The $\beta 1$ Cytoplasmic Domain Regulates the Laminin-binding Specificity of the $\alpha 7 X 1$ Integrin

Ming-Guang Yeh,* Barry L. Ziober,*[†] Baomei Liu,* Galina Lipkina,*
Ioannis S. Vizirianakis,*[‡] and Randall H. Kramer*^{§||}

*Department of Stomatology and [§]Department of Anatomy, University of California at San Francisco, San Francisco, CA 94143-0512

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During muscle development, the laminin-specific $\alpha 7$ integrin is alternatively spliced in the putative ligand-binding domain to yield either the $\alpha 7 X 1$ or the $\alpha 7 X 2$ variant. The relative level of $\alpha 7 X 1$ and $\alpha 7 X 2$ is developmentally regulated. Similarly, the partner $\beta 1$ integrin cytoplasmic domain is converted from the $\beta 1 A$ to the $\beta 1 D$ splice variant. To determine whether $\beta 1 D$ modulates the activity of the $\alpha 7$ receptor, cells were transfected with $\alpha 7 X 1$ and $\beta 1 D$ cDNA. $\alpha 7 X 1$ coupled with $\beta 1 A$ failed to adhere to laminin-1, whereas cotransfectants expressing $\alpha 7 X 1$ and $\beta 1 D$ showed strong adhesion. Interestingly, $\alpha 7 X 1$ complexed with $\beta 1 A$ and $\beta 1 D$ displayed the same level of poor adhesion to laminin-2/4 or strong adhesion to laminin-10/11. These findings indicate that $\alpha 7$ function is regulated not only by $X 1/X 2$ in its extracellular domain but also by $\beta 1$ cytoplasmic splice variants. It is likely that expression of $\beta 1 D$ alters $\alpha 7 X 1$ binding to laminin isoforms by a process related to ligand affinity modulation. Functional regulation of $\alpha 7 \beta 1$ by developmentally regulated splicing events may be important during myogenic differentiation and repair because the integrin mediates adhesion, motility, and cell survival.

INTRODUCTION

The dynamic interaction between myoblasts and laminins is important for skeletal muscle development, regeneration, and myotube stability and survival (Vachon *et al.*, 1997; Gullberg *et al.*, 1999; Colognato and Yurchenco, 2000). The laminin-binding $\alpha 7 \beta 1$ integrin, expressed in mouse skeletal muscle as early as E10.5 d of development, mediates myoblast motility on laminin substrates (Kaufman *et al.*, 1980; Kaufman *et al.*, 1991; Yao *et al.*, 1996a,b; Crawley *et al.*, 1997; von der Mark *et al.*, 2002). In mature skeletal muscle, the $\alpha 7$ receptor is associated with costameres and the myotendinous and neuromuscular junctions (Bao *et al.*, 1993; Belkin *et al.*, 1996; Martin *et al.*, 1996; van der Flier *et al.*, 1997). The

physiological importance of $\alpha 7$ is demonstrated in $\alpha 7$ -null mutant mice, which develop a form of muscular dystrophy with myotendinous junction (MTJ) defects (Mayer *et al.*, 1997), and in humans with mutations in $\alpha 7$ gene, which develop congenital myopathies (Hayashi *et al.*, 1998; Pegoraro *et al.*, 2002).

The $\alpha 7$ integrin subunit is alternatively spliced in both the extracellular ($X 1$ and $X 2$) (Ziober *et al.*, 1993) and the cytoplasmic (A and B) domains (Song *et al.*, 1993; Ziober *et al.*, 1993). During the differentiation of myoblasts to myotubes, the cytoplasmic domain of the $\beta 1$ integrin is converted from isoform A to D (van der Flier *et al.*, 1995; Zhidkova *et al.*, 1995; Belkin *et al.*, 1996). $\beta 1 D$ begins to be expressed in late fetal life and eventually displaces $\beta 1 A$ in mature muscle (Brancaccio *et al.*, 1998). Previous studies have shown that the presence of $\beta 1 D$ is correlated with altered adhesion and is associated with enhanced interaction with actin cytoskeleton (Belkin *et al.*, 1996; Belkin *et al.*, 1997; Pfaff *et al.*, 1998). This complex differentiation-dependent pattern of $\alpha 7$ and $\beta 1$ splicing allows the generation of a unique set of variant $\alpha 7 \beta 1$ complexes that are structurally, and presumably functionally, distinct.

Laminins, the only known ligand for $\alpha 7$, also undergo a complex pattern of expression during muscle development (Gullberg *et al.*, 1999; Colognato and Yurchenco, 2000; Pedrosa-Domellof *et al.*, 2000). Laminin-1 is expressed during embryonic and fetal stages. Interestingly, laminin-1 is

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Present addresses: [†]Department of Otorhinolaryngology, University of Pennsylvania, Philadelphia, PA 19104; [‡]Laboratory of Pharmacology, Department of Pharmaceutical Sciences, Aristotle University of Thessaloniki, GR-54006, Thessaloniki, Greece.

^{||} Corresponding author. E-mail address: rkramer@itsa.ucsf.edu. Abbreviations used: DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MTJ, myotendinous junction; NMJ, neuromuscular junction; pAb, polyclonal antibody; PCR, polymerase chain reaction.

concentrated at the MTJ of fetal human skeletal muscle and persists until birth. As development of muscle continues, laminin-2 and 4 become the dominant isoforms.

The X1/X2 alternative splicing of $\alpha 7$ is developmentally regulated (Ziober *et al.*, 1993). In early muscle differentiation of rodent limb, $\alpha 7X1$ shows a relative increase in expression but postnatally the X2/X1 ratio increases (Hodges *et al.*, 1997). Previously, we transfected mouse $\alpha 7X1$ and $\alpha 7X2$ isoforms into human MCF-7 cells, which normally adhere poorly to laminin-1 (Yao *et al.*, 1996a; Ziober *et al.*, 1997). In the transfectants, $\alpha 7X2$ bound laminin-1 readily, but $\alpha 7X1$ bound only when activated by the $\beta 1$ -activating monoclonal antibody (mAb) TS2/16. However, both X1 and X2 were functional when expressed in the HT-1080 cell line. These results indicated that alternative splicing regulates $\alpha 7\beta 1$ ligand-binding competence in a cell-specific manner. Recently, von der Mark and collaborators reported that the X1/X2 variants have different binding specificity and affinities for laminin isoforms (von der Mark *et al.*, 2002).

We proposed that the X1 isoform is important during dynamic adhesion related to muscle development and repair (motility, fusion, and matrix assembly), whereas the X2 variant performs more stable adhesion functions (mature junctional assemblies) (Ziober *et al.*, 1997). This is consistent with the dynamics of the expression of $\alpha 7$ and $\beta 1$ alternatively spliced variants during development (Ziober *et al.*, 1993) and muscle regeneration (Kaariainen *et al.*, 2001; Kaariainen *et al.*, 2002). Laminin isoform expression also changes during muscle development and repair (reviewed in Colognato and Yurchenco, 2000) and so the expression of integrin and potential ligand is coordinately coupled.

Integrins are capable of changing their function dynamically (Diamond and Springer, 1994; Humphries, 1996; Mould, 1996; Hughes and Pfaff, 1998). It has been proposed that certain intracellular events lead to alterations in integrin conformation, resulting in enhanced adhesion to ligand (inside-out signaling) (reviewed in Liddington and Ginsberg, 2002). Alternatively, the process of ligand binding may regulate activity of the integrin receptor (outside-in signaling). In the case of inside-out signaling, there is strong evidence that the actin cytoskeleton and its link proteins (e.g., talin) are one mechanism that can regulate integrin activation. This process may represent changes in integrin affinity or integrin-clustering events leading to increased avidity (Zent *et al.*, 2000). However, it remains controversial whether events such as lateral rearrangements that may modulate receptor avidity can play a role in integrin function (reviewed in Shimaoka *et al.*, 2002). As stated above, after myoblast differentiation into myotubes and mature muscle, the $\beta 1$ integrin cytoplasmic alternative splice shifts from A to D. Given the consensus that the cytoplasmic domains of β integrins can regulate the conformation and activity of integrin subunits, it is possible that the $\beta 1D$ isoform controls $\alpha 7X1/X2$ function. To investigate this possibility, we examined the functionality of $\alpha 7$ integrin in the presence of the $\beta 1A$ or $\beta 1D$ cytoplasmic domain.

MATERIALS AND METHODS

Materials

For cell adhesion studies, several different ligands were used as described previously. Laminin-1 isolated from the mouse EHS tumor was obtained from Invitrogen (Carlsbad, CA). Human placen-

tal merosin, consisting of laminin-2/4 isoforms purified by EDTA extraction and followed by ion-exchange chromatography was purchased from Invitrogen. Human laminin-10/11, isolated from placenta by mild pepsin digestion and by affinity chromatography on mAb 4C7-coupled Sepharose, was also from Invitrogen. A sample of recombinant laminin-10 was kindly provided by Masayuki Doi (University of Okayama, Okayama, Japan) and Karl Tryggvason (Karolinska Institute, Stockholm, Sweden). Collagen was obtained from Cohesion (Palo Alto, CA). Human vitronectin and fibronectin were gifts from Caroline Damsky (University of California, San Francisco, San Francisco, CA).

Antibodies against integrin subunits, including rat anti-human $\beta 1$ (mAb A1B2), mouse anti-human $\alpha 2$ (mAb VM1), rat anti-human $\alpha 6$ (mAb GoH3), and rabbit anti-mouse $\beta 1D$ cytodomain (polyclonal antibody anti- $\beta 1D$), were kindly provided by Caroline Damsky and Vera Morhenn (SRI International, Menlo Park, CA), Arnon Sonnenberg (Netherlands Cancer Center, Amsterdam, The Netherlands), and Eva Engvall (Burnham Institute, La Jolla Cancer Research Center, CA), respectively. Mouse anti- $\alpha 3$ (mAb J143) was from the American Type Culture Collection (American Type Culture Collection, Manassas, VA), rat anti-mouse $\beta 1$ (mAb MB1.2) and mouse anti-human $\beta 1$ (mAb 2000) were from Chemicon International (Temecula, CA), rat anti-mouse $\alpha 7$ (mAb CY8), mouse anti-human $\alpha 7$ (mAb 9.1), rabbit anti-human $\beta 1$ cytodomain (polyclonal antibody [pAb] 22778), and rabbit anti-mouse $\alpha 7$ light chain (pAb 1211) were from our laboratory, as described previously (Yao *et al.*, 1996b, 1997; Vizirianakis *et al.*, 2001). Fluorescein-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Construction of Mouse $\beta 1D$ cDNA

The full-length mouse $\beta 1D$ cDNA was prepared by reverse transcription followed by polymerase chain reaction (PCR) from a mouse skeletal muscle cDNA library (BD Biosciences Clontech, Palo Alto, CA). The two primers for PCR were reverse primer, 5'-CTAGTCTAGAATTCAGAGACCAGCTTTACGTCCATAG and forward primer, 5'-CGCGGATCCGAATTCGAATGAATTTGCAACTGGTTTCCTG. The sequence of full-length mouse $\beta 1D$ was verified at the Biomolecular Resource Center (University of California, San Francisco). The mouse $\beta 1D$ cDNA was then ligated into *Bam*HI and *Xba*I sites of pcDNA3.1/Hygro (Invitrogen). Full-length mouse $\beta 1A$ cDNA was obtained from Louis Reichardt (University of California, San Francisco) and was cloned into the regulatable expression vector in the same manner as $\beta 1D$ (see below).

Cloning of Human $\alpha 7X1$

Full-length human $\alpha 7X2$ cDNA (Vizirianakis *et al.*, 2001) was used as a template for the generation of full-length human $\alpha 7X1$. The X2 exon was replaced with the X1 exon by uracil DNA glycosylase excision to generate cohesive ends on the PCR product of the X1 exon. Specifically, we designed primers containing uracil that matched the corresponding T in the splicing regions. We then used these primers to generate the X1 exon by PCR from human $\alpha 7$ cDNA by using human fetus limb muscle RNA as template (forward primer, 5'-AAGGGUACUGCCAGGGTGGAGCTCTGTG; reverse primer, 5'-AGAAGCCAAAGUA GCUGTTGGCAGGGA). The full-length cDNA proximal to the splicing site was also generated by PCR (forward primer, 5'-CCGGGATCCATGGCCGCGGGCTCGGAGCCG; reverse primer, 5'-AGTACCCUCCATTATAGGTTTCCTGGGG). The primers for full-length cDNA distal to the splicing site were as follows: forward primer, 5'-AGCTACTTTGGCUUCUCT ATTGACTCGGGGAAAGGTCTG; reverse primer, 5'-CCGCTCGAGCTAGGCGG TGCTTGCCCT. The three pieces of PCR products were first treated with uracil DNA glycosylase according to the manufacturer's protocol (Invitrogen) and then ligated. The ligation product was purified by gel electro-

phoresis followed by cloning into pCDNA3.1/Hygro and verified by sequencing.

Generation of Cell Lines

MCF-7 human carcinoma cells and the α 7-transfected cells were cultured as described previously (Yao *et al.*, 1996a; Ziober *et al.*, 1997). MCF-7 cells do not adhere well to laminin-1 even though they express moderate levels of integrins α 2, α 3, and α 6, which are potential receptors for this ligand. Previously, we generated MCF-7 cells expressing full-length mouse α 7 cDNA containing either the X1 or X2 splice form, which formed heterodimers with the partner β 1A subunit. MCF-7 cells expressing the splicing isoform α 7X2 were active in binding laminin-1, whereas cells expressing α 7X1 lacked ligand-binding activity (Ziober *et al.*, 1997). The original X1/A-p expressing population was further enriched by fluorescence-activated cell sorting (FACS) by using mAb CY8 against mouse α 7 to yield the X1/A cell line used in the current experiments. The X2/A cell line was derived from MCF-7 cells transfected with mouse α 7X2, which heterodimerizes with the endogenous human β 1A (Yao *et al.*, 1996a).

Expression of mouse β 1D in MCF-7 cells was generated in two different systems. In the first, the X1/A-p cells were stably transfected with mouse β 1D cDNA by the calcium phosphate procedure (Mammalian Transfection kit; Stratagene, La Jolla, CA). After selection with hygromycin, the double transfectants (α 7X1/ β 1D) were enriched by FACS after labeling with anti-mouse β 1 antibody (mAb MB1.2; Chemicon International). The enriched population was thereafter called X1/D. In addition, a total of 20 single cell clones were isolated either by single cell sorting by FACS or by limiting dilution into individual wells of 96-well plates. The expression of mouse β 1D was verified by immunoblot analysis with β 1D-specific pAb from Eva Engvall and by flow cytometry with mAb MB1.2. A number of clones with a range of α 7 and β 1D levels, as determined by FACS, were chosen for further study.

In the second approach, MCF-7 cells were transfected with pTet-Off (pUHD15-1 neo; BD Biosciences Clontech), and the expression of tTA-vp16 was verified. The MCF-7 Tet-Off cells were then transfected with the full-length cDNA for either human α 7X1 integrin (as described above) or human α 7X2 integrin (Vizirianakis *et al.*, 2001). We used anti-human α 7 mAb 9.1 to enrich the α 7-expressing population by FACS. Retroviral transduction was then used to introduce the pRevTRE plasmids (BD Biosciences Clontech) with mouse β 1A or mouse β 1D into the human α 7 stable transfectants. In brief, Phoenix Amphi retrovirus packaging cells from American Type Culture Collection were transfected with pRevTRE β 1A/ β 1D via calcium phosphate. The mouse β 1D or mouse β 1A cDNA was cloned downstream of the tetracycline-responsive element and the minimal immediate early promoter of cytomegalovirus (P_{min} CMV). Retroviral supernatants were then used to infect α 7-expressing cells. To test the regulation of expression, cells were cultured with various concentrations of doxycycline. After retroviral induction, cells were screened by FACS with mAb MB1.2 against mouse β 1 ectodomain, and cell lines with high expression of mouse β 1 integrin in the absence of doxycycline and low background in the presence of doxycycline were selected for further study. MCF-7 Tet-Off cells with human α 7X1/mouse β 1A, human α 7X1/mouse β 1D, or human α 7X2/mouse β 1A were designated HuX1/A, HuX1/D, and HuX2/A, respectively. For adhesion assays, cells were typically cultured for 5–7 d in the presence of doxycycline (0.3 ng/ml or less) to stabilize integrin expression levels.

Flow Cytometry

Standard procedures for flow cytometry were followed (Yao *et al.*, 1996a). Briefly, cells (10^6 /ml) were incubated with predetermined optimal concentrations of primary antibodies, washed, and incubated with secondary FITC-conjugated fluorescein-labeled antibodies (affinity-purified goat anti-mouse or anti-rat antibodies; Jackson

Immunoresearch Laboratories). After washing, the cells were stained with propidium iodide and processed for flow cytometry on an FACScan (BD Biosciences, San Jose, CA). Samples without primary or secondary antibody were always included as a control. Data are expressed as the mean fluorescence intensity after subtraction of background staining produced by secondary antibody alone (<5% of signal).

Cell Adhesion Assay

Cell attachment was measured using a published protocol (Yao *et al.*, 1996a; Ziober *et al.*, 1997). Briefly, microtiter plates (96-well Immulon 1B plates; Thermo Labsystems, Franklin, MA) were coated with matrix proteins at the indicated concentrations in phosphate-buffered saline for 1 h at 37°C. Single cell suspensions were prepared and assayed in triplicate in 96-well plates with an incubation period of 30–60 min at 37°C. Normally, cell adhesion assays were performed using a short time of incubation (30 min) at the lower range of ligand-coating concentrations to preferentially study early stages of adhesion. Adherent cells were fixed, stained with crystal violet, and solubilized in 2% SDS. Absorbance was read at 562 nm. Background cell adhesion to 1% bovine serum albumin-coated wells (usually <5% of value) was subtracted from all readings. Values for total cell input per well to represent 100% attachment were determined by seeding cells on a separate microtiter plate coated with collagen type I or polylysine (10 μ g/ml) followed by incubation at 37°C for 120 min. The 100% attachment value was then estimated by fixation on the monolayer and staining with crystal violet as described above. The effect of specific blocking antibodies was tested by preincubating the cells with an optimal blocking concentration of mAb on ice for 30 min before the assay. Optional concentrations of the blocking antibodies were predetermined and used as described previously (Yao *et al.*, 1996a; Ziober *et al.*, 1997). The concentrations of integrin function-blocking mAb were as follows: 10 μ g/ml purified GoH3 (anti- α 6) and J143 (anti- α 3). VM1 (anti-human α 2) ascites were used at a dilution of 1:400. Both CY8 (anti-mouse α 7) and A11B2 (anti-human β 1) ascites were used at a dilution of 1:300. Cytochalasin D was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). Adhesion assays included control cells treated with DMSO alone (final concentration, 0.2%). The cells were processed as described above except that they were pretreated with 20 μ M cytochalasin D or DMSO alone for 20 min on ice before the assay.

Immunoprecipitation and Western Blotting of Cell Lysates

Cell lysates were prepared by extraction of cells with lysis buffer (1% Triton $\times$ 100 in 50 mM Tris-HCl, pH 7.5, 1 mM CaCl_2 , with 2 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, and 2 mM leupeptin as protease inhibitors) for 90 min and centrifuged. The supernatants were precleared with agarose beads coupled with secondary antibodies overnight. For the purpose of depletion analysis, aliquots of cell lysates (600 μ g of protein) were immunoprecipitated with antibody against α 2, α 3, or α 7 for three consecutive rounds. The beads were pooled and washed with wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl_2 , 0.5% Nonidet P-40, and 0.1% bovine serum albumin) three times and heated at 100°C with SDS sample buffer. Samples including controls for whole cell lysates (10 μ g protein/lane) were processed for SDS-PAGE under nonreducing conditions and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were incubated with rabbit antibody against human integrin β 1 (pAb 22778) (Martin *et al.*, 1996; Yao *et al.*, 1997) or mouse β 1 (mAb 2000; Chemicon International). In a different approach to assess the α 7 preference to β 1A or β 1D, equal amounts of cell lysate (500 μ g of protein) prepared from X1/D-27 cells (a clone of X1/D cells) were immunoprecipitated with human β 1A or mouse β 1D antibody for three consecutive rounds. The samples were then processed as

Table 1. Expression levels of mouse $\alpha 7$ in transfectants

Cell line	Integrin pair*	Mean fluorescence intensity		
		$\alpha 7^a$	Hu $\beta 1A^b$	Ms $\beta 1D^c$
MCF-7	— $\beta 1A$	—	78	—
X1/A	Msa7X1/ $\beta 1A$	43	86	—
X1/D	Msa7X1/ $\beta 1D$	32	55	20
X2/A	Msa7X2/ $\beta 1A$	40	58	—

* The parental MCF-7 cells and transfectants were processed for FACS analysis using the indicated antibody as detailed in MATERIALS AND METHODS. The level of expression is expressed as the mean fluorescence intensity of 10,000 cells scanned. The Hu $\beta 1A$ is the endogenous integrin partner.

^a mAb CY8, rat anti-mouse $\alpha 7$.

^b mAb AIIB2, rat anti-human $\beta 1$.

^c mAb MB1.2, rat anti-mouse $\beta 1$.

described above except under reducing conditions for immunoblotting and probed with rabbit antibody (pAb 1211) against mouse $\alpha 7$ (Yao *et al.*, 1997).

RESULTS

Expression and Adhesive Activity of $\alpha 7X1/\beta 1D$ Transfectants

To test the hypothesis that alternatively spliced amino acid sequences in the $\beta 1$ cytoplasmic domain may regulate the activity of the $\alpha 7$ integrin, we transfected human MCF-7 cells with mouse $\alpha 7X1$ alone (X1/A cell line) or with both mouse $\alpha 7X1$ and $\beta 1D$ cDNA (X1/D cell line). After selection, the transfectants expressed high levels of $\alpha 7X1$ and $\beta 1D$ at their surface, as detected by flow cytometry with mAb directed against $\alpha 7$ (CY8) or mouse $\beta 1$ (MB1.2) (Table 1). The level of $\alpha 7$ expression in the X1/A AND X2/A cell lines were found to be similar. In the case of the double transfectant X1/D, the X1 level was somewhat lower than that of the X1/A cell line from which it was derived.

To assess whether X1/D and X1/A cells displayed different adhesion profiles, we used standard adhesion assays to examine their ligand binding to the extracellular matrix components vitronectin and type I collagen. On these immobilized ligands, X1/A and X1/D cells showed a similar ligand dose-response pattern (Figure 1, A and B) over a range of ligand-coating concentrations. Thus, expression of $\beta 1D$ did not alter the adhesion of $\alpha 7X1$ -expressing cells to these components. Previous reports showed that in MCF-7 cells, adhesion to vitronectin and type I collagen was mediated primarily by $\alpha v\beta 1$ (Maemura *et al.*, 1995) and $\alpha 2\beta 1$ integrins (Jones *et al.*, 1995; Maemura *et al.*, 1995), respectively. This result indicated that there was no change in the general adhesion activity toward type I collagen or vitronectin in the transfectants.

We previously reported that MCF-7 cells expressing $\alpha 7X1$ with the endogenous human $\beta 1A$ partner adhered poorly to laminin-1 (Ziobler *et al.*, 1997). However, after transfection with cDNA of the mouse $\beta 1D$ subunit, X1/D cells acquired the capacity to strongly bind laminin-1 (Figure 1C). This increase in adhesion was evident at coating concentrations

Figure 1. Adhesion of X1/A and X1/D transfectants to matrix molecules. Ligand dose-response analysis of adhesion to vitronectin (A), type I collagen (B), and laminin-1 (C). Effect of anti-integrin antibodies on adhesion to laminin-1 (D). Cell adhesion was assayed as described in MATERIAL AND METHODS. Values are the mean of triplicate wells; bars indicate SE. In D, ligand coating concentration was 12 μ g/ml.

above 8 μ g/ml. We also generated 20 single cell clones by limiting dilution and by single cell sorting (see below). A number of representative clones were chosen for the same assay, and the results were similar (our unpublished data). Because the stimulatory effect of β 1D on adhesion was restricted to laminin-1 and not vitronectin or collagen I, this suggested that laminin-binding integrins such as α 7X1, but not other potential β 1 partner subunits, were involved. The poor adhesion of X1/A cells to laminin-1 was not related solely to the level of α 7X1 expressed at the surface, because this cell line had a significantly higher expression level of α 7X1 than did X1/D cells (Table 1).

Enhanced Adhesion of β 1D Transfectants to Laminin-1 Is α 7 Specific

To establish that the β 1D-induced increase in laminin-1 adhesion was attributable specifically to α 7X1, we performed standard adhesion assays with blocking mAb against other expressed α subunits (Figure 1D). The α 7X1-expressing cells (X1/A) showed poor adhesion to laminin-1, and adhesion could be diminished further by treatment with a cocktail of blocking mAb to α 2, α 3, and α 6 integrins. In contrast, the X1/D cells expressing β 1D showed strong adhesion to laminin-1, and most of this activity was resistant to the mixture

of antibodies to α 2, α 3, and α 6 integrins. Importantly, blocking mAb CY8 to α 7 completely ablated adhesion to laminin-1.

Analysis of Integrin Complexes in the β 1D Transfectants

To determine what fraction of the α 7 subunit was partnered with endogenous β 1A or transfectant β 1D, two different strategies were used. In the first, lysates prepared from X1/D cells were exhaustively immunoprecipitated with mAb specific to either human β 1 or mouse β 1, followed by immunoblotting for partner α 7 (Figure 2A). The results show that the fraction of α 7 that heterodimerized with either the mouse β 1D (lane 5) or the human β 1A (lane 6) isoform was approximately equal. Thus, neither subunit seems to have any preferential affinity for α 7 during integrin heterodimerization.

In the second approach to estimate the relative pairing of α 7 integrin subunit with endogenous β 1A or transfectant β 1D, we used the X1/D-27 cell line, which expresses high levels of both α 7X1 and β 1D (Figure 2B). Cells were first processed for immunoprecipitation with anti- α 7 antibody, followed by immunoblotting analysis with antibody specific for either human β 1A (isoform) or mouse β 1D (isoform).

Figure 2. Association of α 7 with β 1D. (A) Immunodepletion of lysates with antibody against β 1. To determine the relative association of α 7 with human β 1 or with mouse β 1, X1/D double transfectant cells were processed for immunodepletion experiments by using antibodies against the β 1D (mouse) or β 1A (human) subunit as described in MATERIALS AND METHODS. Cell lysates were prepared from X2/A cells (lane 1) or X1/D cells (lane 2) as controls for α 7 expression. Equal amounts of X1/D cell lysate were subjected to preclear control (beads and secondary antibody only, lanes 3 and 4). X1/D cell lysate was exhaustively precipitated with mAb against mouse β 1 (mAb MB1.2, lane 5) or against human β 1 (mAb A11B2, lane 6), and after depletion with mouse β 1 (lane 7) or human β 1 (lane 8) of X1/D cell lysates, supernatants were subjected to a final additional round of immunoprecipitation to confirm depletion. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions, followed by Western blotting with anti- α 7 antibody and visualized by chemiluminescence. The position of the 38-kDa α 7 light chain is indicated. (B) Immunodepletion of cell lysates with antibody against α 7. Cell lysate from X1/D-27 cells (a clone of X1/D cells) were immunoprecipitated with mAb to α 7. Immunoprecipitates were processed for immunoblotting under nonreducing conditions and probed with rabbit antibodies specific to β 1A (left) or β 1D (right) and by chemiluminescence. Lane 1, whole cell lysate; lane 2, control anti-rat IgG-beads; lane 3, anti- α 7. The position of the mature β 1 subunit is indicated. (C) Same procedures as in (B) except the lysates (lane 1) were processed for immunoprecipitation with anti- α 2 integrin (mAb VM1 [lane 2]) and anti- α 3 integrin (mAb J143 [lane 3]) and blotted with antibodies against β 1A (left) or β 1D (right).

Figure 3. Adherence to laminin-1 correlates with the surface expression level of $\beta 1D$. MCF-7 transfectants and clones derived from X1/D double transfectants were compared in standard adhesion assay as described in Figure 1. Parental cells (MCF-7), transfectants expressing $\alpha 7X1$ (X1/A), mouse $\beta 1A$ ($\beta 1A$), or mouse $\beta 1D$ ($\beta 1D$) all showed poor adhesion to laminin-1. The $\alpha 7X1$ and $\beta 1D$ double transfectants (X1/D and its derived cell clones) showed increased adhesion to laminin-1 that correlated closely with the relative level of $\beta 1D$ and $\alpha 7X1$ integrin expression.

Again, similar levels of $\beta 1A$ and $\beta 1D$ were detected in the immunoprecipitates (Figure 2B, lane 3, left and right panels). There seemed to be an abundance of $\beta 1D$ precursor (Figure 2B, lane 1; right) compared with the mature, fully processed form of the subunit. In contrast, nearly all of the endogenous $\beta 1A$ subunit was present in the mature form (Figure 2B, lane 1; left). Similarly, we found that $\alpha 2$ and $\alpha 3$ integrin subunits were associated with equivalent proportions of $\beta 1A$ and $\beta 1D$ (Figure 2C). In other studies, we examined the amount of integrin subunits associated with $\beta 1A$ and $\beta 1D$ by immunoprecipitation of [^{35}S]methionine-labeled cells; similar results were observed (our unpublished data). On the basis of these studies, we concluded that $\alpha 7$ as well as $\alpha 2$ and $\alpha 3$ subunits were able to complex with similar efficiency to $\beta 1A$ and $\beta 1D$ subunits.

Adhesion to Laminin-1 Correlates with $\alpha 7X1$ and $\beta 1D$ Levels

To further define the relationship between $\beta 1D$ and $\alpha 7$ activity, we compared the relative adhesion to laminin-1 of a large series of $\alpha 7/\beta 1D$ -expressing clones and cell lines. A three-dimensional scatter plot summarizing these results indicates that adhesion to laminin-1 strictly correlated not only with the expression of $\alpha 7$, but also with the relative level of expression of $\beta 1D$ (Figure 3). The parental cells with endogenous $\beta 1A$ (MCF-7) or cells transfected with $\alpha 7X1$ alone (X1/A) showed poor adhesion to laminin-1. The $\alpha 7X1$ and $\beta 1D$ double transfectants (X1/D and clones 10, 12, 13,

17, 21, and 27) showed a strong correlation between levels of $\alpha 7$ and $\beta 1D$ expression. Thus, the expression of mouse $\beta 1D$ in MCF-7 cells transfected with $\alpha 7X1$ consistently enhanced the expression of $\alpha 7$ in this system. The level of $\alpha 7$ increased in parallel with that of the transfected mouse $\beta 1D$ and correlated with correspondingly greater adhesion to laminin-1. Furthermore, transfection of cells with mouse $\beta 1A$ or $\beta 1D$ in the absence of $\alpha 7$ to yield the $\beta 1A$ or $\beta 1D$ cell lines did not result in enhanced adhesion to laminin-1. This result indicated that the overexpression of mouse $\beta 1A$ or $\beta 1D$ was unable to augment adhesion activity of endogenous $\alpha 2$, $\alpha 3$, or $\alpha 6$ integrins for laminin-1. In other studies, we generated human $\alpha 7X1$ - and $\alpha 7X2$ -expressing MCF-7 Tet-Off cells and found that these transfectants showed a similar adhesion profile to laminin-1, with $\alpha 7X1$ showing poor binding to laminin-1 and $\alpha 7X2$ showing strong binding (our unpublished data).

Functionality of Human $\alpha 7X1$ in Cells Expressing Tetracycline-regulatable $\beta 1D$

In a parallel approach to establish the role of integrin $\beta 1$ cytoplasmic variants on the expression and function of human $\alpha 7$, we created stable cell lines constitutively expressing either the human $\alpha 7X1$ or $\alpha 7X2$ isoform and tetracycline-regulatable mouse $\beta 1A$ or $\beta 1D$ integrin. These cell lines were designated as HuX1/A, HuX1/D, and HuX2/A were selected for further study. To regulate the expression of mouse $\beta 1$ integrin, cells were cultured in medium containing increasing concentrations of doxycycline, and integrin expression was then measured by FACS by using mAb to $\alpha 7$ integrin and mouse $\beta 1$ integrin. Doxycycline suppressed the expression of mouse $\beta 1$ integrin in a dose-dependent manner (Figure 4A) and at the lower range of doxycycline shown the integrin expression was linear. The sensitivity to regulation by doxycycline repression of HuX1/A and HuX1/D differed slightly; the HuX1/D required about twice the doxycycline concentration to suppress mouse $\beta 1$ integrin expression compared with that required for the HuX1/A. Expression of mouse integrin was completely suppressed with a doxycycline concentration of >1 ng/ml for $\beta 1D$ and 0.65 ng/ml for $\beta 1A$. By modulating the levels of doxycycline, comparable levels of mouse $\beta 1$ integrin expression were reached for both cells (Figure 4A). Due to the difference in sensitivity to doxycycline repression, we chose to use 0.1 and 0.3 ng/ml doxycycline for HuX1/A and HuX1/D, respectively, for subsequent adhesion assays on laminin-1. HuX1/A, HuX1/D, and HuX2/D cells were cultured at this low concentration of doxycycline, the integrin profile as detected by FACS is summarized in Table 2. Importantly, for the Tet-Off cells, high levels of human $\alpha 7$ and the mouse $\beta 1$ level are maintained at increasingly higher levels as the doxycycline concentration was lowered. The mouse $\beta 1$ seems to be the dominant subunit compared with the endogenous human $\beta 1A$. As the level of mouse $\beta 1A$ or $\beta 1D$ integrin was increased after induction, we observed a corresponding increase in $\alpha 7$ surface expression (Figure 4B). The levels of induced expression of $\alpha 7$ were 5–6 times that of the background expression at high doxycycline concentrations. If the concentration of doxycycline was further reduced, the increase in integrin expression eventually reached a plateau (our published data). Analysis of integrin profiles revealed that, besides $\alpha 7$ and $\beta 1D$ subunits, other

Figure 4. β 1D expression promotes adhesion of human α 7X1 Tet-Off cells to laminin-1. MCF-7 cells expressing human α 7X1 and transfected with tetracycline-regulatable mouse β 1A or β 1D, were tested for adhesion to laminin-1. The double transfectants (HuX1/A and HuX1/D) were cultured with the indicated concentration of doxycycline (dox) and then processed for adhesion. Expression levels of mouse β 1 (A) and human α 7X1 (B) was determined by FACS analysis as detailed in the text. Data is expressed as the mean fluorescence intensity (MFI) of 10,000 cells scanned. (C) Expression levels of endogenous α 2, α 3, and β 1A integrins were determined for HuX1/D cells as in (A). (D) Corresponding adhesion efficiency to laminin-1. Values are the mean of triplicate wells; bars indicate SE. x-axis, the concentration of dox used for HuX1/D and HuX1/A are indicated. Cell adhesion was performed as described in Figure 1 with a ligand-coating concentration of 12 μ g/ml.

Table 2. Expression levels of human $\alpha 7$ in tetracycline-regulatable double transfectants

Cell line	Integrin pair $\alpha 7/\beta 1$	Mean fluorescence intensity*		
		Human $\alpha 7^a$	Human $\beta 1A^b$	Mouse $\beta 1A/D^c$
HuX1/A	$\alpha 7X1/\beta 1A$	125	45	130
HuX1/D	$\alpha 7X1/\beta 1D$	110	36	120
HuX2/A	$\alpha 7X2/\beta 1A$	86	40	116

* The transfected cell lines were processed for FACS analysis using the indicated antibody as detailed in MATERIALS AND METHODS. The level of expression is expressed as the mean fluorescence intensity of 10,000 cells scanned. The cells were cultured in low levels of doxycycline as indicated in the text.

^a mAb 9.1, mouse anti-human $\alpha 7$.

^b mAb AIIIB2, rat anti-human $\beta 1$.

^c mAb MB1.2, rat anti-mouse $\beta 1$.

major endogenous integrins (human $\alpha 2$, $\alpha 3$, and $\beta 1A$) remained relatively stable as the regulatable $\beta 1D$ was modulated (Figure 4C and Table 2). It is interesting that as the level of regulatable mouse $\beta 1D$ expression was increased there was no significant increase in the surface expression of $\alpha 2$, $\alpha 3$ integrins (Figure 4C). This may be due to the high level of $\alpha 7$ precursor that is able to compete for available $\beta 1$. Presumably, as more mouse $\beta 1$ is synthesized, the $\alpha 7$ heterodimers continue to be preferentially assembled and expressed at the surface.

We also analyzed the influence of relative $\alpha 7X1$ and $\beta 1$ expression on the adhesion of doxycycline-regulatable cells to laminin-1 in standard attachment assays (Figure 4D). Mouse $\beta 1A$ -expressing cells showed poor adhesion at all levels of $\beta 1A$ integrin expression even though $\alpha 7X1$ was increased several fold over the range of doxycycline treatment (Figure 4B). In contrast, induction of $\beta 1D$ at low doxycycline concentrations resulted in a strong adhesive response to laminin-1 that corresponded to elevated $\beta 1D$ and $\alpha 7X1$ expression levels. In fact, the elevation in adhesion to laminin-1 correlated closely to the increase in $\alpha 7X1$ expression. For comparison, we also analyzed the adhesion efficiency of Tet-Off cells expressing $\alpha 7X2/A$ and $\alpha 7X2/D$. We found no significant difference in adhesion to laminin-1 between $\alpha 7X2$ cells expressing either $\beta 1A$ or $\beta 1D$ (our unpublished data). Both double transfectants adhered strongly to laminin-1 (70–85%). This result indicated that $\alpha 7X2$ is fully functional for adhesion to laminin-1 and that exogenous $\beta 1D$ did not enhance the adhesion further.

Expression of $\beta 1D$ Does Not Alter Adhesion of $\alpha 7X1$ to Laminin-2/4 or Laminin-10/11

The above-mentioned results indicate that $\beta 1D$ was able to modify the binding of $\alpha 7X1$ to laminin-1. We compared the relative adhesion efficiency of $\alpha 7X1$ -expressing cells in the presence of $\beta 1A$ or $\beta 1D$ to available laminin isoforms. We first tested the adhesion of $\alpha 7$ -expressing cells on preparations of human placental merosin. Previous analyses have indicated that these preparations contain primarily human

Figure 5. Expression of $\beta 1D$ does not enhance the adhesion of $\alpha 7X1$ to laminin-2/4. HuX1/A, HuX1/D, and HuX2/A cells were tested for their adhesion to laminin-2/4. (A) Ligand dose-response analysis was done in the presence of a mixture of blocking antibodies to integrins $\alpha 2$, $\alpha 3$, and $\alpha 6$. (B) $\alpha 7$ integrin mediates adhesion of HuX2/A to laminin-2/4. The addition of $\alpha 7$ -perturbing antibody completely blocked the adhesion of X2/A cells to laminin-2/4. The ligand-coating concentration was 15 $\mu\text{g/ml}$. Values are the mean of triplicate wells; bars indicate SE.

laminin-4 ($\alpha 2\beta 2\gamma 1$, S-merosin) with lesser amounts of laminin-2 ($\alpha 2\beta 1\gamma 1$, merosin) (Delwel *et al.*, 1994; Spinardi *et al.*, 1995; Yao *et al.*, 1996a). Thus, we tested the relative adhesion of HuX1/A, HuX1/D, and HuX2/A to human merosin (laminin-2/4) (Figure 5A). Interestingly, cells expressing $\alpha 7X1/\beta 1A$ (HuX1/A) or $\alpha 7X1/\beta 1D$ (HuX1/D) adhered poorly. In contrast, cells expressing $\alpha 7X2/\beta 1A$ (HuX2/A) showed strong attachment. Adhesion by HuX2/A cells to laminin-2/4 was not sensitive to a mixture of mAbs to $\alpha 2$, $\alpha 3$, and $\alpha 6$ integrins, but adhesion was completely blocked by anti- $\alpha 7$ mAb 9.1 (Figure 5B).

In a similar manner, we examined the adhesion capacity of these three cell lines to laminin-10/11 (Figure 6A). In contrast to adhesion on laminin-2/4 substrates, HuX2/A adhered poorly to laminin-10/11. Whereas HuX1/A and

Figure 6. Expression of β 1D does not alter adhesion of α 7X1 to laminin-10/11. HuX1/A, HuX1/D, and HuX2/A cells were tested for their adhesion to laminin 10/11. (A) Ligand dose-response analysis was done in the presence of a mixture of blocking antibodies to integrins α 2, α 3, and α 6 (Figure 5 legend). (B) Addition of α 7-perturbing antibody blocked the adhesion of HuX1/A and HuX1/D cells to laminin 10/11. The coating concentration was 7 μ g/ml. Values are the mean of triplicate wells; bars indicate SE.

HuX1/D both attached well to this substrate, HuX2/A displayed poor adhesion even at high ligand coating concentrations. Treatment of cells with anti-human α 7 blocking mAb effectively inhibited adhesion confirming that this receptor is mediating binding (Figure 6B). Similar results were obtained using recombinant human laminin-10 (our unpublished data). Together, these results show that replacing β 1A with β 1D confers activity of α 7X1 for laminin-1 but does not alter binding of the double transfectants to laminin-2/4 or laminin-10/11 substrates.

Cytochalasin D Modulates HuX1/D Adhesion to Laminin-1

Integrin-cytoskeletal interactions have been shown to influence both integrin clustering and extracellular domain conformation. It is well established that integrins connect to the cytoskeleton through the cytoplasmic domain of the β subunit and evidence indicates that talin may play an important role in regulating integrin activity by binding to the β 1

Figure 7. Cytochalasin D modulates adhesion of HuX1/D cells to laminin-1. HuX1/A, HuX1/D, and HuX2/A cells were assayed for adhesion to laminin-1 in the presence or absence of cytochalasin D. Cells were preincubated with cytochalasin D (20 μ M) or DMSO carrier (0.2%) on ice for 20 min before the adhesion assay. The ligand coating concentration was 12 μ g/ml. For the HuX1/A or HuX2/A cells, treatment with cytochalasin D did not alter the binding to laminin-1. However, for HuX1/D cells, cytochalasin D treatment significantly reduced adhesion to laminin-1. Values are the mean of triplicate wells; bars indicate SE.

cytoplasmic domain (Liddington and Ginsberg, 2002). Furthermore, it has been shown that the β 1D tail binds talin better than the β 1A cytoplasmic domain (Belkin *et al.*, 1997; Pfaff *et al.*, 1998). To test the potential importance of cytoskeleton linkage to the adhesion activity of α 7X1-expressing cells, we assayed binding of MCF-7 Tet-Off double transfectants to laminin-1 substrates in the presence of cytochalasin D, an inhibitor of actin polymerization. Treatment of cells with 20 μ M cytochalasin D did not affect the adhesion of the HuX1/A and HuX2/A cell lines to laminin-1 substrates (Figure 7). However, the presence of the drug reduced HuX1/D cell adhesion to laminin-1 by nearly 70%. Adhesion of all cell lines to type I collagen was unaffected by cytochalasin D (our unpublished data). These results suggest that the formation of polymerized actin during the adhesive process was necessary for the β 1D to confer enhanced binding of α 7X1 to laminin-1.

DISCUSSION

We demonstrate that when the α 7X1 alternatively spliced integrin heterodimerizes with the muscle β 1D subunit, the ligand specificity is enhanced. If α 7X1 is coupled to β 1A, it is unable to induce cell adhesion to laminin-1 but can efficiently mediate adhesion to laminin-10/11. After the exchange of β 1A for β 1D, the receptor can then also promote cell binding to laminin-1. These results show that the replacement of β 1A with β 1D may induce a higher order of activation of α 7, thereby permitting the receptor to bind not only to laminin-10/11 but also to laminin-1. This effect seems to represent an example of ligand affinity modulation of an integrin by the companion β cytoplasmic domain.

The alternative splicing of α 7X1/X2 occurs between the III and IV homology repeat domains, a region implicated in ligand specificity, and affinity. Springer originally proposed that the extracellular portion of integrin α chain is organized

in a cyclic- β propeller structure (Springer, 1997), and this has been confirmed by x-ray crystallography (Xiong *et al.*, 2002). In this model, the alternatively spliced segment of $\alpha 7$, which is predicted to be located outside the "blade" structure joined by the two-homology repeat domains, may modulate folding of these adjacent β sheets, thereby controlling integrin conformation and function. We speculate that $\alpha 7X1$, when bound to the $\beta 1D$ variant, forms a modified active ligand-binding pocket capable of binding laminin-1 and may partially resemble the structure formed by the constitutively active $\alpha 7X2/\beta 1A$. Yet both integrins are functionally distinct because they differentially bind laminin-2 and -10/11.

Exogenous $\beta 1D$ expression in MCF-7 cells did not enhance cell adhesion to laminin-1 in the absence of $\alpha 7$ and, in the presence of $\alpha 7X1$ and $\beta 1D$, the enhancement of adhesion was mainly attributed to $\alpha 7$ and not to the other moderately expressed $\alpha 2$, $\alpha 3$, or $\alpha 6$ integrins that could potentially bind laminin-1. Because there is no preference in the coupling of these α subunits to $\beta 1A$ or $\beta 1D$ (Belkin *et al.*, 1997; Pfaff *et al.*, 1998; this study), our results indicate that $\beta 1D$ -induced functional augmentation in MCF-7 cells is specific for the $\alpha 7X1$ integrin. MCF-7 cells have $\beta 1$ integrin receptors for other ligands, including $\alpha 2\beta 1$ for collagen 1 (Jones *et al.*, 1995; Maemura *et al.*, 1995) and $\alpha v\beta 1$ for vitronectin (Wong *et al.*, 1998). Adhesion to these two ligands was not altered by the expression of $\beta 1D$. In addition, the forced expression of $\beta 1D$ in the fully functional $\alpha 7X2$ cells did not further enhance the adhesion to laminin-2/4 or -10/11 (our unpublished data). These results suggest that the $\beta 1D$ partner subunit can selectively alter $\alpha 7X1$ functionality. However, it is possible that in these cases that the integrin (e.g., $\alpha 2$ or $\alpha 7X2$) may already be at a fully activated level and that binding activity cannot be further enhanced by coupling with $\beta 1D$.

Affinity modulation can occur by inside-out signaling via transmembrane conformational transitions (Faull *et al.*, 1994; Schwartz *et al.*, 1995; Hughes *et al.*, 1998; Longhurst and Jennings, 1998; Liddington and Ginsberg, 2002). Previous studies with cells transfected with $\beta 1D$ indicated that the subunit induced a number of cellular alterations that included decreased spreading and migration, increased binding of fibronectin, and incorporation into matrix (Belkin *et al.*, 1997). These results are consistent with stabilized integrin-cytoskeleton interaction that seems to be mediated by increased binding of $\beta 1D$ with talin and elevated levels of integrin activation. In the context of the current studies, $\alpha 7X1/\beta 1A$ seems to exist in a partially active conformation as indicated by the finding that the TS2/16 mAb could switch this integrin to a functional laminin-1 receptor (Ziobler *et al.*, 1997). Formation of the $\alpha 7/\beta 1D$ complex seems to induce a similar conversion from an intermediate level of activation to a fully active state. There is precedence for intermediate affinity states (Takagi *et al.*, 2002).

Alternatively, $\beta 1D$ -induced activation of $\alpha 7X1$ may be related to postreceptor occupancy events such as receptor clustering and avidity regulation. For example, enhanced tethering of integrins to the cytoskeleton and localized clustering may lead to strengthened adhesion as a result of increased avidity. However, as Springer and colleagues (Shimaoka *et al.*, 2002) have summarized, the data supporting this mechanism of increased integrin activity (avidity modulation) remain controversial. Furthermore, the cellular en-

vironment can also modulate integrin function (Zhang *et al.*, 1996; Hughes *et al.*, 1997). Thus, in contrast to MCF-7 cells, when HT-1080 cells that express only $\beta 1A$ were transfected with $\alpha 7$ splice variants, both $X1$ and $X2$ isoforms were fully active for binding to laminin-1 (Ziobler *et al.*, 1997), indicating that factors related to cell-type specificity are important in regulating $\alpha 7$ integrin function. Additional studies are needed to further define the specific mechanism responsible for $\beta 1D$ -induced functional modulation of $\alpha 7X1$.

Differences in the interaction of $\beta 1$ alternatively spliced cytoplasmic tails with the cytoskeleton have been reported. Previous work by Belkin *et al.* (1997) has shown that overexpression of $\beta 1D$ physically displaces endogenous $\beta 1A$ normally found at adhesion sites with the extracellular matrix. As a result of this displacement of $\beta 1A$ by $\beta 1D$ integrins, cells exhibited a dominant positive phenotype and formed a stronger interaction with the underlying matrix. This activity is apparently related to differences in how $\beta 1D$ and $\beta 1A$ interact with the cytoskeleton. The muscle-specific $\beta 1D$ splice variant has been shown to bind talin with higher affinity than $\beta 1A$ (Belkin *et al.*, 1997; Pfaff *et al.*, 1998). Conversely, the $\beta 1A$ tail has been reported to bind filamin and α -actinin more strongly than does $\beta 1D$ (Belkin *et al.*, 1997; Pfaff *et al.*, 1998). It is possible that in the current experiments the effect of $\beta 1D$ on $\alpha 7X1$ activity is a result of this increased interaction with the actin cytoskeleton. In support of this possibility we found that treatment of HuX1/D cells with cytochalasin D resulted in the loss of $\beta 1D$ induced stimulation of adhesion to laminin-1 (Figure 7). Yet the effect seems to be specific to $\alpha 7$ since other integrins, e.g., $\alpha 2$, which were shown to associate with $\beta 1D$, did not show any enhanced adherence to collagen I. As mentioned above, this may be explained by the possibility that the $\alpha 2$ integrin is already fully activated and adhesion cannot be enhanced further by complexing with $\beta 1D$. Interestingly, Gimond *et al.* reported that replacement of $\beta 1A$ with $\beta 1D$ in embryonic stem cells did not lead to either faster rates or a higher extent of cell adhesion to either fibronectin or laminin 1 substrates via the $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrin, respectively (Gimond *et al.*, 2000). In fact, on both substrates, the $\beta 1D$ cells bound much less efficiently than the $\beta 1A$ cells. This may suggest that depending on the α chain that is complexed with $\beta 1D$, the effect on integrin function may vary.

We found that cells expressing $\alpha 7X1/\beta 1A$ (HuX1/A) adhered poorly to laminin-2/4. In contrast, cells expressing $\alpha 7X2/\beta 1A$ (HuX2/A) showed strong attachment to this ligand. On laminin-10/11, HuX2/A cells adhered poorly whereas HuX1/A cells showed strong affinity. von der Mark and collaborators recently showed that soluble double truncated forms of $\alpha 7X1$ and $\alpha 7X2$ bind differently to various laminin isoforms (von der Mark *et al.*, 2002). Our results are similar in that $\alpha 7X1$ expressing cells bound to laminin-10/11 substrates whereas $\alpha 7X2$ bound preferentially to laminin-1. However, our data differ with regard to $\alpha 7X1$ and laminin-2/4 substrates. In the previous study, both receptors showed intermediate affinities to purified laminin-2. The divergent results may be due to the nature of the laminin preparations used.

We have investigated the mechanism underlying functional regulation of the alternatively spliced $\alpha 7$ variants by its partner $\beta 1$ integrin. The physiological importance of the $\alpha 7$ and $\beta 1$ splice variants is related to the varied roles of $\alpha 7$

as the major laminin-binding receptor in skeletal muscle, where it has multiple functions not only during myoblast motility and differentiation but also in maintenance of mature fiber anchorage and assembly of its underlying basement membrane. When myoblasts begin to differentiate into myotubes, the partner integrin switches from the β 1A to the β 1D cytoplasmic form. Our results indicate that coupling of the α 7X1 variant with the β 1D isoform modifies the receptor's ligand binding specificity and converts α 7X1 to a promiscuous receptor that can mediate adhesion to laminin-1 and other laminin isoforms.

In summary, we have demonstrated that the cytoplasmic domain of the β 1 integrin can modulate the functionality and ligand specificity of the α 7 receptor. We show that when coupled to the β 1D partner, α 7X1 splice variant shows a broader spectrum of ligand specificity and can efficiently bind and mediate adhesion to laminin-1. The formation α 7X1/ β 1D in vivo seems likely given that both subunits are present during the late embryonic stage of skeletal muscle development. This suggests that the simultaneous expression of α 7X1 along with β 1D could facilitate the organization and stability of the MTJ during the early stages of muscle fiber maturation.

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