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Laminins promote the locomotion of skeletal myoblasts via the alpha 7 integrin receptor

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SUMMARY

The α7β1 integrin is specifically expressed by skeletal and cardiac muscles, and its expression and alternative mRNA splicing at the cytoplasmic domain are developmentally regulated. We analyzed the role of α7 integrin in mediating myoblast adhesion and motility on different laminin isoforms. Mouse C2C12 and MM14 myoblast cell lines were found by flow cytometry and immunoprecipitation to express high levels of the α7 integrin. Overall expression of α7 increased as the C2C12 myoblasts differentiated; myoblasts expressed only the α7B cytoplasmic variant whereas in differentiating myotubes α7A increased markedly. Function-perturbing monoclonal antibodies generated to α7 integrin efficiently blocked both adhesion and migration of MM14 and C2C12 mouse myoblasts on laminin 1. Other studies with MM14 myoblasts showed that α7 is also a receptor for laminin 2/4 (human placental merosins) but not for epithelial-cell-specific laminin 5. Blocking antibody to α7 only partially inhibited adhesion to laminin 2/4 but almost completely blocked motility on this substrate. Finally, to assess the potential role of the α7 cytoplasmic domain, CHO cells were stably transfected to express chimeric α5 cDNA constructs containing the wild-type α5 or the α7A or α7B cytoplasmic domain; all forms of the integrin showed identical activities for adhesion, migration, proliferation, and matrix assembly on fibronectin substrates. These results established that α7β1 receptor can promote myoblast adhesion and motility on a restricted number of laminin isoforms and may be important in myogenic precursor recruitment during regeneration and differentiation.

Key words: α7 Integrin, Laminin, Myoblast, Adhesion, Motility

INTRODUCTION

Laminins are a major component of the basement membrane that surrounds individual skeletal muscle myofibers and are important ligands that promote diverse cellular responses. Interaction of cell adhesion receptors with laminin-rich basement membrane is important in maintaining normal tissue organization and in tissue renewal and repair. Recent studies indicate there are at least 10 trimeric isoforms of laminin formed from different combinations of α, β, and γ subunit chains (Timpl and Brown, 1994). Furthermore, the different laminin isoforms are tissue-specific and are expressed in a developmentally regulated pattern. There are at least three different laminin isoforms located at different domains on skeletal muscles: laminin-2 (merosin: α2, β1, and γ1) between muscle fibers; laminin-3 (s-laminin: α1, β2, and γ1) at neuromuscular junctions; and laminin-4 (s-merosin: α2, β2, and γ1) at myotendinous junctions (Engvall et al., 1990). However, the functional importance of this distribution and what determines the differential distribution of laminin isoforms are still unknown.

It has been shown that laminin 1 specifically stimulates rodent myoblast proliferation and locomotion (Goodman et al., 1989; Ocalan et al., 1988) and also promotes myogenesis in cultured skeletal myoblasts (Foster et al., 1987; von der Mark and Ocalan, 1989). In addition to laminins, other ECM components and their receptors, as well as specific growth factors, play an important role in regulating myoblast proliferation and differentiation (Sastry et al., 1996; McDonald et al., 1995). Different cell types may have a special biological response to laminin, and this may result from the specific cellular environment and the different repertoire of integrin receptors expressed by the individual cells (Chan et al., 1992). At least eight members of the integrin family, α1β1, α2β1, α3β1, α6β1, α7β1, αvβ3, and α6β4, have to date been reported to bind different laminin isoforms. Several α subunits associated with β1 are present in muscle tissue including α1, 3, 4, 5, 6, 7 and 9 (Bronner-Fraser et al., 1992; Duband et al., 1992; Enomoto et al., 1993; George-Weinstein et al., 1993; Hirsch et al., 1994; Lakonishok et al., 1992; McDonald et al., 1995; Steffensen et al., 1992). It has been shown that anti-β1 antibody inhibits myoblast migration on laminin (von der Mark et al., 1991) and it has been suggested that α7β1 integrin directs motility of satellite-cell-derived myoblasts along laminin-rich basement membranes during skeletal muscle regeneration (von der Mark et al., 1991).

In the present study, we generated monoclonal antibodies against α7 integrin to study its distribution and function. We found that on laminin 1 substrates, myoblasts attached and spread rapidly and α7 integrin became concentrated in focal adhesion sites. Function-perturbing antibodies against the α7 integrin blocked myoblast adhesion to laminin 1. Moreover, α7β1 integrin bound not only to laminin 1 but also to a mixture of laminin 2 and 4. α7 did not bind efficiently to laminin 5. Finally, we examined whether chimeric constructs of the α5 extracellular domain joined with the cytoplasmic A or B variant domains of α7 could differentially contribute to cellular events in transfected cells.

MATERIALS AND METHODS

Cell culture and reagents

Rat-2 cells (Topp, 1981) were grown in DMEM H-21 with 10% FBS (Cell Culture Facility, University of California, San Francisco, CA). The C2C12 mouse myoblast cell line (kindly provided by Dr Rik Derynck, University of California, San Francisco, CA) was maintained in DMEM H-21 with 20% FBS (Yaffe and Saxel, 1977). C2C12 cells were induced to differentiate into myotubes by growing in DMEM with 2% horse serum. The MM14 mouse myoblast cell line (kindly provided by Dr Stephen Hauschka, University of Washington, Seattle, WA) was maintained in Ham's F10 medium ($CaCl_2$ adjusted to 1.2 mM) with 15% horse serum and 2 ng/ml basic fibroblast-growth factor (bFGF) (Campbell et al., 1995). The CHO α5β1-integrin-deficient B2 variant cell line (Bauer et al., 1993) was kindly provided by Dr Rudolph Juliano (University of North Carolina, Chapel Hill, NC) and maintained in MEM α without nucleosides with 10% FBS.

Laminin 1 was purified from mouse EHS tumor as previously described (Kramer et al., 1991). Human placental laminin (a mixture of laminin 2 and 4: Delwel et al., 1994; Spinardi et al., 1995) was purchased from Gibco-BRL (Gaithersburg, MD). Purified human laminin 5 was kindly provided by Dr Robert Burgeson (Cutaneous Biology Research Center, Boston, MA). Human plasma fibronectin was purchased from Collaborative Biomedical Products (Bedford, MA).

Antibodies against integrin subunits included hamster anti-mouse α1 monoclonal antibody (mAb) Ha31/8, anti-mouse α2 mAb Ha1/29 and anti-mouse β1 mAb Ha2/11 (Mendrick et al., 1995); rabbit anti-mouse α3 cytoplasmic region antiserum, kindly provided by Dr Hannu Larjava (University of British Columbia, Vancouver, Canada); rat anti-human α5 mAb B2G2, kindly provided by Dr Caroline Damsky (University of California, San Francisco, CA), and mouse anti-human α5 mAb 6F4, a kind gift from Dr Ralph Isberg (Tufts University, Boston, MA). Rat anti-mouse α4 mAb R1-2, rat anti-mouse α5 mAb IIA1 and rat anti-mouse β4 mAb 346-11A were purchased from Pharmingen (San Diego, CA); rat anti-human α6 mAb GoH3 was purchased from AMAC (Westbrook, ME). The rabbit polyclonal antibodies (pAb) 22780 and 1211 were against peptide sequences specific to the α7A cytoplasmic region (NSPSSSFRTNYHR) and to the α7B cytoplasmic region (GTIQRSNWGNSQWEGSDAH), respectively, as described (Martin et al., 1996; Yao et al., 1996). Mouse mAb against vinculin (hVIN-1) was purchased from Sigma (St Louis, MO). Fluorescein-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Streptavidin-HRP and ECL kit were purchased from Amersham (Arlington Heights, IL). All reagents were purchased from Sigma (St Louis, MO) unless specified.

Production of monoclonal antibodies to mouse α7

Mouse α7 X2B cDNA (Ziober et al., 1993) cloned in a RSV expression vector (Invitrogen, San Diego, CA) was transfected into Rat-2 cells by using the calcium phosphate method (Mammalian Transfection Kit from Stratagene, La Jolla, CA). Individual clones were isolated and analyzed by northern blotting, western blotting and enhanced adhesion to laminin substrates. Two clones (6 and 20) that were high expressors of mouse α7 were selected and used for mAb production in syngeneic Fisher rats. Rats were injected with live cells (5×10^6) once, either intraperitoneally or subcutaneously. Titers of tail bleeds were monitored by FACS using α7 MCF-7 transfectants; the MCF-7 transfectants were generated by transfecting with the mouse α7 X2B cDNA in a CMV promoter construct (Yao et al., 1996). The fusions were performed as described (Harlow and Lane, 1988) with polyethylene glycol 1500 (from Boehringer Mannheim, Indianapolis, IN) using isolated spleen cells and Sp2/0 mouse myeloma cells at the ratio of 3:1. The cells were plated in 96-well tissue culture plates in Opti-MEM medium (from Gibco BRL, Gaithersburg, MD) supplemented with 20% heat-inactivated fetal bovine serum, thymidine, adenine, Na-pyruvate, glycine, glutamine, oxaloacetate, hypoxanthine, insulin, transferrin, sodium selenite, Mito+ serum extender (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) and IL-6 (Gibco BRL). Hybridoma supernatants were screened by using whole-cell ELISA on α7-expressing clone 23 of K1735 mouse melanoma cells. Positive clones were further screened by immunoprecipitation of lysates from biotin surface-labeled K1735 melanoma cells. Screening for function-perturbing antibodies was performed in adhesion assays for α7 MCF-7 transfectants on laminin-1 substrates. Selected hybridomas were further subcloned by limiting dilution and were subtyped by using an isotyping system for rat immunoglobulins (Gibco BRL). Monoclonal antibodies were purified from hybridoma conditioned medium by passage over a Protein G-Sepharose 4 Fast Flow column (Pharmacia, Uppsala, Sweden) and elution with 0.1 M glycine-HCl, pH 2.7. Rat mAbs CA5 (IgG2b), CY4 (IgG2b) and CY8 (IgG2a) were found positive against α7 integrin for immunoprecipitation and FACS; CY4 and CY8 were function-perturbing mAbs that inhibited α7-integrin-mediated adhesion on several cell types.

Production of monoclonal antibodies to cytoplasmic regions of α7 integrin

Mouse mAb 3F4 (IgG2b) was generated in our laboratory against a peptide sequence of the α7B cytoplasmic region GTIQRSNWGNSQWEGSDAH coupled to cationized BSA (Pierce, Rockford, IL). Details of all procedures have been described (Harlow and Lane, 1988). Briefly, the conjugated peptide was injected into A/J mice intraperitoneally with complete Freund's adjuvant once, and then with incomplete Freund's adjuvant three times with 2 weeks between injections. Serum titer was monitored by ELISA. The final booster was injected with peptide solution in PBS intravenously 3 days before fusion. After fusion was performed, the supernatants of hybridomas were screened by ELISA and positive clones were verified by immunoprecipitation of myoblasts and of MCF-7 α7B transfectants. The hybridoma subtyping was performed with Isostrip (Boehringer Mannheim).

Flow cytometry

Subconfluent cells were detached with 2 mM EDTA. Single-cell suspensions (10^6/ml) were incubated with optimal concentrations of primary mAb in wash buffer (2% normal goat serum in PBS) for 1 hour on ice, washed three times, and incubated with the goat anti-rat or anti-mouse secondary fluorescein-labeled antibodies for 30 minutes on ice. After washing again three times, the cells were stained with propidium iodide (1 μg/ml) to identify non-viable cells. Flow cytometry was performed on a FACscan flow cytometer (Becton Dickinson). Control samples consisted of cells with or without secondary antibody binding. Any non-viable cells stained with propidium iodide were eliminated from the analysis.

Immunoprecipitation of surface biotin-labeled cells

Confluent cultures of cells were washed twice with PBS and then labeled with NHS-LC-Biotin (Pierce), 1 mg/ml in cold PBS at 4°C for 90 minutes. Cells were washed twice with 50 mM glycine blocking buffer and incubated in this buffer for 10 minutes at 4°C. The cells were lysed in lysis buffer (PBS with 0.1 M Tris, pH 7.5, 2% NP-40, 2 mM PMSF and 1 mM *N*-ethylmaleimide). After preclearing with Protein A beads, the lysate was mixed by rotation for ≥3 hours with primary antibody and Protein A beads. The beads were washed with the wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% NP-40, 0.1% BSA) three times and heated at 100°C in SDS sample buffer for 5 minutes. The supernatant was divided into two aliquots: one for non-reducing samples and one for reducing with 2-mercaptoethanol. Samples were separated by 7.5% SDS-PAGE under reduced and non-reduced conditions. The biotinylated proteins were detected by streptavidin-horseradish peroxidase and then enhanced chemiluminescence (ECL).

Immunofluorescence staining

Cells were seeded on laminin-1 coated coverslips for 1 hour, washed with PBS and fixed with 2% paraformaldehyde in PBS. Cells were permeabilized with 0.4% Triton X-100 for 5 minutes and blocked with 10% normal goat serum in PBS for at least 1 hour. Samples were incubated with primary antibodies for 1 hour at room temperature, washed with PBS, and then incubated with FITC- or Rhodamine-labeled anti-rat or anti-mouse IgG for 1 hour at room temperature. After washing with PBS, the samples were mounted in Vectashield (Vector) and observed under a Nikon epifluorescence microscope.

Cell adhesion assay

Microtiter plates (96-well Immulon plates, Dynatech) were coated with matrix proteins at the indicated concentrations in PBS for 1 hour at 37°C in a humidified atmosphere. Plates were washed with PBS and incubated with medium containing 0.1% BSA for 60 minutes in a CO_2 incubator to block nonspecific adhesion. Single-cell suspensions were prepared in DMEM with 0.1% BSA at 4×10^5 cells/ml, added in triplicate to 96-well plates, and then incubated for 40-90 minutes at 37°C. Non-adherent cells were removed by shaking on a titer plate shaker (Lab-line Instruments) and washing with PBS. Cells were fixed with 1% formaldehyde, stained with 1% crystal violet, and solubilized in 2% SDS, and absorbance was then read at 562 nm. Cells bound to wheat germ agglutinin (10 μg/ml) or collagen type I (100 μg/ml) on a separate 96-well plate were used to indicate 100% attachment. Background cell adhesion to 1% BSA-coated wells was subtracted. The effect of specific antibody was tested by pre-incubating the cells with the hybridoma supernatants or dilutions of purified antibody on ice for 30 minutes prior to the assay.

Migration assay

Cell migration was assayed in a modified Boyden chamber (Neuroprobe, Bethesda, MD) as described previously (Matsumoto et al., 1994). Briefly, an 8-μm-porosity polyvinylpyrolidone-free polycarbonate filter (Nucleopore, Pleasanton, CA) was precoated with ligand at the indicated concentration. The lower well of the chamber was filled with serum-free medium containing 0.1% BSA. In some studies, the lower chamber contained medium with or without bFGF as indicated. Cell suspensions were prepared from subconfluent cultures and resuspended to a final concentration of 4×10^5 cells/ml in serum-free medium containing 0.1% BSA. A 50 ml aliquot of cell suspension was added to the upper chamber and then incubated for the indicated time at 37°C. Cells on the top of the filter were removed by wiping, and the filter was then fixed in 1% formaldehyde in PBS. Migrating cells were stained with 1% crystal violet, and nine randomly chosen fields from triplicate wells were counted at ×400 magnification.

Generation of α5/7 integrin chimeras

Full-length human α5 cDNA in PECE expression vector was obtained from Dr Erkki Ruoslahti (Cancer Research Center, La Jolla). The *Sal*I-*Xba*I fragment was cloned into Bluescript plasmid (Stratagene). The *Hin*dIII-*Xba*I cytoplasmic region was deleted and replaced with PCR-generated α7A or α7B cytoplasmic fragments designed to have cohesive *Hin*dIII-*Xba*I ends. The cytoplasmic fragments were amplified by using DNA templates from reverse transcription of C2C12 myotube RNA with primer sets J1 (5′AATCTAGACCCAAGGAGCCATCTTGGAA3′) and J36 (5′CCCCCAAGCTTGGCTTCTTCCGTCGGAAC3′) for α7A and primer sets J1 and J37 (5′CCCCCGGGCTTGGATTCTTCAAGCGGGCG3′) for α7B for 20 cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds). In order to generate an AAGCTT *Hin*dIII site for cloning purposes, a cysteine was replaced by a leucine at the α7A cytoplasmic domain near the transmembrane region (from KCGFFRR to KLGFFRR...). The α7B amino acid sequence at the cytoplasmic region remained unchanged (KLGFFKR...). The new cDNA constructs were sequenced through the altered regions. The chimeric human α5-mouse α7 constructs were further cloned into pRc/CMV expression vectors (Invitrogen).

Transfection and selection of CHO α5-deficient variants

For transfection with α5/7 chimeric constructs, a total of 6×10^6 CHO B2 variant cells were resuspended in 0.8 ml Ca^{2+}/Mg^{2+}-free PBS and electroporated with 10 μg *Pvu*I linearized cDNA at 25 μF, 600 V. Stable transfectants were selected in MEM α without nucleosides with 10% FBS and 1 mg/ml G418. A panning procedure was devised for enrichment of positive α5 expressers. Dishes were precoated with goat anti-mouse IgG (3 μg/ml in 50 mM Tris-HCl, pH 9.5, at 4°C overnight) and then nonspecific binding was blocked with 1% BSA in PBS for 1 hour at room temperature. After detachment with 2 mM EDTA, cells were incubated in mAb 6F4 (mouse anti-human α5), 5 μg/ml in PBS containing 2% FBS for 45 minutes on ice. Then cells were plated onto antibody-coated dishes at 37°C and incubated for 30 minutes. Floating cells were removed by gentle washing with PBS containing 2% FBS. The cells remaining on the dish were expanded in medium containing G418. This ‘panning’ was repeated a second time after 3 days, and the resultant cells were sorted using 6F4 antibody and FITC-labeled goat anti-mouse secondary antibodies. Typically, nearly 60% of the population were positive. Sorted cells were expanded in medium with 1 mg/ml G418, and aliquots of these low-passage cells were stored frozen. For experiments, cells from frozen stocks were cultured and the expression level of α5 was measured by FACS; assays were done within 2 weeks. Expression levels of α5 in the transfectants were confirmed by immunoprecipitation with both mAb to α5- and α7 cytoplasmic-specific antisera.

Cell growth assays

Cells were plated at 2,000 cells/well in four 96-well tissue culture plates in triplicate. Four conditions were applied to each cell line: low-serum or high-serum supplement with or without fibronectin substrates. For each plate, wells in half of the plate were precoated with 10 μg/ml fibronectin for 1 hour before seeding. Cells were divided and resuspended in 10% serum or in 0.2% serum and seeded onto wells with or without fibronectin coating. Two hours after seeding and on days 2, 3 and 4, plates were washed and fixed with 1% formaldehyde in PBS. On day 4 the cells were stained with 1% crystal violet, and solubilized in 2% SDS and absorbance was then read at 562 nm.

Fibronectin matrix assembly assays

Transfectants were plated at approximately 40% confluence in Lab-Tek 8-chamber culture slides (Nunc) in medium containing 50 μg/ml of purified human fibronectin. On day 3, the cell monolayer was fixed with 1% formaldehyde and stained with a rabbit anti-fibronectin antiserum followed by fluorescein-conjugated goat anti-rabbit IgG

antibodies as previously described (Wu et al., 1995). The cells were observed under a Nikon epifluorescence microscope.

RESULTS

MM14 and C2C12 myoblasts express a similar repertoire of laminin-binding integrins

Laminin induces myoblast motility and subsequently promotes differentiation and myogenesis (Ocalan et al., 1988; von der Mark and Ocalan, 1989). To identify the specific integrins responsible for the laminin-induced response, we first identified the repertoire of integrins expressed on the two murine myoblast lines, MM14 and C2C12. Both cell lines were derived from adult muscle satellite cells, express muscle-specific markers, are competent for differentiating into myotubes and can be incorporated into functional muscle fibers after transplantation in vivo (Linkhart et al., 1981; Yaffe and Saxel, 1977). The MM14 cells have been reported to exhibit different migratory phenotypes on laminin verses fibronectin substrates (Ocalan et al., 1988; von der Mark and Ocalan, 1989), and the C2C12 cells have been widely used for studies of myoblast differentiation and the fate of myoblast transplantation (Blau and Hughes, 1990; Pavlath et al., 1989).

Flow cytometry using rodent-specific mAbs indicated a limited number of integrin α chains on the two myoblast cell lines (Fig. 1). For both MM14 and C2C12 cells, we found no detectable α1 or α2 integrins, but significant levels of α5 and α6 were present. For analysis of α7 expression, several anti-α7 extracellular domain-specific mAbs were generated. The approach was to use a syngeneic system of rat-2 cells that were transfected with the mouse α7 cDNA. These transfectants, when used as immunogen in syngeneic Fisher rats, generated a specific immune response and resulted in at least six different anti-α7 mAb-producing hybridomas, several of which (CY4, CY8) exhibited potent function-blocking activity. For flow cytometry, the CY8 anti-α7 mAb was used (Fig. 1) and the result showed that both MM14 and C2C12 myoblasts express high levels of α7. For both cell lines, α7 appeared to be the predominant α chain integrin.

Integrin expression by the MM14 and C2C12 myoblasts was also analyzed using immunoprecipitation of surface-biotinylated cells (Fig. 2A and B). While the efficiency of biotinylation between the integrin chains may vary, significant levels of α1, α2, or α4 were not detected in the immunoprecipitates from either myoblast cell line. Consistent with the capacity of MM14 and C2C12 cells to adhere to fibronectin (Goodman et al., 1989 and unpublished results), moderate levels of α5β1 were present (Fig. 2A and B, lane 6). The immunoprecipitation pattern confirmed the high expression of α7 which migrates with its characteristic M_r 35,000 and M_r 70,000 cleavage products after reduction (Fig. 2A and B, lane 8). In addition, significant α6-immunoreactive material was immunoprecipitated from both cell lines. While the C2C12 cells expressed the α6β1 heterodimer, the MM14 cells displayed a unique α6-positive pattern with a M_r 200,000 band that was not present in the C2C12 myoblast immunoprecipitates (Fig. 2A and B, lane 7). Additional experiments established that this band represented the β4 subunit that paired with the α6 chain (Fig. 2C). Both anti-α6 (GoH3) and anti-β4 (346-11A) mAbs immunoprecipitated the same complex of surface-biotinylated MM14 cell lysates (Fig. 2C, lanes 1, 2, 4, and 5). Furthermore, after the lysate was exhaustively precleared with anti-β1 pAb, the unique β4 band still could be immunoprecipitated with GoH3 mAb (Fig. 2C, lanes 3 and 6). The abundance of the α6β4 integrin suggested that only a small fraction of α6 subunit is complexed with β1 in the MM14 cells. In contrast, β4-reactive material was never detected in the C2C12 myoblasts.

Since no extracellular-domain-specific mAb for mouse α3 is available, we estimated α3 integrin levels by immunoprecipitation with pAb specific for the α3 cytoplasmic domain. Only moderate amounts of α3 were present in either cell type (Fig. 2A and B, lane 4). Therefore, C2C12 and MM14 myoblasts have a similar limited repertoire of integrins on their cell surface, with α7 as the predominant receptor.

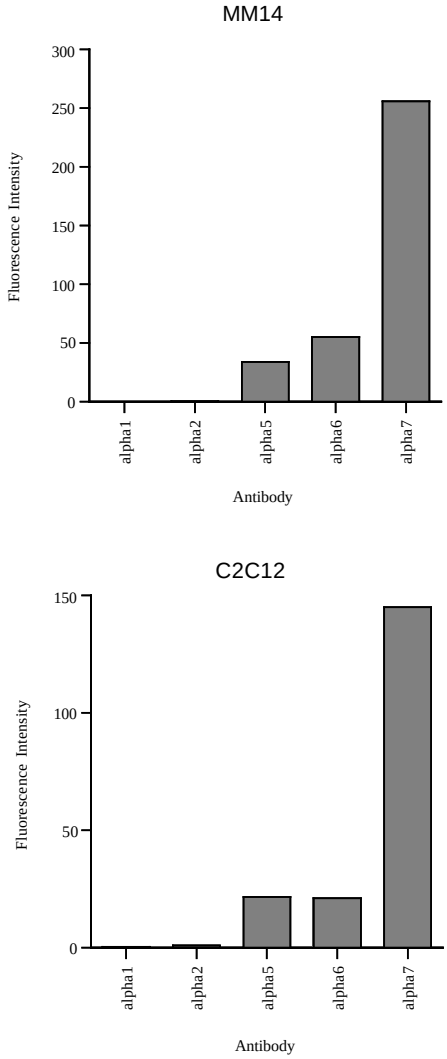

Fig. 1. Cell surface expression of integrin subunits on MM14 and C2C12 myoblasts. Flow cytometry analysis of the MM14 and C2C12 myoblasts was performed with optimal concentrations of mAbs Ha1/29 (anti-α1), Ha31/8 (anti-α2), IIA1 (anti-α5), GoH3 (anti-α6) and CY8 (anti-α7), followed by incubation with FITC-labeled goat anti-hamster (for Ha1/29 and Ha31/8) or goat anti-rat (for IIA1, GoH3 and CY8) IgG. Values from controls of secondary antibody alone were subtracted to give the mean fluorescence intensity.

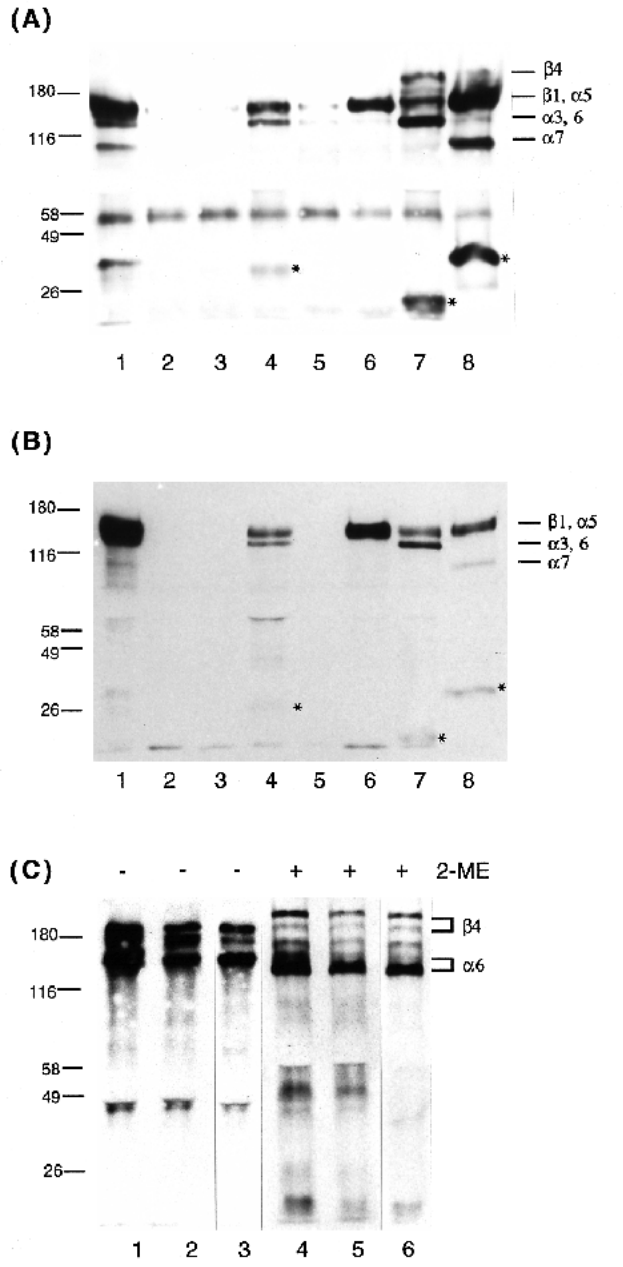

Fig. 2. Association of α6 integrin with β4 subunits in MM14 myoblasts. (A) Surface-biotinylated MM14 myoblast or (B) C2C12 myoblast lysates were immunoprecipitated with rabbit pAb anti-β1 (anti-β1, lane 1), mAb Ha31/8 (anti-α1, lane 2), mAb Ha1/29 (anti-α2, lane 3), pAb anti-α3 (lane 4), mAb R1-2 (anti-α4, lane 5), IIA1 (anti-α5, lane 6), GoH3 (anti-α6, lane 7) or CY8 (anti-α7, lane 8). (C) Surface-biotinylated MM14 myoblast lysates were directly immunoprecipitated with anti-α6 mAb GoH3 (lanes 1 and 4) or anti-β4 mAb 346-11A (lanes 2 and 5) or sequentially immunoprecipitated with rabbit pAb against β1 followed by anti-α6 mAb GoH3 (lanes 3 and 6). Immunoprecipitates were resolved by 7.5% SDS-PAGE under reducing (2-ME +) for A and B and under either non-reducing (2-ME –) or reducing (2-ME +) conditions for C and transferred to Immobilon-P membranes. Proteins were visualized by incubation with streptavidin conjugated to horseradish peroxidase and then detected by ECL. Size standards are shown at left (kDa). Cleavage products of α3, α6, and α7 subunits upon reduction are marked (*).

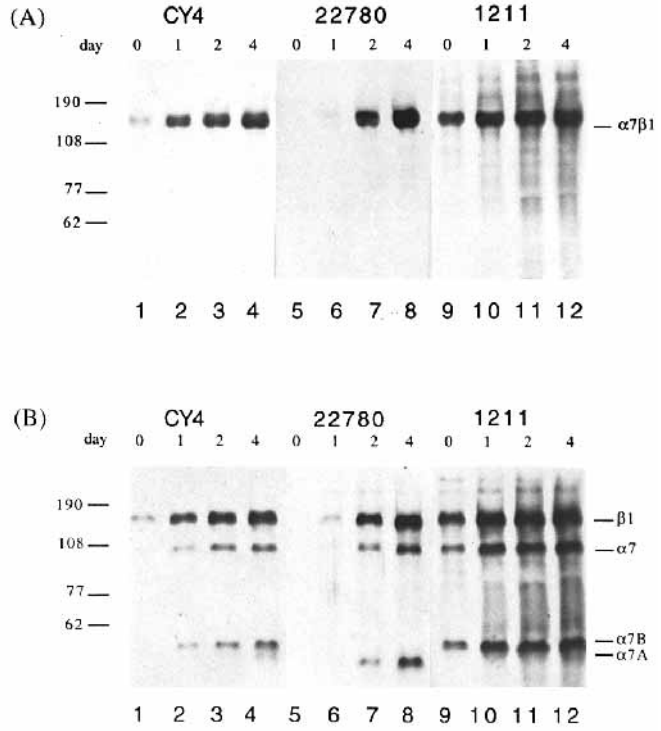

Fig. 3. Immunoprecipitation analysis of α7 cytoplasmic domain isoforms on C2C12 myoblasts during differentiation to myotubes. C2C12 cells were incubated in differentiation medium for 0 (lanes 1, 5, 9), 1 (lanes 2, 6, 10), 2 (lanes 3, 7, 11), and 4 days (lanes 4, 8, 12). Substantial amounts of myotubes were formed after 3 days in differentiation medium. Surface-biotinylated lysates were immunoprecipitated with either anti-α7 extracellular domain mAb CY4 (lanes 1-4), anti-α7A cytoplasmic domain pAb 22780 (lanes 5-8), or anti-α7B cytoplasmic domain pAb 1211 (lanes 9-12). Immunoprecipitates were resolved by 7.5% SDS-PAGE under both non-reducing (A) and reducing (B) conditions and transferred to Immobilon-P membranes. Proteins were visualized by incubation with streptavidin conjugated to horseradish peroxidase and then detected by ECL. Size standards (kDa) are shown on the left and the migration positions of the integrin chains are shown on the right. Note that following reduction, the α7 subunit is cleaved into its heavy and light chain fragments.

Expression of α7A and α7B integrin isoforms is differentiation-dependent

RT-PCR analysis previously suggested that alternative mRNA splicing occurs at the cytoplasmic domain of α7 integrin in skeletal muscle (Collo et al., 1993; Song et al., 1993; Ziober et al., 1993). We analyzed the levels of α7 and its A and B alternatively spliced cytoplasmic isoforms that are expressed at the surface of C2C12 cells at different stages of their differentiation from myoblasts to myotubes, using surface biotinylation and immunoprecipitation with specific pAb and mAb (Fig. 3A,B). A third potential alternatively spliced isoform, α7C, has been identified (Song et al., 1993). However, this highly truncated cytoplasmic isoform appears to be expressed at relatively low levels in myoblasts (Song et al., 1993) and was not evaluated in the current study. Immunoprecipitation of cell lysates with CY4, a mAb generated against the extracellular domain of α7, yielded the α7 subunit, expression of which increased greatly following myoblast differentiation to

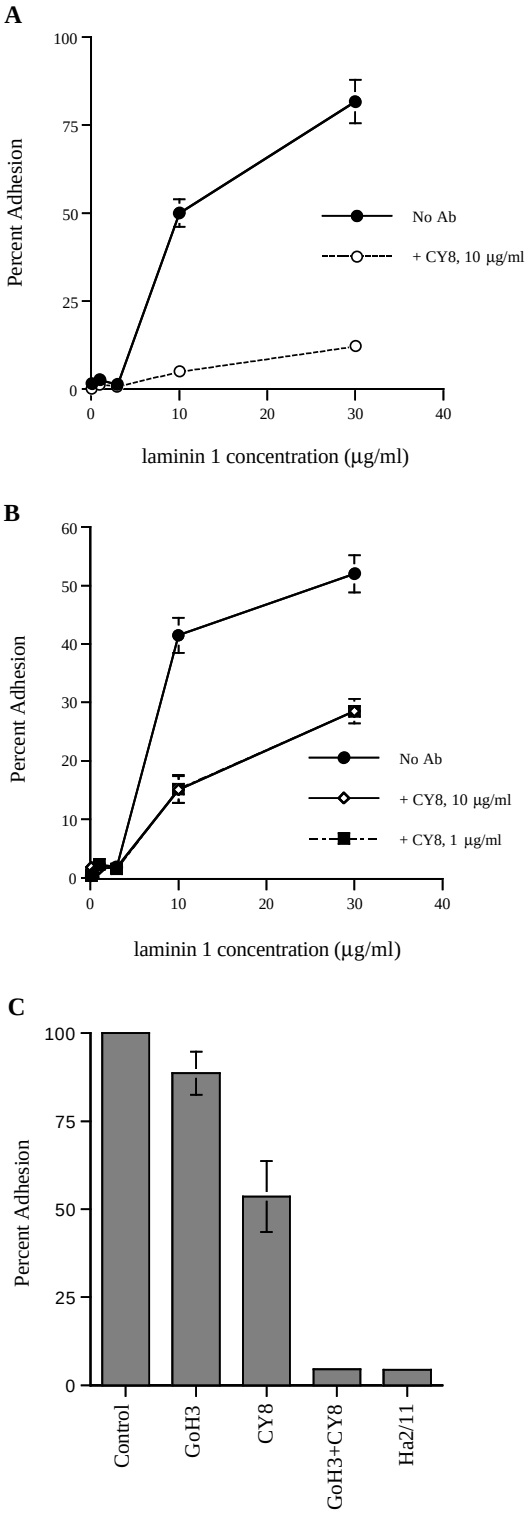

Fig. 4. Inhibition of myoblast adhesion on laminin 1 by function-perturbing mAbs to α7. MM14 cells (A) or C2C12 cells (B) were pre-incubated with mAb CY8 (anti-α7) for 30 minutes on ice, added to laminin-1-coated plates in triplicate, and allowed to adhere to the substrate at 37°C for 50 minutes as described in Materials and Methods. Two different concentrations of CY8 (1 μg/ml and 10 μg/ml) showed the same degree of inhibition. (C) Adhesion of C2C12 cells on laminin 1 (10 μg/ml) was assayed with mAb GoH3 (anti-α6), CY8 (anti-α7), or Ha2/11 (anti-β1). Data are presented as a percentage of the total cells added to each well in A and B; maximum adhesion in the control wells in C was taken as 100% adhesion; bars show s.d.

myotubes; under non-reducing conditions the β1 subunit partner comigrated with the α7 subunit (Fig. 3A, lanes 1-4) (Kramer et al., 1989, 1991). Following reduction, the β1 subunit exhibited decreased mobility while the α7 subunit was cleaved to yield a M_r 100,000 fragment and an ~30,000 fragment containing the cytoplasmic tail (Fig. 3B, lanes 1-4); the light chain was frequently resolved into doublet bands of M_r 35,000 and 25,000. Immunoprecipitation showed that the 35,000 fragment was recognized by rabbit pAb 1211 generated to α7B (Fig. 3B, lanes 9-12) and the 25,000 fragment was recognized by pAb 22780 generated to α7A (Fig. 3B, lanes 5-8). In C2C12 myoblasts, the α7A variant was not detectable whereas α7B was strongly expressed (Fig. 3A,B, lanes 5 and 9). However, after induction of differentiation to myotubes, levels of both α7B and α7A were markedly elevated. Monoclonal Ab 3F4 was also prepared against the α7B cytoplasmic region and verified the expression pattern (data not shown). Thus, the increase in the α7 subunit at the cell surface following the conversion of proliferating myoblasts to non-proliferative myotubes was correlated with the elevation of α7B and the induction of α7A expression.

Myoblast α7 integrin mediates adhesion and migration on laminin 1

Since both MM14 and C2C12 myoblast cell lines expressed high levels of α7, we tested their capacity to attach and migrate on laminin 1 substrates in the presence of different concentrations of function-perturbing mAb CY8. The CY8 mAb to α7 inhibited up to 90% of MM14 cell adherence to a range of coating concentrations of laminin 1 (3 to 30 μg/ml) (Fig. 4A). Adhesion of C2C12 myoblasts to laminin 1 was also blocked but to a lesser extent, especially at high ligand-coating concentrations (Fig. 4B). This inhibition of adhesion of C2C12 cells varied but reached 60% at a laminin coating of 10 μg/ml. At 30 μg/ml of coating, the inhibitory effect of mAb CY8 decreased to ~45%. Therefore, although C2C12 and MM14 myoblasts have a similar repertoire of laminin-binding integrins on their cell surfaces, they differ somewhat in their dependency on α7 for adherence to laminin 1.

The resistance of C2C12 cells to function-blocking mAb to α7 suggested that the α6 integrin could be involved in binding to laminin 1. Though the percentage of blocking by GoH3 or CY8 varied slightly between experiments, slight to no inhibition by GoH3 and substantial inhibition by α7 were always noticed. The combination of GoH3 and CY8 consistently blocked adhesion completely as well as Ha2/11 (Fig. 4C). Interestingly, α6 integrin, which was also detected by FACS on MM14 cells at similar levels, did not appear to have an important role in binding to laminin. This may be a consequence of its pairing to β4 in the MM14 cells. This suggested that in the case of MM14 myoblasts, α7β1 is the dominant receptor and that α6β4 is not effective in mediating adhesion to laminin 1. Although α7β1 has been shown by affinity chromatography on fibronectin-Sepharose columns to bind fibronectin (Gu et al., 1994), in our study function-perturbing

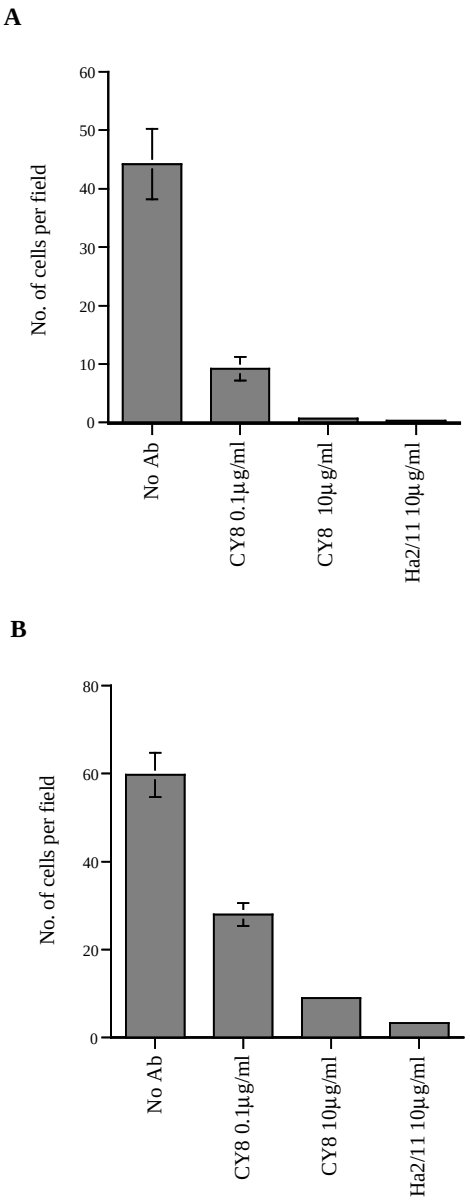

Fig. 5. Inhibition of myoblast migration on laminin 1 by function-perturbing mAbs to α7. (A) Motility of MM14 myoblasts on laminin 1 was measured in the presence of function-perturbing mAbs in a modified Boyden chamber assay. MM14 cells were resuspended in F10 medium containing 0.1% BSA; 2×10^4 cells were added to the upper chamber and incubated for 5 hours. Some samples were pre-incubated with CY8 (0.1 μg/ml or 10 μg/ml) or Ha2/11 (anti-β1, 10 μg/ml). (B) C2C12 cells in DMEM H-21 containing 0.1% BSA were tested in the motility assay but incubated for 3.5 hours. Motility was quantified by counting the number of cells that migrated to the undersides of the membranes. The results are averages of at least nine random ×400 microscopic fields; bars show s.d.

mAb to α7 did not block C2C12 myoblast adhesion to fibronectin substrates, which appears at least partially to depend on the α5β1 receptor (data not shown) which is expressed at moderate levels (Figs 1 and 2).

Migration on laminin 1 substrates was effectively blocked in a dose-response manner for both MM14 and C2C12 myoblasts with anti-α7 mAb (CY8) in modified Boyden chamber assays (Fig. 5A,B). CY8 mAb at 10 μg/ml blocked MM14 cell migration by nearly 100% and C2C12 cell migration by more than 85%. This inhibitory effect was similar to that generated by the anti-mouse β1 function-perturbing mAb (Ha2/11), and to that produced by a second blocking mAb to mouse α7 (CY4) (data not shown). Thus, although the C2C12 myoblasts can use α6β1 to adhere to laminin 1, migration is much more dependent on the α7 integrin.

Myoblast α7 binds differentially to specific laminin isoforms

We next tested the effect of function-perturbing mAb to α7 on adherence of MM14 cells to the available laminin isoform preparations. Monoclonal Ab to α7 (CY8) inhibited 90% of MM14 cell adherence to laminin 1 and 45% of adherence to laminin 2/4, but failed to block the modest adhesion of MM14 cells to laminin 5 (Fig. 6A). In a more detailed study of MM14 myoblast adherence to laminin 2/4, the substantial inhibition of adhesion by α7 mAb was not duplicated by α6 mAb (Fig. 6B), but was sensitive to blocking β1 mAb (Ha2/11). Therefore, on MM14 myoblasts, α7 is required for mediating binding to laminin 1 and laminin 2/4 but not to laminin 5. Adhesion to laminin 5, although weak, may be mediated by α3β1 or α6β4 integrin, which is expressed at low levels on these cells. In additional experiments, neither mAb GoH3 (anti-α6) nor mAb CY8 (anti-α7) could block MM14 myoblast adhesion to laminin 5. The combination of these two mAbs produced a slight (<10%) inhibition whereas mAb Ha2/11 (anti-β1) substantially blocked adhesion to laminin 5 (data not shown). The results suggest that the α7 on myoblasts is not an effective receptor for epithelium-specific laminin 5 but binds with high efficiency to laminin 1 and laminin 2/4. It is noteworthy that although the adhesion of C2C12 to laminin 1 could be completely blocked by the combination of α6 and α7 mAbs (Fig. 4C), the adhesion to laminin 2/4 was only partially blocked by this combination (data not shown). Interestingly, the adhesion was not completely dependent on β1 integrins. We next examined the motility of MM14 cells on laminin 2/4 substrates. Even though anti-α7 mAb only partially blocked adhesion in short term assays on laminin 2/4 (Fig. 6B), the mAb was very effective in blocking motility on this ligand (Fig. 6C). As expected, anti-β1 mAb effectively inhibited MM14 motility on laminin 2/4.

Functional analysis of α5/α7 cytoplasmic domain chimeras

The differentiation-dependent alternative splicing of the α7 integrin (Fig. 3) suggests that the α7A and 7B cytoplasmic regions may regulate integrin-dependent activities, as has been suggested for the closely related α6A and 6B integrin variants (Shaw and Mercurio, 1994). We tested potential functional differences between α7A and α7B isoforms in a chimera context by transfecting α5 subunit chimeric molecules into α5-deficient CHO cells. CHO B2 cells were transfected with expression vectors containing (i) wild-type human α5 cDNA (α5/α5), (ii) extracellular human α5/cytoplasmic mouse α7A cDNA (α5/7A), or (iii) extracellular human α5/cytoplasmic mouse α7B cDNA (α5/7B) (Fig. 7). Stable transfectants were selected by 'panning' with immobilized anti-α5 mAb (6F4) and by sorting with the same mAb. The mean expression levels

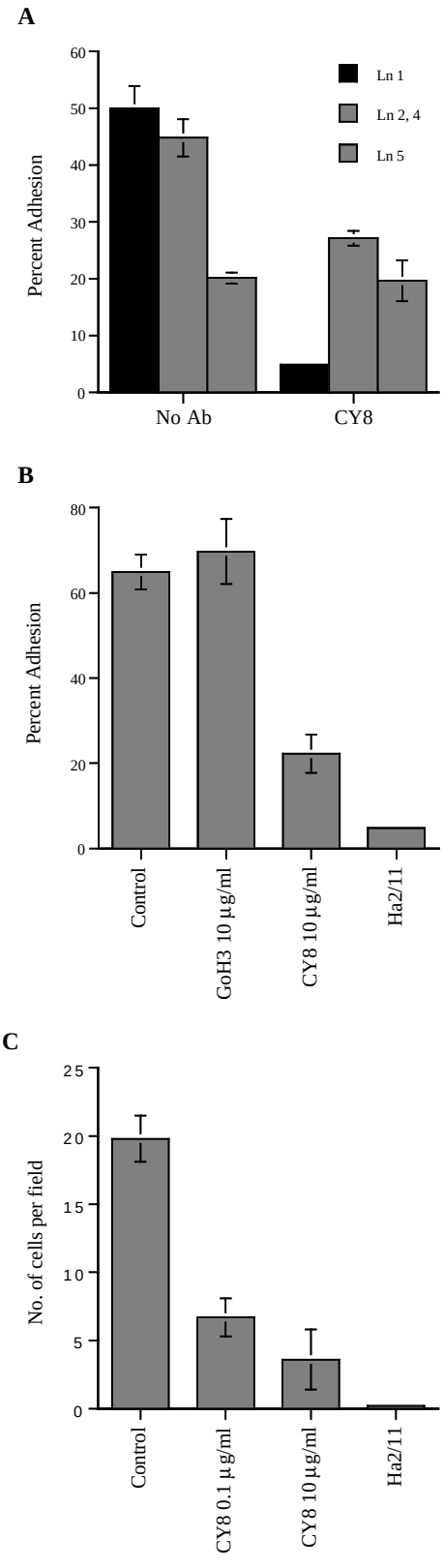

Fig. 6. (A) Adhesion of MM14 myoblasts to laminin isoforms. Dependency of α7 integrin on MM14 cell adhesion to laminin 1 (10 μg/ml), laminin 2/4 (30 μg/ml) or laminin 5 (3 μg/ml) was assessed at the coating concentration giving 20-50% cell adhesion. Monoclonal Ab CY8 (anti-α7) at 10 μg/ml was able to inhibit adherence to laminin 1 and laminin 2/4, but had no effect on adherence to laminin 5. Data are presented as a percentage of the total cells added to each well. (B) Adhesion of MM14 cells to laminin 2/4 (10 μg/ml) was dependent on α7β1 integrin but not on α6. (C) Migration of MM14 myoblasts on laminin 2/4 was assayed with function-perturbing mAbs and was almost completely blocked with anti-α7 mAb (CY8) as well as anti-β1 mAb (Ha2/11). Assays were performed as described in Fig. 5 (A) using human placental merosin at the coating concentration of 20 μg/ml. Bars show s.d.

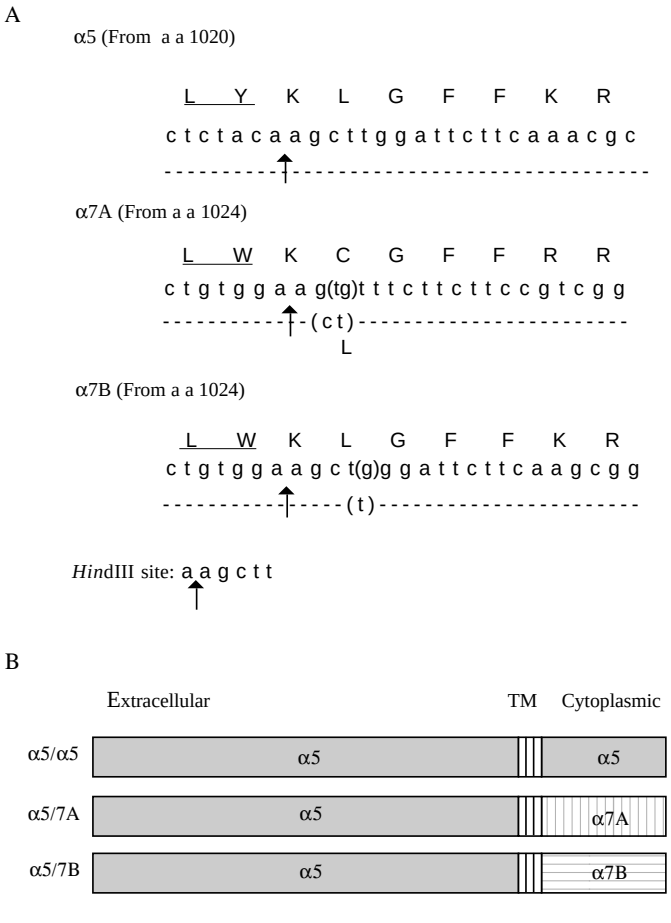

Fig. 7. Construction of chimeric α5/7A and α5/7B molecules. (A) The cytoplasmic regions of the α5, α7A, α7B subunits are aligned to show amino acid sequence and nucleotide substitution (shown in brackets). (B) Schematic presentation of chimeric cDNAs containing the α5 extracellular domain and α5, α7A or α7B cytoplasmic domain.

of the transfectant cell lines were compared by FACS (Fig. 8A) using anti-human α5 mAb (B2G2). Similar expression levels were obtained for the wild-type human α5 and the α5/7A and α5/7B chimeras.

We then tested the transfectants for their ability to adhere to fibronectin substrates. Both the α5/7A and the α5/7B transfectants adhered well to fibronectin, as efficiently as the wild-type human α5 transfectants (Fig. 8B). Initial binding was detected at coating concentrations of ≥1 μg/ml fibronectin, while maximum adhesion of cells to fibronectin was obtained at a coating concentration of 30 μg/ml.

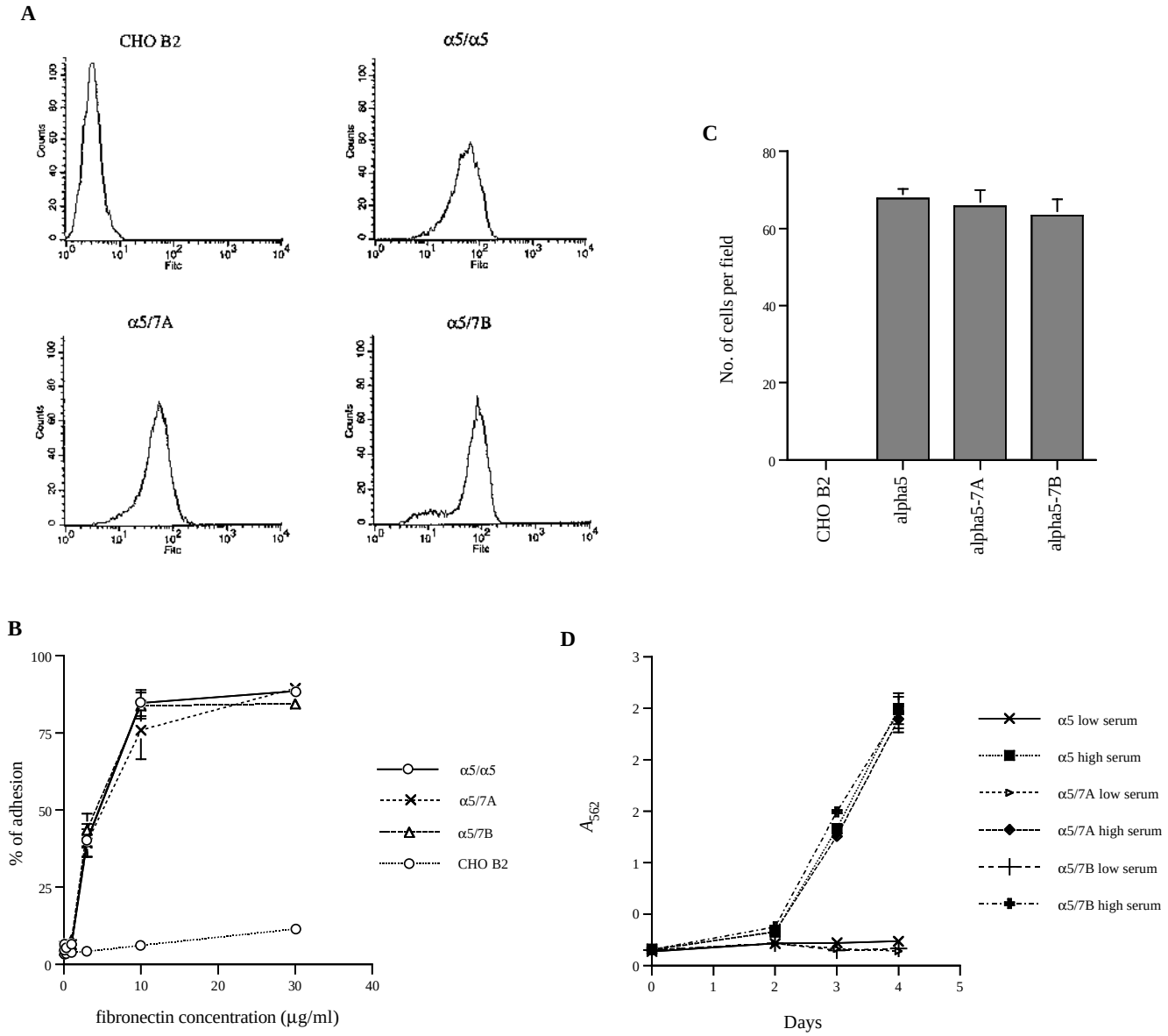

Fig. 8. Characterization of wild-type α5 and chimeric α5/7A and α5/7B transfectants. (A) Surface expression of wild-type α5, chimeric α5/7A and α5/7B in CHO B2 transfectants. α5-deficient CHO B2 cells were transfected with cDNA encoding human α5 or chimeric human α5/mouse α7A or human α5/mouse α7B constructs as described in Materials and Methods. Transfectants were enriched for expression of the α5 extracellular domain by panning and FACS using 6F4, a mAb specific for the human α5 integrin subunit. The surface expression was analyzed by flow cytometry using B2G2 (anti-human α5). (B) Adhesion of transfectants to fibronectin-coated substrates. Cells (2×10^4) were resuspended in medium containing 0.1% BSA and added to plates coated with fibronectin at different concentrations. Adherence of cells in 1% BSA-coated wells was treated as background binding and subtracted. Data are presented as percentage of the total cells added to each well. Values are means and s.d. of the triplicate wells. The parental CHO B2 cells are a negative control for wild-type α5, α5/7A and α5/7B transfectants. (C) Motility of transfectants on fibronectin. In the modified Boyden chamber assay, filters were coated with fibronectin (10 μg/ml) as described in Materials and Methods. Cells were added to the upper chamber and incubated for 5 hours. Motility was quantified by counting the number of cells that migrated to the undersides of the membranes. The results are averages of at least 9 random ×400 microscopic fields. CHO B2 cells are a negative control for wild-type α5, α5/7A and α5/7B transfectants. (D) Analysis of cell proliferation. Cell growth assays were performed as described in Materials and Methods. Transfectants had similar growth rates under the same conditions. In low-serum conditions, cells grew poorly. In high-serum conditions, cells grew rapidly. Data from low-serum and high-serum conditions with fibronectin coating are shown. Values are means of triplicate wells; bars show s.d.

Using a modified Boyden chamber assay, we examined the contribution of the α7A and α7B cytoplasmic domains to cellular haptotaxis on fibronectin substrates (Fig. 8C). After 5 hours of incubation, the α5/7A and α5/7B transfectants migrated on fibronectin to a similar extent as the wild-type α5 transfectants. As expected, the parental CHO B2 cells, which lack the α5 integrin, did not migrate on fibronectin. Therefore, the α7A and α7B cytoplasmic domains can equally support the

α5 extracellular domain in inducing CHO cells to adhere, spread and migrate on fibronectin.

Previous studies have shown that the α5β1 integrin plays a role in the control of CHO cell growth (Giancotti and Ruoslahti, 1990; Schreiner et al., 1991). We evaluated whether the 7A or B transfectants could replace the α5 cytoplasmic domain in mediating this function. Proliferation of transfectants under high (10%) or low (0.2%) serum conditions with or without a fibronectin substrate was measured at different times after plating. Analysis of growth rate indicated that in the high-serum conditions all transfectants grew at a similar rate after an initial log period following cell plating (Fig. 8D). Low-serum conditions failed to support growth of any of the cell lines. No differences in growth rate were detected between the α5, α7A or α7B cytoplasmic tails. Finally, because α5β1 integrin functions in fibronectin matrix assembly, we tested the α5/7A and α5/7B CHO transfectants for ability to support the functional role of α5 in the assembly of a fibronectin matrix. The results indicated that cells expressing either of the chimeric molecules were able to assemble exogenous fibronectin molecules into a complex network-type matrix, like the cells expressing the α5 wild-type receptor (data not shown). In aggregate, then, these data suggest that α7A and α7B cytoplasmic domains are functionally similar with regard to a number of integrin-dependent activities.

DISCUSSION

Laminin has been shown to enhance proliferation, migration and differentiation of myoblasts (Goodman et al., 1989; Ocalan et al., 1988; von der Mark and Ocalan, 1989). Using function-perturbing antibodies, we show for the first time that α7 is the major receptor mediating skeletal myoblast adhesion and migration on laminins1 and 2/4. Recently Echtermeyer et al. (1996) showed that transfection of human 293 carcinoma cells with cDNA to α7 integrin increased the motility but apparently not adhesion to laminin 1. This result would suggest that α7 may selectively induce cell motility. However, in another study, MCF-7 carcinoma cells transfected with α7 promoted both adhesion and motility on laminins (Yao et al., 1996).

In the present study we also examined the ligand specificity of the α7 receptor in myoblasts, using available laminin isoforms. In MM14 myoblasts, both adhesion and migration to preparations of laminin 1 and to human placental laminins (a mixture of laminin 2 and 4) were inhibited by function-perturbing antibody to α7β1. However, the binding to laminin 5 was not affected by the mAb to α7. It is not possible to attribute the binding of α7β1 to laminin 2 or laminin 4 in this mixed preparation. A more detailed analysis of the ligand specificity of the α7 receptor awaits the availability of purified preparations of different members of the laminin superfamily, especially laminin 2 (merosin), laminin 3 (s-laminin) and laminin 4 (s-merosin) which are present in skeletal muscle basement membranes (Engvall et al., 1990; Sanes et al., 1990). Although the adhesion of MM14 cells to laminin 1 and laminin 2/4 were perturbed by mAb to α7, the binding to laminin 5 was not altered either by the mAb to α7 or the mAb to α6 (data not shown). It has been shown previously that α6β4 receptor can be resistant to function-perturbing mAb GoH3 (Sonnenberg et al., 1993). In the case of MM14 myoblast adhesion to laminin 5, it is likely that α3β1 and/or α6β4 integrins are involved since these receptors have been shown to bind to the laminin 5 isoform (Delwel and Sonnenberg, 1995).

Our finding that blocking mAb to α7 only partially inhibited MM14 cell adhesion to a mixture of laminin 2 and 4 indicates that additional laminin-binding receptors besides α7 are involved in myoblast adhesion to these laminin isoforms. There may be redundant mechanisms for myoblasts to bind laminin 2 and laminin 4, which are localized at the basement membrane surrounding individual muscle fibers and at myotendinous junctions, respectively. Of the β1 integrins expressed in cultured myoblasts, both α3 and α6 could also contribute to the binding of laminin 2/4 (Delwel et al., 1994; Spinardi et al., 1995). In previous studies, low levels of α3 and α6 integrins were detected on surface-iodinated C2C12 and G7 murine myoblasts (von der Mark et al., 1991). However, using surface-biotinylated cells we found significant amounts of α3 and α6 integrins in both MM14 and C2C12 myoblasts. These differences may reflect the experimental protocols used or variations in the cell lines.

It is likely that during early developmental stages, both α6 and α7 integrins may mediate interactions with laminin and promote migration and differentiation; in adult muscle, α6 expression is completely down-regulated while α7 shows increased expression (Ziober and Kramer, 1996) and localizes to the myotendinous and neuromuscular junctions. However, the detection of α6β4 on MM14 myoblasts is unexpected since α6β4 expression usually is restricted to epithelia (Natali et al., 1992). Whether α6β4 is expressed at specific time points during muscle development awaits further study. On the other hand, myoblasts do express additional non-integrin laminin-binding receptors, including α-dystroglycan (Ervasti and Campbell, 1993; Klietsch et al., 1993) and 5′ nucleotidase (Mehul et al., 1990, 1992), which can also contribute to interactions with basement membrane. Eventually, it will be important to define the individual and the cooperative contributions of each other potential laminin-binding receptor to adhesion and migration on specific laminin isoforms.

The dynamic interaction between myoblasts and specific laminin isoforms is important for both muscle development and muscle regeneration. Recently Vachon et al. (1996) showed that merosin is specifically required for myotube stability and survival. During development, there are several waves of myogenic precursor migration from myotome to the sites where muscle eventually forms (Van Swearingen and Lance-Jones, 1995). In mature muscle, satellite cells are responsible for repair of injured tissue (Schultz and McCormick, 1994). It has been shown that α7 integrin is expressed not on primary but on secondary myoblasts, and also on all terminally differentiated myotubes (George-Weinstein et al., 1993). α7 integrin is also highly expressed in all the cell lines derived from satellite cells of adult muscle (von der Mark et al., 1991, and the current results). Following muscle injury, degenerating cells are removed by scavanger cells but the basement membrane remains largely intact (Alameddine et al., 1991). Satellite cells can pass through the residual muscle basement membrane, undergo extensive migration, proliferate, differentiate, and be incorporated within the preexisting myofibers (Hughes and Blau, 1990). Therefore, cell motility on extracellular matrix is required in both normal muscle development and the repair following injury.

Upon interacting with extracellular ligands, different cytoplasmic domains of α7 may trigger differential downsteam events. We found that α7 integrin condensed into vinculin-positive focal contacts when myoblasts were plated on laminin 1 substrates (not shown). Focal adhesion sites have been shown to contain not only cytoskeletal proteins as structural components but also other signaling complexes. It is possible that upon binding the ligand, α7 integrin orchestrates downstream signaling events that lead eventually to specific proliferative, motile, and differentiated phenotypes on laminin.

Several α subunits of integrins have alternative RNA splicing forms, including α3, α6 and α7 (Collo et al., 1993; Cooper et al., 1991; Hogervorst et al., 1991, 1993a; Song et al., 1993; Tamura et al., 1990; Ziober et al., 1993). However, differential functions of the α chain-cytoplasmic isoforms have not been well delineated. Studies searching for functional differences between α6A and α6B have obtained conflicting results (Delwel et al., 1993; Hogervorst et al., 1993a,b; O'Toole et al., 1994; Shaw et al., 1993; Shaw and Mercurio, 1994). In the current chimeric construct experiments with CHO cells, we found that α7A and α7B exhibited similar adhesive activities. The functional studies we chose, however, might not detect all potential differences. It is also possible that α7A/B integrins will show differential functions only in the endogenous muscle microenvironment. Skeletal muscle cells have uniquely organized cytoskeletal-sarcolemmal associations that function to transfer force from the contractile apparatus to the extracellular anchorage. The recent identification of the differentiation-dependent switching in β1 integrin cytoplasmic tail isoforms also argues that α7 cytoplasmic variant function may require a skeletal muscle environment. Following myoblast differentiation to myotubes, β1D completely replaces the β1A isoform (van der Flier et al., 1995; Zhidkova et al., 1995) and pairs with α7 subunits. It is possible that differential functions of α7 cytoplasmic domain isoforms will only be detected when α7 is associated with β1D.

In summary, we show for the first time that α7β1, the major laminin receptor on myoblasts, specifically mediates both cell adhesion and migration on laminin 1. In addition we have demonstrated that α7 in myoblast can bind to a mixture of laminin 2 and 4, but not to laminin 5. These results strongly support the role of the α7 receptor in mediating interactions with specific laminin isoforms not only in mature muscle fibers but also during the recruitment and expansion of resident satellite myoblasts during muscle regeneration.

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