmic domain of integrin β1 chain (provided by Dr. A. F. Horwitz), partially purified by ammonium sulfate precipitation, was used at a 1:100 dilution. Rabbit Igg purified by ammonium sulfate precipitation and SP2/0 culture fluid were used as negative controls. Fluorescein- or rhodamine- conjugated donkey anti-mouse and donkey anti-rabbit IgG (Jackson Immuno-Research Laboratories Inc., Avondale, PA) were used as secondary antibo- dies. In the double label experiments with H36 and 04-1 antibodies, live cells were first reacted with 04-1 antibody, then with fluorescein-anti-mouse IgG, followed by purified normal mouse IgG. Biotinylated anti-H36 antibody was then added, followed by rhodamine-avidin and the cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde. Immunofluorescence and staining with DAPI were performed as described (Kaufman et al., 1991). Cell nuclei were stained with DAPI. Rhodamine- and fluorescein-conjugated secondary antibodies were visualized with an Axiosplan microscope (Zeiss).
followed by fluorescein-anti-rabbit IgG. The coverslips were mounted in glycerol/PBS 9/1 (vol/vol), pH 8.5, containing 10 mM p-phenylenediamine (Eastman, Rochester, NY), sealed with Flu-tex (Fisher Scientific Co., Pittsburough, PA), and examined with a photomicroscope III (Carl Zeiss, Inc., Oberkochen, Germany) equipped with epi-illumination optics and an HBO 100-W mercury lamp.

Immunoblot Analysis

The samples of purified H36 protein or myotubes lysates were separated in 0.75-mm thick, 8% polyacrylamide minigels (Laemmli, 1970). Cell lysates were prepared by sonication (three times, for 10 s, at 4°C) in PBS containing 1 mM PMSE. Electrophoresis was at 100 V for 50 min at room temperature. The gels were equilibrated in 25 mM Tris, 200 mM glycine, pH 8.8, and 20% methanol for 10 min at 4°C, and the proteins transferred to nitrocellulose at 100V for 60 min. The filters were blocked with 2% gelatin in TBS (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20), reacted with primary antibodies, followed by alkaline phosphatase conjugated goat anti-mouse immunoglobulin (Jackson Immunoresearch). Immunoreactive proteins were visualized with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 200 mM NaCl, 5 mM EDTA, and 100 mM Tris-HCl, pH 9.5.

RNA Analysis

Poly(A)+ RNAs were purified according to Badley et al. (1988). Cultured cells were collected, pelleted, and lysed in 200 mM NaCl, 1.5 mM MgCl2, 2.0% SDS, 0.2 M Tris-HCl, pH 7.5, and 200 μg/ml proteinase K. The poly(A)+ RNAs were purified using oligo dT cellulose (Collaborative Research Inc., Waltham, MA), electrophoresed in 1% agarose gels containing 2.2 M formaldehyde, and transferred onto GeneScreen plus (DuPont-NEN Products, Wilmington, DE) by capillary action (Sambrook et al., 1989). The filters were prehybridized at 65°C for 2-3 h in 1% gelatin, 7% SDS, 100 μg/ml salmon sperm DNA, 1 mM EDTA, 0.55 M sodium phosphate buffer, pH 7.2, and then hybridized with probes labeled by random primer labeling (Oligolabeling Kit; Pharmacia Fine Chemicals, Piscataway, NJ) using [α-32p]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL). The filters were washed three times with 0.2x SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% SDS at 65°C and exposed to x-ray film (Dupont Co.) for 12-72 h.

DNA Sequencing

pBluescript SK+, rescued from lambda UniZap XR positive clones detected by immunoscreening, were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using [α-32p]dATP (1,000 Ci/mmol, Amersham Corp.). The nucleotide sequences of independent subclones of unidirectional deletions of clones OSA, OSE, and OSE, made with Exo/Mung Bean nuclease (Henikoff, 1984; Stratagene, La Jolla, CA), were determined. The cloned PCR fragment was sequenced from both ends. Analyses of the nucleotide and amino acid sequences were performed using DNASTAR, MacVector, and the University of Wisconsin Genetics Computer Group software, version 7.

Antigen Cross-linking Protocol

L8E63 cells, plated on 60-mm dishes and grown for 3 d, were reacted with mAbs H36, O26, or A5. H36 and O26 antibodies react with purified H36 protein and H36 on live cells; A5 reacts with a non-crossreacting cell surface protein (B. and Kaufman, 1981). Rabbit antimouse immunoglobulin (Jackson Immunoresearch) was added as secondary antibody. Cells were washed three times with cold Dulbecco's PBS and once with cold buffer B (100 mM KCl, 3 mM MgCl2, 1 mM CaCl2, 300 mM sucrose, and 10 mM Hepes, pH 6.9). The cells were extracted with 1% Triton X-100 in buffer B for 5 min on ice, and the supernatants were collected. The residue was solubilized in 1% SDS. The effect of cross-linking H36 with antibody on its extractability in Triton X-100 was determined by immunoblot analysis.

Extracellular Matrix Protein Affinity Chromatography of H36-α7 Integrin

L8E63 myotubes and myoblasts on three 100-mm dishes (≈1.5 x 106 nuclei) were collected using a rubber policeman and extracted with 2% Triton X-100 for 30 min, at 4°C, with 150 mM octylglucoside buffer (200 mM octyl-ß-D-glucopyranoside, 1 mM PMSE, and 100 mM Tris-HCl, pH 7.4). The extracts were adjusted to a final concentration of 2 mM MnSO4 and passed through a 1-ml column of Affigel-10 (Bio-Rad Laboratories) conjugated with either 1.08 mg purified Engelbreth-Holm-Swarm sarcoma laminin (provided by Dr. B. Rainich (University of Illinois, Urbana, IL) and Dr. A. F. Horwitz (University of Illinois, Urbana, IL)), 1.10 mg purified fibronectin (provided by Mr. K. McDonald (University of Illinois, Urbana, IL) and Dr. A. F. Horwitz), 1.94 mg type I rat tail collagen (Sigma Chemical Co., St. Louis, MO) or 1.68 mg type IV calf skin collagen (Sigma Chemical Co.).
The columns were washed with buffer B (50 mM octyl-β-D-glucopyranoside, 1 mM MnSO₄, 50 mM Tris-HCl, pH 7.4), followed by buffer B containing 250 mM NaCl and eluted with 50 mM octyl-β-D-glucopyranoside, 10 mM EDTA, 50 mM Tris-HCl, pH 7.4. The eluate was adjusted to 20 mM MgCl₂, concentrated using a Centricon-30 tube (Amicon Corp., Danvers, MA) and analyzed by immunoblotting.

**Results**

**Preparation of L8E63 Myotube cDNA Library**

Poly(A)⁺ RNA was purified from L8E63 myotubes using oligo dT cellulose affinity chromatography. The poly(A)⁺ RNA migrated in a 1% agarose, 2.2 M formaldehyde gel as a broad band with a heterogeneous mobility corresponding to ~0.5 to 10 kb. This RNA promoted, in a stoichiometric fashion, the synthesis of protein in an in vitro wheat germ system. The poly(A)⁺ RNA also promoted the synthesis of H36 upon injection into *Xenopus* oocytes, indicating that the specific mRNA of interest was present in the mixture.

A myotube unidirectional cDNA library was constructed in lambda UniZap XR (Stratagene) using the poly(A)⁺ RNA and oligo dT priming. The original library contained 1.4 × 10⁷ primary plaques and was subsequently amplified. The cDNA inserts were excised by coinfection of XL-1 blue cells with the lambda phage and VCSM13 helper phage. The phagemid was isolated and used to transform XL-1 blue cells. The pBluescript SK⁻ plasmid was purified and linearized using Xhol. Translationaly active RNA was transcribed, capped and microinjected into *Xenopus* oocytes. Immunofluorescence of H36, detected after injection of three independent RNA preparations, indicated that the cDNA of interest was contained in the cloned library (data not shown).

**Isolation of H36 cDNA Clones and Sequence Analysis**

Since the original H36 mAb does not readily react with the denatured protein, it was necessary to prepare additional antibodies to use to screen the lambda expression library. H36 protein isolated from 5-d-old rat pups by immunoaffinity chromatography was used as the immunogen. One of the antibodies, 05 antibody, identified bands at 120,000 and 70,000 D in the unreduced purified H36 complex and at 35,000 D and 100,000 D upon reduction. (B) To confirm that the proteolytic cleavage of the 120,000-D protein yields the 100,000-, 70,000- and 35,000-D fragments, affinity purified H36 was electrophoresed under nonreducing conditions, the 120,000-, 100,000-, and 70,000-D bands were cut out, incubated with 100 mM DTT, electrophoresed again on 8% polyacrylamide SDS gels and immunoblotted with 05 antibody. The results demonstrate the origin of the fragments from the 120,000-D protein. (C) Cleavage at either or both of the proteolytic cleavage sites RRQ and RRRE (determined by sequence analysis) completely accounts for the generation and sizes of the fragments detected with the 05 antibody. The apparent molecular weights of prestained β-galactosidase (116,000), phosphorylase b (106,000), bovine serum albumin (71,000), ovalbumin (44,000), and carbonic anhydrase (28,000) are indicated.
**Figure 2.** Crosslinking H36 protein on the cell surface with antibodies alters extractability by detergent. L8E63 cells were grown for 3 d, reacted with mAbs 026, A5, or H36. The cells were then reacted with rabbit antimouse immunoglobulin and extracted with 1% Triton X-100. The supernatants (S) and detergent insoluble cell pellets (P) were collected, subjected to SDS-PAGE under reduced conditions, followed by immunoblot analysis using 05 antibody. In the absence of primary antibody (No 1°) or primary and secondary antibodies (No 1°, No 2°) most H36 protein detected with 05 antibody is solubilized (S). Cross-linking with 026 and H36 antibodies promotes association of H36 protein with the cytoskeleton, rendering it insoluble in Triton X-100 (P). A5 antibody does not react with H36, but with another protein on the L8E63 cell surface (Lowrey and Kaufman, 1990), and therefore does not effect extraction.

mAbs, denoted 05, immunoblotted with the 120,000-D protein in the purified H36 preparation and in lysates prepared from L8E63 cells, under unreduced conditions (Fig. 1 A). The additional high molecular weight bands seen in the immunoblot of unreduced purified H36 protein are due to the reaction of secondary antibody with H36 mAb that elutes during the immunoaffinity purification. When the H36 protein was in its reduced state, 05 antibody identified bands at 35,000 D and 100,000 D in immunoblots. The 35,000-D band was most prominent upon analysis of the reduced purified H36 protein whereas the 100,000-D protein predominated in the immunoblots of the reduced myotube lysate. A 70,000-D protein in the unreduced preparation of purified H36 also reacted with the 05 antibody. Two-dimensional polyacrylamide gel analysis demonstrates that the 35,000-, 70,000-, and 100,000-D proteins originate from the proteolytic cleavage of the unreduced 120,000-D protein (Fig. 1, B and C). In this experiment, purified H36 protein was electrophoresed under non-reducing conditions and the 120,000-, and 100,000-, and the 70,000-D proteins were isolated, reduced and rerun. As shown in Fig. 1, B and C, reactivity with one mAb (05) and two proteolytic cleavage sites, account for all the peptides detected in the immunoblots. As will be discussed, sequence analysis confirms the location of these protease cleavage sites. To further demonstrate reactivity of O5 antibody with H36 we cross-linked H36 protein on L8E63 cells using H36 antibody followed by rabbit anti-mouse antibody. Normally, most H36 is readily extracted from the cells by Triton-X 100, however, cross-linking with antibody promotes its association with the cell cytoskeleton, rendering it relatively unextractable (Lowrey and Kaufman, 1989). As shown in the immunoblot in Fig. 2, crosslinking H36 protein with anti-H36 antibody specifically alters the extractability of the protein reactive with 05 antibody. Likewise, O26 antibody also reacts with H36 and also promotes its association with the cytoskeleton. Cross-linking with antibodies reactive with proteins not in the H36 complex, for example A5, does not alter the association of H36 with the cytoskeleton nor its extractability.

The lambda Unizap XR cDNA library was screened with the O5 antibody. Three independent cDNA clones were isolated from assays of 5 x 10^5 plaques. The pBluescript SK+ plasmids were isolated as indicated above and the sizes of the inserts were determined by cleaving with EcoRI and XhoI and electrophoresis in 0.8% agarose gels. The sizes of the three cDNA inserts, O5A, O5B, and O5C, are 2.5, 2.3, and 1.8 kb, respectively (Fig. 3). Nucleotide sequence analysis indicates that these cDNAs encode the same protein. The 2.3-kb insert codes for an 80,000-D fusion protein, (determined by immunoblotting) of which, ~15,000 D is β-galactosidase.

A PstI fragment representing 293 bp from the 5'-end of the O5B clone was prepared, labeled by random primer labeling, and used to screen the library for a full-length cDNA insert. The O5E cDNA clone was isolated and the size of the gene insert was determined to be 3.4 kb. As a full-length cDNA insert could not be identified in the library, a 544-bp cDNA fragment overlapping the O5E clone and encoding the amino terminal end of the protein was synthesized, cloned, and sequenced.

DNA sequencing was performed by the dideoxy chain termi-
H36 Is a Novel Integrin Alpha Chain

Alignment and comparison of the inferred H36 amino acid sequence (Fig. 5) demonstrates that H36 shares extensive structural homologies with integrin alpha chains (Tamura et al., 1990; Albelda and Buck, 1990; Hemler, 1990; Humphries, 1990) that include: (a) The GFFKR sequence (residues 1032–1036) in the cytoplasmic domain of H36 is identical with the characteristic of all reported integrin alpha chains with the exception of the Drosophila PS2 protein in which the lysine is replaced by an asparagine residue (Bogaert et al., 1987); (b) the single membrane–spanning region and single cytoplasmic domain inferred from hydrophobicity analysis of the H36 sequence are consistent with that found in all other integrin alpha chains; (c) there is extensive conservation of the locations of cysteine residues in H36 and the other integrin alpha chains. 16 of 22 cysteines identified in H36 align with those in all other alpha integrins and eighteen of the twenty-two cysteines are at conserved positions in H36 and the non-1 domain alpha chains; (d) H36 has a protease cleavage recognition site at residues 882–886 in the extracellular domain that satisfies the consensus sequence, K/RR/EE/D, identified in other integrin alpha chains (de Curtis et al., 1991). (e) the five conserved repeats of glycine/alanine/proline (GAP) found in the amino-terminal half of all the other integrin alpha chains are also present in H36.

Using DNASTAR, a range of homologies was found between H36 and the other integrin alpha chains (Fig. 5). Sequence alignment shows that H36 shares 24% identity in a 1,008 amino acid overlap with α6 integrin (Argarves et al., 1987), 31% identity in a 1,008 amino acid overlap with the αIIbβ3 chain (Poncz et al., 1987) and 35% identity in a 1,052 amino acid overlap with galactoprotein b3 chain (Tsuij et al., 1990) as well as the other basic features cited above. In general H36 exhibits greater homology with those integrin alpha chains that have the characteristic protease cleavage site compared with those that contain the I domain.

The greatest homology of the deduced H36 sequence is with human α6 integrin (Tamura et al., 1990) (Fig. 5). There is 47% identity in a 1,047 amino acid overlap and the transmembrane domains of these two proteins are even more similar (70% identity). Differences between H36 and the human α6 chain include: (a) H36 has a unique cytoplasmic domain comprised of 77 amino acids. With the exception of the GFFKR consensus sequence, the cytoplasmic domain of H36 is as divergent from those of the other integrin alpha chains as they are from each other; (b) The protease cleavage site in the extracellular domain of H36 (RRRRE, residues 882–886) is distinct from that in human α6 (RRKRRE). Cleavage at this site yields the 100,000-D fragment detected with the α5 antibody in immunoblots and a smaller fragment, ~28,000 D, which would not be detected by this antibody. Such a peptide was detected using an antiserum reactive with purified H36 protein (data not shown). There is a second potential proteinase cleavage site (RRQ) at residues 575–577 in H36. Cleavage here generates the 70,000-D fragment and cleavage at both sites yields the 70,000- and 35,000-D fragments detected with H36-α5 antibody (Fig. 1). Two additional potential dibasic cleavage sites, KK and KR, one on each side of the RRQRE site, could provide heterogeneity in the cleavage products; (c) There are ten insertions in the H36 amino acid sequence, including one of seven amino acids, and one double and seven single amino acid deletions. The homology of the human α6 amino acid sequence with other integrin alpha chains is between 18–26% identity whereas the homology between α6 chains from human and chick is 73% (de Curtis et al., 1991). Thus the H36 and α6 are amongst the most closely related integrin alpha chains, yet they are distinct. We conclude that H36 is a novel integrin alpha chain. In keeping with the nomenclature of the alpha integrin chains, we suggest H36 be designated α7.

H36-α7 Is Associated with Integrin β1 Chain and Binds to Laminin

To determine which integrin beta chain is associated with H36-α7, rabbit antisera raised against the β1, β3, β4, β5, or β6 cytoplasmic domains were reacted in immunoblots against the purified H36 complex. Two independently produced antisera specific for the cytoplasmic domain of β1 and one monoclonal anti-β1 antibody (O14-1) identified the integrin β1 chain in the H36 complex: the other antisera were negative (Fig. 6 A). Immunoblots using extracts prepared from myotubes also indicated integrin β1 chain in these lysates (Fig. 6 B).

The extensive homology between H36-α7 and α6 integrins and the binding of α6β1 to laminin suggested that H36-α7β1 also might bind this extracellular matrix protein. The heterodimer extracted from L8E63 myotubes selectively bound to a laminin column and was eluted with EDTA (Fig. 6 C). There was much less binding of H36-α7 to fibronectin and no association with types I and IV collagen was detected. The H36-α7 that bound and eluted from the laminin column was complexed with β1 integrin (data not shown). These results were confirmed by immunoblots of the material that did not bind to each extracellular matrix protein column. Immunofluorescence localizations using anti-H36 and anti-β1 antibodies indicate both coincident and distinct distributions of these determinants on myoblasts and myotubes.
Figure 4. Nucleotide sequence and deduced amino acid sequence of H36-a7. The DNA sequence of H36-a7 and the corresponding open reading frame of 1106 amino acids are shown. The single transmembrane domain inferred from hydrophobicity analysis is indicated by a solid line. Three potential calcium binding sites in the amino-terminal half of the molecule are enclosed in open boxes. The conserved repeats of glycine/alanine/proline (GAP) found in the amino-terminal half of H36-a7 are indicated in bold. The single transmembrane frame of 1106 amino acids are shown. Two potential asparagine glycosylation sites are indicated by asterisks (*). The two shaded boxes mark the consensus sequences of potential protease cleavage sites. As discussed, cleavage at these sites could generate the polypeptides depicted in Fig. 1C. The presumed polyadenylation recognition sequence, ATTAA, is indicated in bold. DNA Star, Mapseq, and MacVector Protein Subsequence, and Protein Toolbox were used in this analysis. The sequence data are available from EMBL/GenBank/DDJB/Swiss-Prot under accession number X65036.
In mixed cultures of myoblasts and fibroblasts prepared from newborn thigh, localization of H36-α7 is restricted to the myogenic cells whereas β1 integrin is found on both cell types (Fig. 7, A–E). The greatest coincident localization of the two chains on replicating myoblasts appears at the periphery of the cells, although for the most part their distributions are disparate, especially on the upper surface of the cells. Coincidence in staining of H36-α7 and β1 is most evident on the attached surface of fusing myoblasts (Fig. 7, F and G) and newly formed myotubes, especially in those areas where localization of the alpha chain is most linear (Fig. 7, H and J). Many myotubes, especially those that are well developed, are largely devoid of β1 staining, in contrast with H36-α7 which is strongly evident at this stage of development (Fig. 7, D and E). This decrease in β1 localization on myotubes was seen with five distinct antibody preparations, two reactive with the cytoplasmic domain and three with extracellular determinants. Immunoblot analyses confirm that an increase in H36-α7 and decrease in β1 accompanies myogenic differentiation (Fig. 8). An antiserum reactive with the cytoplasmic domain of β1 was used in the experiment shown and identical results were obtained using two additional antisera reactive with the β1 chain. Since integrins are assembled as heterodimers, the absence of more complete coordinate localization of H36-α7 and β1 obtained using antibodies that react with extracellular determinants suggests that the H36-α7 may associate with more than one form of beta chain. These results also indicate that the expression of H36-α7 and β1 integrins are developmentally regulated.

**Developmental Regulation of H36-α7 mRNA Expression**

To determine whether the expression of H36-α7 mRNA is developmentally regulated, poly(A)+ RNAs were purified from L8E63 cells at different stages of development and analyzed by northern blots using random primer labeled clone 05B as probe. H36-α7 mRNA corresponding in size to 4.0 kb was detected and appeared identical in size in myoblasts and a quantitative increase occurs upon terminal differentiation. This increase appears to approximately coincide with the onset of myogenin expression (Fig. 9) and is consistent with immunofluorescence detection of the H36 protein (Kaufman et al., 1985) and immunoblots using H36-05 antibody (Fig. 8). Myosin heavy chain mRNA is expressed relatively late compared to the increase in H36-α7 mRNA.

The amount of H36-α7 mRNA detected in the developmentally defective fu-1 mutant of L8E63 myoblasts is markedly reduced (Fig. 10 A) and these cells also express very little H36-α7 protein as determined by immunofluorescence photometry (Kaufman et al., 1985). Expression of H36-α7 mRNA was also found to accompany the development of H36+ myogenic precursor cells in the day 12 embryonic limb bud into H36+ cells. Immunofluorescence indicates that cultures prepared from embryonic day 12 rat myofibroblasts treated with PDGF were positive for H36-α7 but negative for β1 integrin (Fig. 10 A).

**Figure 5. Alignment of H36-α7, α6, α5, αv, αIIb, and α4 integrin chain sequences.** Alignment of the amino acid sequences of H36-α7 and the integrin alpha chains which have a characteristic protease cleavage site is shown. This common cleavage site (RRRRE, at residues 882–886 in H36-α7) and the homologous sites in the other chains are indicated in shaded boxes, as is a second potential cleavage site (RRQ, at residues 575–577) in H36-α7. The sequence data of integrin alpha chains were obtained from the Swiss Protein database, version 17, with the following accession numbers: α5, fibronectin receptor (p08648); αv, vitronectin receptor (p06756); αIIb, gpIIb (p08514); α4, VLA-4 (p3612); and α6 (Tamura et al., 1990). Paired alignments were initially done using the AAalign program of DNASTAR and the GAP program of University of Wisconsin Genetics Computer Group. The multiple alignment was obtained using the LINEUP program from the Wisconsin Group. The amino acids conserved in all six integrin alpha chains are indicated in bold. The presumed calcium binding sites are enclosed by boxes. The highly conserved sequence in the cytoplasmic domain, GFFKR, is indicated in the box with a dashed line. These integrins all have a single cytoplasmic domain; each begins two residues to the amino terminal side of the GFFKR sequence and each is distinct in length and composition.
Figure 8. Expression of H36-α7 and β1 integrin during L8E63 cell differentiation. Cell lysates prepared from L8E63 cells at different stages of development (Days 2, 3, and 8) were electrophoresed in 8% polyacrylamide SDS gels under nonreduced (−) and reduced (+) conditions and immunoblotted using 05 mAb and an antiserum against the cytoplasmic domain of β1 chains. Fusion was evident on day 4 and extensive by day 8 of culture. The amount of H36-α7 increases and β1 decreases during differentiation.

hindlimb buds do not initially express H36 or desmin but do so upon serial passage in vitro (Kaufman et al., 1991). Likewise, H36-α7 mRNA was not detected in RNA from passage 1 (P1) cells but was present in poly(A)+ RNA from P3 and P4 cells (Fig. 11). As will be discussed, activation of transcription of the H36-α7 gene at this early stage in the myogenic lineage appears to be distinct from later stages of development. The expression of H36-α7 mRNA also parallels the conversion of 10T1/2 cells to myogenic cells. H36-α7 mRNA is not detected in 10T1/2 cells but is present in myotubes derived from myoblasts obtained by azacytidine treatment or transfection of 10T1/2 cells with MRF4 (Fig. 10 C).

Cell Specificity of H36-α7 Integrin

Both hybridization analyses and immunofluorescence indicate that the expression of H36-α7 is highly restricted and developmentally regulated during the development of skeletal muscle. The detection of H36 was first reported to be limited to cardiac and skeletal muscle that were grown in vitro (Kaufman et al., 1985). It has since been used to identify cells at distinct stages in the myogenic lineage both in vivo and in vitro (Kaufman and Foster, 1988; Kaufman et al., 1991). As shown in Fig. 12, H36-α7 also is present in adult skeletal muscle in vivo. H36-α7 has also been detected by immunofluorescence on dorsal root ganglion cells in the developing rat embryo (George-Weinstein, M., manuscript submitted for publication). As noted above, H36-α7 integrin can be detected on myoblasts but not fibroblasts in mixed cultures of hindlimb cells (Fig. 7), in myogenic cells derived from precursors (Kaufman et al., 1991), and in myotubes derived from 10T1/2 cells (Fig. 10 C), further indicating that expression of this protein is highly selective. Northern blot analyses demonstrate that H36-α7 mRNA is expressed in myogenic cells of rat (L8E63 and RMo) and mouse (C2, 23A2, MRF4) origin including the BC3H1 line that does not fuse (Fig. 10). H36-α7 mRNA is detected at reduced levels in human PCI myotube RNA. No H36-α7 mRNA was evident in poly(A)+ RNA from myotubes that developed from primary cultures of chick embryo hindlimbs or from the quail QM7 myogenic line indicating that transcripts with high homology are absent in avian skeletal muscle. H36-α7 mRNA was not detected in either mouse 10T1/2 cells or BHK cells (Fig. 10).

Discussion

Molecular cloning and sequencing identify H36 as a new integrin α chain, α7. H36-α7 shares extensive features and homologies common to all known α chains, especially the members of the non-I region subgroup that also...
have a characteristic protease cleavage site. Amongst these, 
H36-α7 most closely resembles α6 integrin.

Like all the integrin alpha chains, H36-α7 has a unique cytoplasmic domain. This portion of the molecule contains the sequence GFFKR that is characteristic of all known alpha chains. The remainder of the cytoplasmic domain (with exception of residues 1081-1083, SDA) is distinct. The interactions of integrin αβ heterodimers with extracellular matrix proteins, with other cell surface ligands, with complement, and perhaps with additional molecules may transmit or initiate signals between and within cells, promote cell movement, or in other cases, cell localization. It is generally believed that the transduction of such signals is mediated through interactions of the integrins with the cell cytoskeleton and perhaps with other cytoplasmic components as well. Some molecules involved in these interactions, for example talin and vinculin, have been identified (Horwitz et al., 1986; Singer and Paradiso, 1981). The role of beta chains in the interactions of the integrins with the cytoskeleton seems preeminent (Hibbs et al., 1991) and phosphorylation of specific residues appears to have a regulatory role (Hirst et al., 1986; Dahl and Grabel, 1989; Hillery et al., 1991; Horvath et al., 1990). The functions of the cytoplasmic domains of the integrin alpha chains have not yet been established. Since the association of the H36-α7 complex with the cell cytoskeleton can be promoted by cross-linking its extracellular domains with antibodies (Lowrey and Kaufman, 1989), it is reasonable to predict that ligands in vivo may also have this effect. Laminin may be one such ligand. The unique size and sequence of the H36-α7 cytoplasmic domain suggests that it may participate in novel molecular interactions. Unlike most of the other integrin alpha and beta cytoplasmic domains, there are no potential sites for phosphorylation in the H36-α7 cytoplasmic domain. In contrast, numerous sites of potential phosphorylation and four potential glycosylation sites exist in the extracellular domain (Fig. 4).

The regulation of expression of the H36-α7 integrin chain during the development of skeletal muscle appears to be complex. During development of the myogenic lineage the expression of H36-α7 is regulated at least twice, by apparently distinct mechanisms (Kaufman and Foster, 1988, 1989; Kaufman et al., 1991). During early limb bud formation...
Figure 12. Immunofluorescence detection of H36-α7 integrin in adult thigh muscle. Cryosections prepared from adult rat thigh muscle stained with anti-H36 antibody and fluorescein anti-mouse IgG (A and C). (A and B) cross sections; (C and D) longitudinal sections. Bar, 26 μm.

Expression of H36-α7 is highly select. Immunofluorescence analysis of a variety of cells grown in vitro (including primary cultures of skeletal muscle fibroblasts, cardiac endothelial cells, liver and kidney cells, brain glial cells and neurites, and stomach and aorta smooth muscle) detected H36 only on skeletal and cardiac muscle (Kaufman et al., 1985). This has since been confirmed using immunoblots (unpublished data). H36-α7 expression in the developing rat embryo likewise appears highly select and developmentally regulated as shown by immunofluorescence staining of cryosections prepared from day 12 to 18 rat embryos (Kaufman et al., 1991). In contrast, the increased expression of H36-α7 that takes place during terminal differentiation of secondary myoblasts (Fig. 9) is accompanied by expression of myogenin. Inhibition of terminal myogenesis by a variety of means including incorporation of BrdUrd, growth in low concentrations of calcium, by an α-amanitin sensitive mutation in RNA polymerase II, or by expression of the ras, src, or SV40 large T antigen oncogenes, all inhibit the increased expression of H36-α7 without altering its basal level of expression (Kaufman et al., 1985, 1990; Haider and Kaufman, manuscript in preparation). These results further indicate that expression of H36-α7 at different stages of myogenesis is regulated by distinct mechanisms.
associate with more than one isoform of beta chain. The
immunofluorescence staining of beta chain on myocytes corresponds with the loss of membrane-associated fibronectin (Hynes et al., 1976; Chen, 1977) and with the increased synthesis (Olwin and Hall, 1985) and assembly of laminin on the myotube surface (Kuhl et al., 1982; Sanes and Lawrence, 1983). A complete analysis of integrin alpha and beta chain expression and their association with specific extracellular matrix proteins is needed to give a full account of what appears to be complex developmental regulation of alpha and beta chains during myogenesis.

The role of integrins during myogenesis may be diverse as they can mediate the interactions of cells with extracellular matrix proteins, with other cells and with additional molecules, such as complement (for reviews see Akiyama et al., 1990; Albeda and Buck, 1990; Hemler, 1990; Humphries, 1990). Cell surface attachment (CSAT) antibody (i.e., anti-beta) and other anti-integrin antibodies can detach chick myoblasts from the in vitro culture substrate (Neff et al., 1982; Greve and Gottlieb, 1982; Horwitz et al., 1985). Growth of myoblasts in vitro on laminin, in contrast to growth on collagen or fibronectin, will selectively maintain myoblast proliferation in vitro, and thereby influence in a quantitative way the extent of myogenesis (Foster et al., 1987; Ocalan et al., 1988). The appearance of laminin during the development of the limb in vivo also corresponds with the appearance of secondary myoblasts that express H36-alpha7 (Weinstein, M., manuscript submitted for publication) and with the onset of increased muscle mass. This coincidence in the expression of H36-alpha7 on secondary myoblasts, its binding to laminin and the consequent effect on myoblast proliferation strongly suggest that H36-alpha7 modulates this stage of myogenesis in vivo through cell-extracellular matrix interactions. Myogenesis may also be inhibited by CSAT antibody (Menko and Boettiger, 1987), by growth of myogenic lines on laminin (Foster et al., 1987), and by addition of fibronectin to these cells (Podleski et al., 1976), further indicating that extracellular matrix receptor occupancy can profoundly regulate myogenic differentiation. In addition, mutant myoblasts (for example, fu-1 cells; Fig. 10) that express reduced levels of H36-alpha7 do not differentiate, have often lost the normal control of replication that is essential to myogenesis, and are tumorigenic (Kaufman and Parks, 1977; Kaufman et al., 1980). Other myoblasts, selected on the basis of low expression of H36-alpha7 fail to interact with each other and do not fuse to form myotubes, but do biochemically differentiate (unpublished data). These results further imply a role for H36-alpha7 in cell to cell and extracellular interactions during muscle differentiation. The integrins on skeletal muscle fibers may also play a pivotal role in assembly of the myofibril. Drosophila embryos with the lethal myospheroid mutation do not express beta9 integrin, undergo defective myogenesis and form defective Z-bands (Volk et al., 1990). The localization of CSAT antibody at myotendinous and neuromuscular junctions suggests that integrins also stabilize interactions between the sarcolemma and extracellular matrix as well as neuromuscular junctions (Bozyczko et al., 1989).

Non-integrin extracellular matrix binding proteins may also mediate interactions with muscle (Clegg et al., 1988). The expression of H36-alpha7 at different times during the formation of primary and secondary fibers and its persistence on adult muscle (Fig. 12) suggests that this alpha chain may have different functions at different stages of muscle development.

Since the submission of this manuscript, Kramer et al. (1991) reported the sequence of the first 25 residues at the amino terminal end of an integrin isolated from mouse melanoma cells and von der Mark et al. (1991) reported the sequence of 17 amino acids in an integrin expressed by the Rugli line of rat myoblasts. These sequences are identical to that in H36-alpha7 and both these proteins associate with beta1 chains and bind laminin. Based on these similarities it is likely that these proteins are related or identical to the H36-alpha7 integrin chain reported here.

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