Title
The beta1 cytoplasmic domain regulates the laminin-binding specificity of the alpha7X1 integrin

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THE BETA1 CYTOPLASMIC DOMAIN REGULATES THE LAMININ-BINDING SPECIFICITY OF THE ALPHA7X1 INTEGRIN

by

Ming-Guang Yeh

DISSERTATION

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ABSTRACT

THE β1 CYTOPLASMIC DOMAIN REGULATES THE LAMININ-BINDING SPECIFICITY OF THE α7X1 INTEGRIN

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Chairperson of the Supervisory Committee: Professor Randall H. Kramer

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The laminin-binding integrin α7 is alternatively spliced in the putative ligand-binding region to yield either the α7X1 or the α7X2 form of the receptor. Similarly, the partner β1 integrin cytoplasmic domain is converted from β1A to β1D. To determine whether β1D modulates the activity of the α7 receptor, we transfected MCF-7 cells expressing mouse α7X1 with β1D cDNA. Cells expressing mouse α7X1 coupled with β1A and failed to adhere to laminin-1, whereas transfectants expressing α7X1 and β1D showed strong adhesion. Contrarily, α7X2 adhered to laminin-1 readily when coupled with β1A. We also generated human α7X1 transfectants in which the expression of β1A or β1D was regulated by the tetracycline promoter system. β1D overexpression induced adhesion of α7X1 cells to laminin-1, whereas induction of β1A expression did not. Attachment to other ligands (e.g., collagen type I) was unaltered. Interestingly, α7X1 whether complexed with β1A or β1D displayed the same level of poor adhesion to laminin-2/4 or

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strong adhesion to laminin-10/11.These findings indicate that α7 function is regulated not only by X1/X2 in its extra cellular domain but also by β1 cytoplasmic splice variants. Expression of β1D appears to alter the integrin ligand specificity of α7X1 by enhancing its binding to laminin-1 but not to laminin-2/4 or laminin-10/11. Functional regulation of α7β1 by developmentally regulated splicing events may be important during myogenic differentiation and repair, because the integrin participates in multiple activities related to cell proliferation and survival, motility, and formation of stable myotendinous and neuromuscular junctions.

Randall H. Kramer, Ph.D.

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Most importantly, I want to thank my wife, Yen-Ping, for all her unending loves and supports.

LIST OF ABBREVIATIONS

BSA, bovine serum albumin

cDNA, complementary DNA;

Col, collagen

DMEM, Dulbecco's modified Eagle's medium

DMSO, dimethyl sulfoxide

Dox, doxycycline

dpc, days post conception

GUS, beta-glucuronidase

LN, laminin

mAb, monoclonal antibody

MTJ, myotendinous junction

NMJ, neuromuscular junction

pAb, polyclonal antibody

PAGE, polyacrylamide gel electrophoresis

PCR, polymerase chain reaction

SDS, sodium dodecyl sulfate

UDG, uracil DNA glycosylase

Chapter 1

Introduction and the Goals of Study

The importance of integrins

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 muscle-specific laminin-binding α7β1 integrin mediates myoblast motility on laminin substrates (Kaufman *et al.*, 1991; Yao *et al.*, 1996a; Yao *et al.*, 1996b; Crawley *et al.*, 1997; von der Mark *et al.*, 2002). In mature skeletal muscle, the α7 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 α7 is demonstrated in α7-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 α7 gene, which develop congenital myopathies (Hayashi *et al.*, 1998; Pegoraro *et al.*, 2002).

Splice variants of integrin α7β1

The α7 integrin subunit is alternatively spliced in both the extracellular (X1 and X2) (Ziober *et al.*, 1993) and the cytoplasmic (A, B and C) domains (Song *et al.*, 1993). One report showed that there was also a D splice variant of α7 in adult human striated muscle

(Leung *et al.*, 1998). However, the function of the D isoform has not been widely studied (Vignier *et al.*, 1999). NMJs have all A, B, and C isoforms of α7; the MTJs have both A and B isoforms of α7; and the non-junction sites have only C isoform of α7 (Hodges *et al.*, 1997). However, α7C splice variant was shown to be non-existent in vivo in one report (Vignier *et al.*, 1999). The purpose of this study is to know the functionality of the α7X1 and α7X2 extracellular splice isoforms. In terms of adhesion to laminin-1, both cytoplasmic A and B isoforms of α7 are equally competent (Yao *et al.*, 1996b). This thesis addresses the difference between α7X1 and α7X2 in the context of the B cytoplasmic tail.

The β1 subunit also has several cytoplasmic splice variances-A, B, C, and D isoforms. They are all derived by alternative splicing from exactly the same position in cytoplasmic tail. β1A is ubiquitously expressed. β1B mRNA is coexpressed at a low level together with β1A mRNA in many tissues, but the protein has been detected only in skin and liver (Balzac *et al.*, 1993). β1B is found only in humans and does not localize to focal contacts and exhibits a dominant negative effect on adhesive events specific to only β1 integrins (Cali *et al.*, 1998; Armulik *et al.*, 2000). β1C has two subtypes: β1C-1 (Languino and Ruoslahti, 1992) and β1C-2 (Svineng *et al.*, 1998). β1C-1 was shown to be expressed in placenta, normal platelets and several blood cells lines (Languino and Ruoslahti, 1992). Both β1C-1 and C-2 were shown to be growth inhibitory (Fornaro *et al.*, 1998; Fornaro *et al.*, 1999; Meredith *et al.*, 1999). β1D is present only in cardiac and skeletal muscles in vertebrates. In early embryogenesis, the only β1 isoform present in

striated muscle is β1A. Late in development, β1D is expressed and replaces β1A completely in adult striated muscle (van der Flier *et al.*, 1995; Zhidkova *et al.*, 1995; Belkin *et al.*, 1996). The detailed temporal expression pattern is discussed elsewhere under the section of "integrin profile during myogenesis". The switch from β1A to β1D subunit in cardiac muscle cells coincides with the expression of α7 in mouse (van der Flier *et al.*, 1997).β1D is present in myotendinous junctions and costameres in skeletal muscles, and in intercalated discs of cardiac muscle. The amount of β1D in different types of skeletal muscle (fast, slow, and mixed type) is similar, but cardiac muscle expresses about five times as much as skeletal muscle (van der Flier *et al.*, 1997). This thesis addresses the different effects of β1A and β1D.

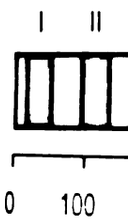
This complex differentiation-dependent pattern of α7 and β1 splicing allows the generation of a unique set of variant α7β1 complexes that are structurally, and presumably functionally, distinct.

Structure of α7 integrin

The α subunits of integrins can be classified into two major groups. The first one contains a 180 amino acid insert between the second and the third homology repeat (see below). This insert is called I domain and is homologous to the collagen binding site found in von Willebrand factor. The crystal structure of this domain has been solved (Lee *et al.*, 1995). This group includes α1, α2, αM, αL, and αX. The second group does not have the I-domain; α3, α4, α5, α6, α7, αIIb, and αV belong to the non-I-domain group.

Besides the presence of I-domain, integrin α subunits are highly conserved. The N-terminus of extracellular domain of all α subunits has 7 homologous repeats (see Figure 1).

Figure 1.



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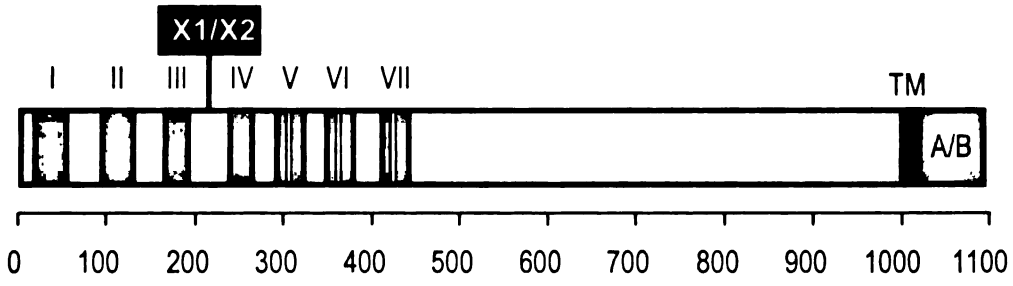

Figure 1. The α7 integrin subunit. The relative positions of the identified alternatively spliced sites in the extracellular domain (X1 and X2) and the cytoplasmic domains (A and B) are indicated. Also shown are the seven homology repeat domains (I-VII, gray boxes) and divalent-cation-binding sites (white slots), and the transmembrane domain (TM).

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According to one model, the 7 homology repeats region forms a 7-bladed β-propeller (Springer, 1997). According to this model, each blade is composed of 4 invariable regions and 4 alternate variable regions. The nomenclature for these regions has also been coined according to this model. Basically, only the invariable regions are designated alphabetically from N to C terminus. There are 4 invariable regions in each blade, so it starts from A, then followed by variable region, then invariable region B, then variable region, then invariable region C and so on. To distinguish the invariable regions from each blade, a number is put before the alphabet to denote its blade position. For example, 3C is the third invariable region in the homologous repeat III (see Figure 2). As shown in figure 2, regions 3B, 3C, and 3D are the 3 invariable regions of blade III, and 4A is the first invariable region of blade IV. In the transition from 3D to 4A, a highly conserved motif (CUP) of this blade constitute one seventh of the cup region which forms the top of propeller and holding an arginine residue from β_1. Recently, this model has been confirmed by X-ray crystallography (Xiong *et al.*, 2001). Crystal structure of the extracellular segment of integrin αVβ3 in complex with an Arg-Gly-Asp ligand has also been reported (Xiong *et al.*, 2002). The crystal structure reveals that the ligand peptide inserts into a crevice formed between the propeller of α7 and the βA domain of β1. More precisely, the arginine side chain inserts into a narrow groove at the top of the propeller domain, formed primarily by the homology repeat III and IV. The asparagine of the ligand peptide contains a carboxylate group, which protrudes into a cleft between the βA loops (A'-α1 and C'-α3) of β1. The rest of the α subunits consist of β-sandwich domain, transmembrane region, and cytoplasmic domain.

Figure 2.

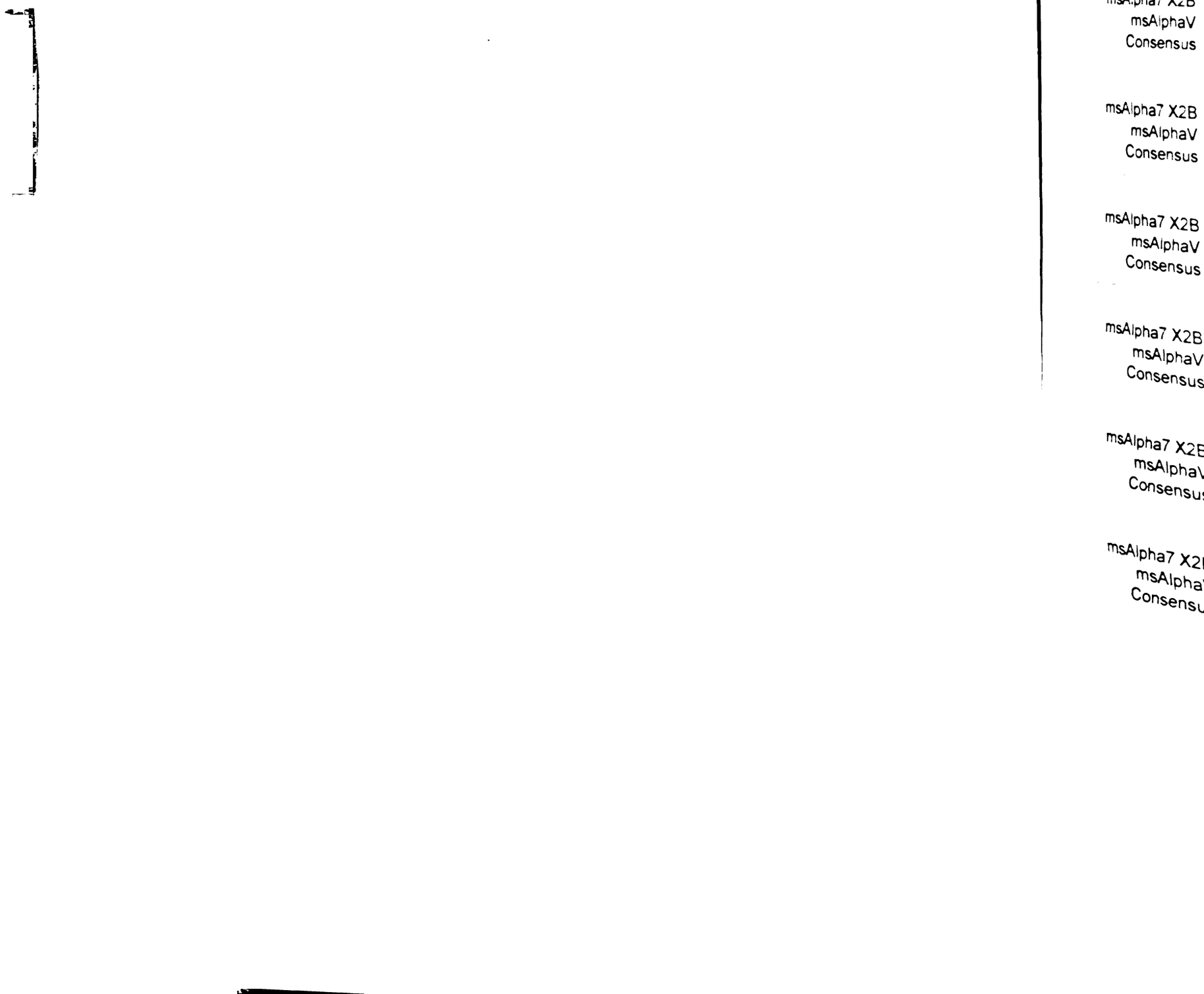
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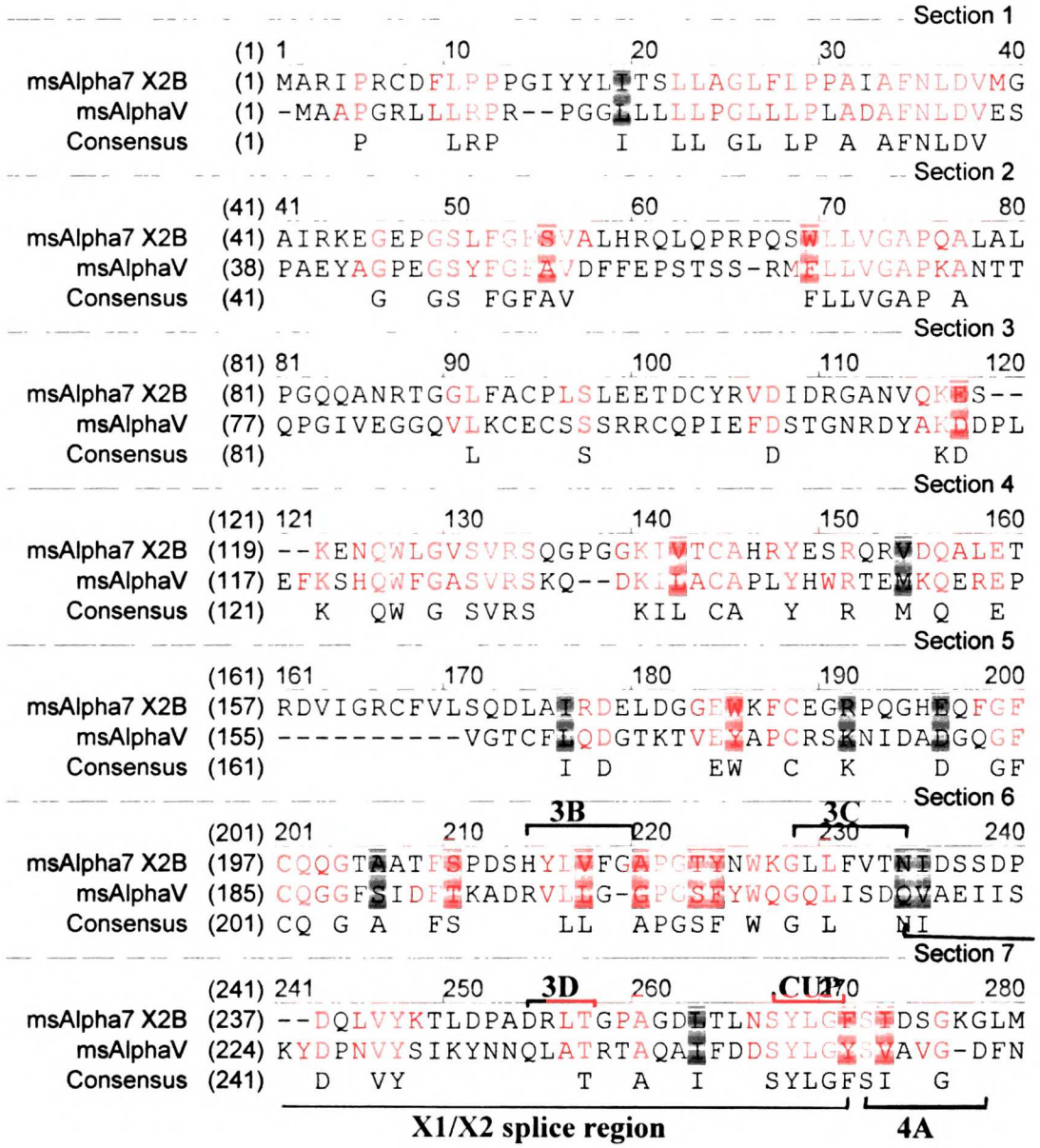
Figure 2.

N-terminus protein sequence alignment of αV and X2B



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The X1/X2 alternative splicing in the α7 mRNA occurs in the variable region between the III and IV homology repeat domains near the putative ligand-binding site, where it may define ligand specificity, affinity, or ligand-binding competence (Irie *et al.*, 1995; Kamata *et al.*, 1995; Tozer *et al.*, 1999). Aligning the α7 sequence to the αV sequence published in the crystal structure paper with sequence alignment tool (Vector NTI, version 6 from InforMax), the X1/X2 alternative splice region spans the invariable region 3C to the 4A (see Figure 2). Amino acid residues in the αIIb subunit those are critical for ligand binding to integrin αIIbβ3 are identified in the β-propeller model (Kamata *et al.*, 2001). Using alanine scanning strategy, they found several hot spots for ligand binding. Most of them are within the 3B to 4A regions. According to alignment by Vector NTI program, this region includes the X1/X2 splice region.

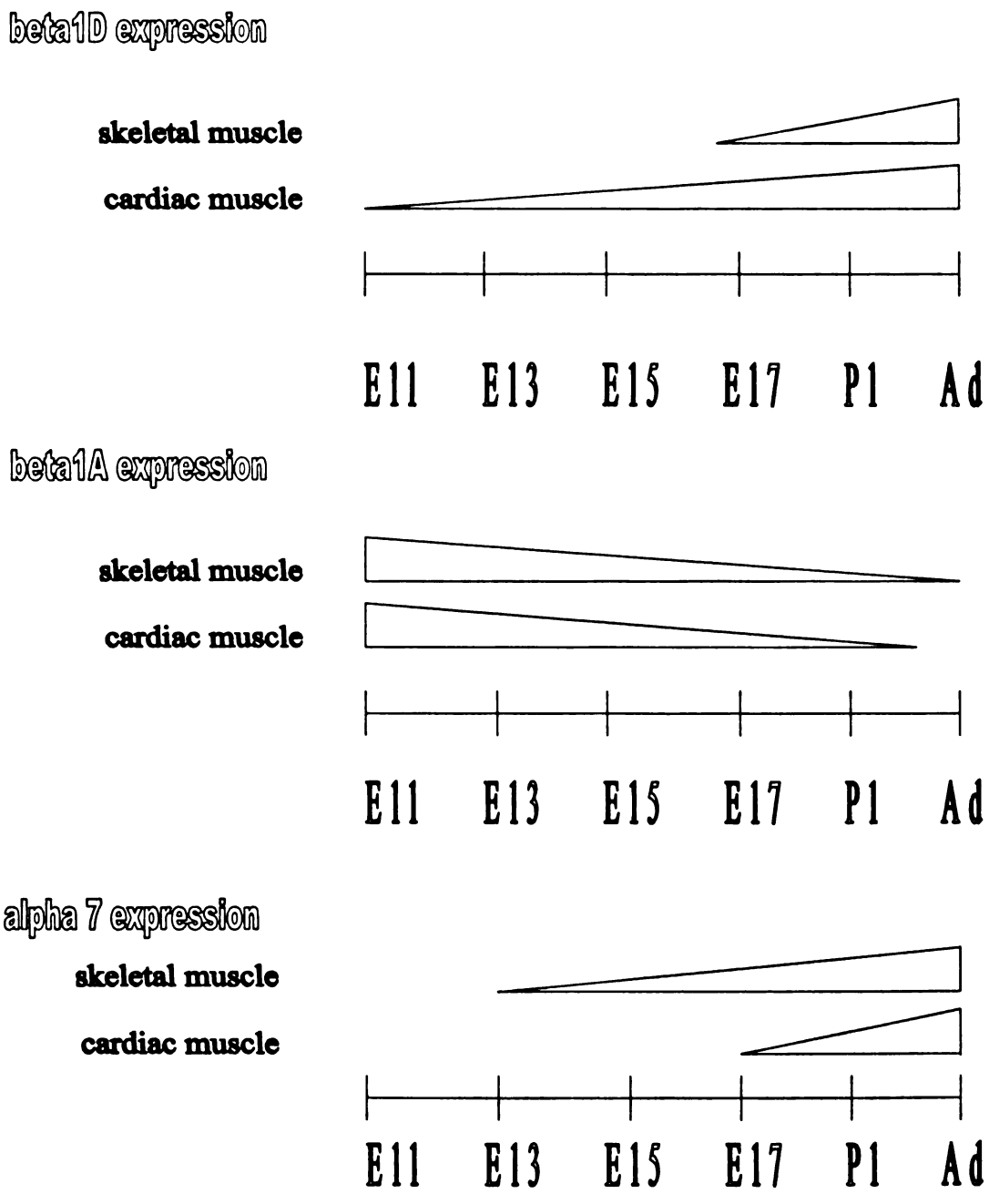
Developmentally regulated expression of α7β1 variants

The formation of a multinucleated muscle fiber is an extraordinary dynamic process involving multiple cell adhesive interactions consisting of homotypic and heterotypic cell-cell interactions as well as interactions with extracellular matrix (ECM) molecules. Early studies of integrins on myogenic cells revealed that β1 integrins localized to focal contacts, costameres, neuromuscular junctions, and myotendinous junctions. One major ECM component in the basement membrane (BM) of striated muscle tissue is laminin. Integrin α7β1 has been shown to be the receptor for this ligand in skeletal muscle (Vachon *et al.*, 1997). As been mentioned earlier, α7β1 integrin has splice isoforms whose expression is developmentally regulated. Data gathered from

studies on mouse myogenesis is summarized by Gullberg *et. al.* (Gullberg *et al.*, 1998) and by Brancaccio e*t. al.* (Brancaccio *et al.*, 1998). The major focus of this study is the α7 X1/X2 and β1 A/D splice variants and the developmental expression patterns of these isoforms are schematized in the following figure (Figure 3).

Figure 3.

Timing of expression of integrin in developing mouse tissue, based on western and immunochemistry, adopted from Brancaccio *et al. (1998).*



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Spatial and temporal distribution of laminin isoforms

The basement membrane (BM) surrounding muscle cells can be classified into three domains. First, the domain where muscle end-points attach to tendons is called the myotendinous junction (MTJ). During development and growth, the MTJ is subject to dynamic changes. According to electron microscopic studies on developing MTJs in avians, basement membrane deposition is one of the first steps in MTJ formation (Tidball and Lin, 1989). Later, junctional folds and myofibrial insertion occur. In the adult, the MTJ is the major site for the transmission of force from the muscle cell interior to the surrounding ECM. Second, the specialized structures where motor neurons attach are called neuromuscular junctions (NMJ). Third, the basement membrane surrounding the majority of the sarcolemma, excluding the MTJs and the NMJs, is referred to as the sarcolemma BM.

Laminin is a heterotrimer of α, β and γ chains. The laminin family currently consists of at least 12 heterotrimers that are formed from five α chains, three β chains and three γ chains (Timpl and Brown, 1994; Timpl, 1996; Colognato and Yurchenco, 2000). The detection of temporal and spatial distribution of laminin heterotrimers is complicated by the presence of multiple chains and not much information has been reported. In developing and mature mouse muscle, the expression of laminins is complex, and is summarized in Table I (Gullberg *et al.*, 1999). Due to the lack of a full battery of antibodies to detect human laminin α chains (even the specificity of detecting mAb 4C7 has previously been erroneously attributed to α1) and to the difficulty of sampling, knowledge about laminin isoforms in human muscle has been only fragmentary (Pedrosa-

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Domellof *et al.*, 2000). In human muscle, laminin α1 chain is expressed at myotendinous junctions as early as 8 weeks of gestation and is evident at 22 weeks (Pedrosa-Domellof *et al.*, 2000). In the MTJ of adult human, only a rudimentary amount of the laminin α1 chain is detected. Similarly, in mouse muscle formation, the α1 chain is present at the MTJ from E11 through the late fetal stage, but not at the sarcolemma or neuromuscular junction basement membrane (Patton *et al.*, 1997). The laminin α5 chain has a similar expression pattern as that of the α1 chain. It was also detected at 8-10 weeks in the MTJ, but it is also expressed at other two BM compartments. Laminin α2 chain has a totally different expression than α1 or α5. It was detected at all the BM compartments from 12 weeks onward.

In summary, the early accumulation of the laminin α2 and α5 chains at the MTJ is followed by expression of these isoforms along the entire length of the developing myotube. On the contrary, the α1 chain was restricted only to MTJs. In the adult, α1 and α5 staining was diminished at the MTJ but is still weakly detectable whereas α2 chain was strongly expressed.

Table I. Tempo

α7	
α7X1[5]	
α7X2[5]	
β1D	
Laminin-1	
Laminin-2 4	
Laminin-10 11	

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Table I. Temporal distribution of α7, β1D, and laminin isoforms in mice

	Early embryo	Fetus	Late Fetus	New Born	Adult
α7	-*	+**	+++	+++++	++++++++
α7X1$	-	+/-***	++	+	+/-
α7X2$	+/-	+	+++	+++++	++++++++
β1D	+/-	+	+++	++++	+++++++
Laminin-1	+++++	+++	++	+	+/-
Laminin-2/4	+	++	+++	++++	++++
Laminin-10/11	+++	+++	+	+	+/-

*Not detected

**Weak expression.

***The more + sign, the stronger the expression.

$Detect mRNA by PCR

Goals of the study

Integrins are capable of changing their function dynamically (Diamond and Springer, 1994; Humphries, 1996; Mould, 1996; Hughes and Pfaff, 1998). It has been proposed (reviewed in (Liddington and Ginsberg, 2002)) that certain intracellular events lead to alterations in integrin conformation, resulting in enhanced adhesion to ligand (inside-out signaling). In addition, the process of ligand binding may regulate the 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 linker proteins (e.g., talin) provide one mechanism that can regulate integrin activation. This process may result in changes in

integrin affinity or integrin-clustering events leading to increased avidity (Calderwood *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, following myoblast differentiation into myotubes and mature muscle, the β1integrin 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 (Schwartz *et al.*, 1995; Hughes *et al.*, 1996; Crommie and Hemler, 1998; Calderwood *et al.*, 1999; Woodside *et al.*, 2001; Liddington and Ginsberg, 2002), it is possible that the β1D isoform controls α7X1/X2 function. To investigate this possibility, we examined the functionality of α7 integrin in the presence of the β1A or β1D cytoplasmic domain. The ligands studied were laminin-1, laminin-2/4, and laminin-10/11 because expression of these laminin isoforms switches during the time period when β1A switches to β1D and because α7β1 is the major receptor for laminin. The cell line used for this study was the breast carcinoma cell line MCF-7, because this cell line neither express α7 nor bind to laminin-1 and because α7X1 and α7X2 binds differently to laminin-1 when expressed in MCF-7. There are several non-integrin receptors also known to bind laminin. Dystroglycan is the most important one in this category. It is now apparent that dystroglycan plays a critical role in the pathogenesis of several muscular dystrophies and is involved in early development, organ morphogenesis, and synaptogenesis. Dystroglycan interacts with a wider repertoire of extracellular ligands other than laminin. The interaction of the cytoskeleton with dystroglycan is not as efficient as that with integrin. In the MCF-7 tissue culture system, the adhesion to ECM mainly is attributed to integrin but not dystroglycan or other

receptors in sho

laminin-binding

Chapter 2

Materials and Methods

ECM proteins

For cell adhesion studies, several different ligands were used as described previously. Laminin-1 isolated from the mouse EHS tumor was obtained from Gibco-BRL (Life Technologies, Invitrogen, Carlsbad, CA). Human placental merosin, consisting of laminin-2/4 isoforms purified by EDTA extraction followed by ion-exchange chromatography was purchased from Gibco-BRL. Human laminin-10/11, isolated from placenta by mild pepsin digestion and by affinity chromatography on mAb 4C7-coupled Sepharose, was also from Gibco-BRL. Collagen was obtained from Cohesion (Palo Alto, CA). Human vitronectin and fibronectin were gifts from Caroline Damsky (University of California, San Francisco; UCSF).

Antibodies

Antibodies against integrin subunits—rat anti-human β1 (mAb AIIB2), mouse anti-human α2 (mAb VM1), rat anti-human α6 (mAb GoH3), and rabbit anti-mouse β1D cytodomain (polyclonal antibody anti-β1D)—were kindly provided by Caroline Damsky, Vera Morhenn (SRI International, Menlo Park, CA), Arnond Sonnenberg (Netherlands Cancer Center, Amsterdam), and Eva Engvall (Burnham Institute, La Jolla Cancer Research Center, CA), respectively. Mouse anti-human α3 (mAb J143) was from the

American Type Culture Collection (ATCC) (Manassas, VA); rat anti-mouse β1 (mAb MB1.2) and mouse anti-human β1 (mAb 2000) were from Chemicon (Temecula, CA); hamster anti-mouse/human β1 (mAb Ha2/5) was from Pharmingen (BD Bioscience, San Diego, CA); rat anti-mouse α7 (mAb CY8), mouse anti-human α7 (mAb 9.1), rabbit anti-human β1 cytodomain (pAb 22778), and rabbit anti-mouse α7 light chain (pAb 1211) were from our laboratory, as previously described (Yao *et al.*, 1996b; Yao *et al.*, 1997; Vizirianakis *et al.*, 2001). Fluorescein-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Construction of Mouse β1D cDNA

The full-length mouse β1D cDNA was prepared by reverse transcription followed by polymerase chain reaction (PCR) from a mouse skeletal muscle cDNA library (Clontech, Palo Alto, CA). The two primers for PCR were: reverse primer, 5′-CTAGTCTAGAATTCAGAGACCAGCTTTACGTCCATAG and forward primer, 5′-CGCGGATCCGAATTCAAGATGAATTTGCAACTGGTTTCCTG. The sequence of full-length mouse β1D was verified at the Biomolecular Resource Center (UCSF). The mouse β1D cDNA was then ligated into *Bam*HI and *Xba*I sites of pcDNA3.1/Hygro (Invitrogen, Carlsbad, CA). Full-length mouse β1A cDNA was obtained from Louis Reichardt (UCSF) and was cloned into the regulatable expression vector in the same manner as β1D (see below).

Cloning of human α7X1

Full-length human α7X2 cDNA (Vizirianakis *et al.*, 2001) was used as a template for the generation of full-length human α7X1. The X2 exon was replaced with the X1 exon by uracil DNA glycosylase (UDG) excision. The generation of cohesive ends on the PCR product of the X1 exon was performed by UDG-mediated excision of uracil. 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 α7 cDNA using human fetus limb muscle RNA as template (forward primer: 5′ -AAGGGUACUGCCAGGGTGGAGCTCTGTG; reverse primer: 5′ -AGAAGCCAAAGUAGCUGTTGGCAGGGA). The full-length cDNA proximal to the splicing site was also generated by PCR (forward primer: 5′ -CCGGGATCCATGGCCGGGGCTCGGAGCCG; reverse primer: 5′ -AGTACCCUUCCAATTATAGGTTCCTGGGG). The primers for full-length cDNA distal to the splicing site were as follows: forward primer, 5′ -AGCTACTTTGGCUUCUCTATTGACTCGGGGAAAGGTCTG; reverse primer, 5′ -CCGCTCGAGCTAGGCGGTGCCTGGCCCT. The three PCR products were first treated with UDG according to the manufacturer's protocol (Gibco-BRL) and were then ligated. The ligation product was purified by gel electrophoresis followed by cloning into pCDNA3.1/Hygro and verified by sequencing.

Generation of Cell Lines

MCF-7 human carcinoma cells and the α7-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 α2β1, α3β1, and α6β1, which are potential receptors for this ligand. Previously we generated MCF-7 cells expressing full-length mouse α7 cDNA containing either the X1 or X2 splice form, which formed heterodimers with the partner β1A subunit. MCF-7 cells expressing the splicing isoform α7X2 were active in binding laminin-1, whereas cells expressing α7X1 lacked ligand-binding activity (Ziober *et al.*, 1997). The original α7X1-expressing population was further enriched by fluorescence-activated cell sorting (FACS) using mAb CY8 against mouse α7 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 α7X2 which heterodimerizes with the endogenous human β1A (Yao *et al.*, 1996a).

Experimental approaches

Expression of mouse β1D in MCF-7 cells was generated in two different systems (see Flow-chart I). In the first, the X1/A-p cells were stably transfected with mouse β1D cDNA by the calcium phosphate procedure (Mammalian Transfection Kit; Stratagene, La Jolla, CA). Following selection with hygromycin, the double transfectants (α7X1/β1D) were enriched by FACS after labeling with anti-mouse β1 antibody (mAb MB1.2; Chemicon). 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 β1D was

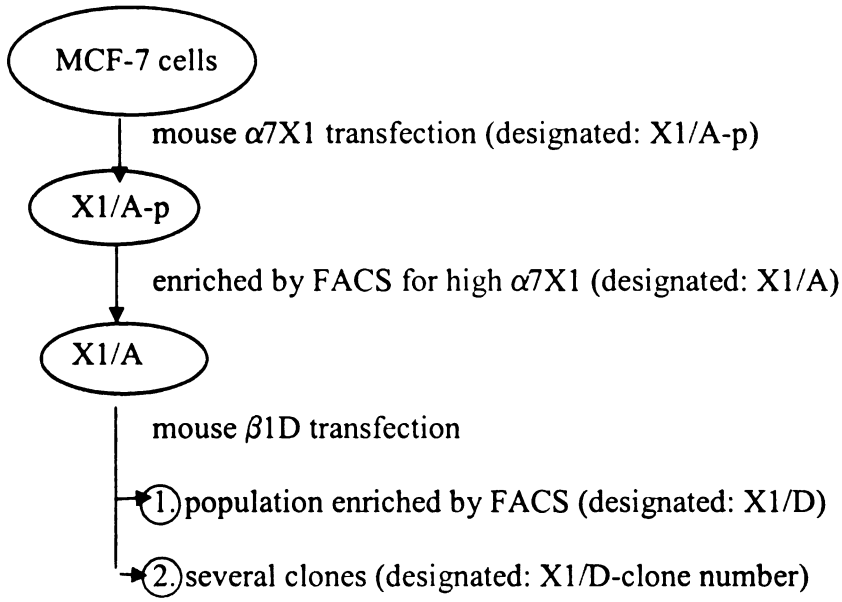
verified by immunoblot analysis using a β1D-specific pAb from Eva Engvall and by flow cytometry using mAb MB1.2. A number of clones with a range of α7 and β1D levels, as determined by FACS, were chosen for further study.

In the second approach, MCF-7 cells were transfected with pTet-Off (pUHD15-1 neo; 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 α7X1 integrin (as described above) or human α7X2 integrin (Vizirianakis *et al.*, 2001). We used anti-human α7 mAb 9.1 to enrich the α7-expressing population by FACS. Retroviral transduction was then used to introduce the pRevTRE plasmids (Clontech) with mouse β1A or mouse β1D into the human α7 stable transfectants. In brief, Phoenix Ampho retrovirus packaging cells from the ATCC were transfected with pRevTRE β1A/β1D via calcium phosphate. The mouse β1D or mouse β1A 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 α7-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 β1 ectodomain, and cell lines with high expression of mouse β1 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 α7X1/mouse β1A, human α7X1/mouse β1D, human α7X2/mouse β1A, or human α7X2/mouse β1D were designated HuX1/A, HuX1/D, HuX2/A, and HuX2/D,

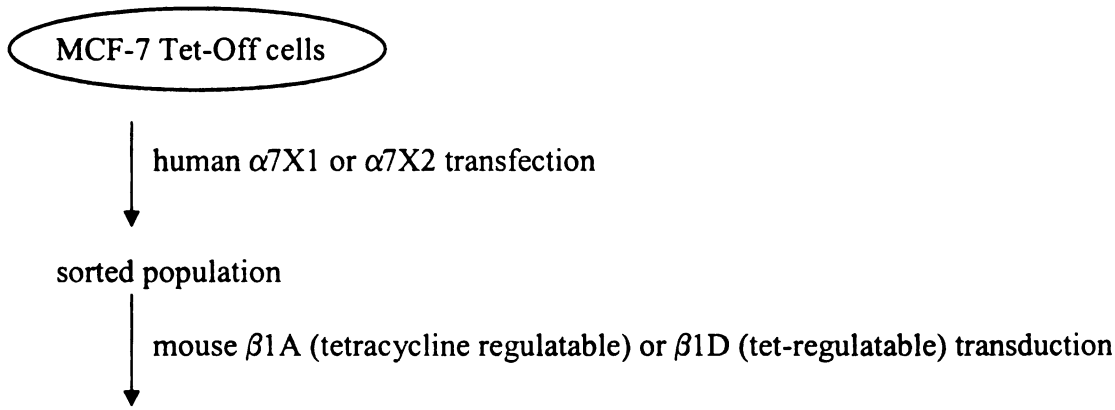
Flow-Chart I.

EXPERIMENTAL APPROACH:

System 1



System 2



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 a FACScan (Becton Dickinson, San Jose, CA). Samples without primary or secondary antibody were always included as a control. Data are expressed as the mean fluorescence intensity (MFI) after subtraction of background staining produced by secondary antibody alone (<5% of signal). The concentration of primary and secondary antibodies was indicated at the beginning of material and methods section.

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 PBS 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 to 60 min at 37°C. Adherent cells were fixed, stained with crystal violet, and solubilized in 2% SDS. Absorbance was read at 562 nm. Background cell adhesion to 1% BSA-coated wells (usually <5% of value) was subtracted from all readings. The effect of specific blocking antibodies was tested by preincubating the cells with an optimal blocking concentration of mAb on ice for 30 min

prior to the assay. Optional concentrations of the blocking antibodies were predetermined and used as previously described (Yao *et al.*, 1996a; Ziober *et al.*, 1997). The concentrations of integrin function-blocking mAb were as follows: 10 μg/ml of purified GoH3 (anti-α6) and J143 (anti-α3). VM1 (anti-human α2) ascites were used at a dilution of 1:400. Both CY8 (anti-mouse α7) and AIIB2 (anti-human β1) 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) at 5 mg/ml. 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 μM cytochalasin D or DMSO alone for 20 min on ice before the assay was begun.

Immunoprecipitation and Western Blotting of Cell Lysates

Cell lysates were extracted with lysis buffer (1% Triton-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, three aliquots of 600 μg of total protein of cell lysates were immunoprecipitated with antibody against α2, α3, or α7 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% BSA) three times and heated at 100^0C in SDS sample buffer. Samples were processed for SDS-PAGE under nonreducing conditions and then transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membranes were incubated with rabbit antibody against

human integrin β1 (pAb 22778) (Martin *et al.*, 1996; Yao *et al.*, 1997) or mouse β1 (mAb 2000; Chemicon). In a different approach to assess the α7 preference to β1A or β1D, equal amounts of cell lysate were immunoprecipitated with human β1(A) or mouse β1(D) antibody for three consecutive rounds. The samples were then processed as described above except under reducing conditions for immunoblotting and probed with rabbit antibody (pAb 1211) against mouse α7 (Yao *et al.*, 1997).

Quantitative PCR

HPLC purified salt-free primer for the target gene mouse α7X1 (forward primer, GCAAGACCCCGCCTC; reverse primer, CTATCCTTGCGCAGAATGAC), for mouse α7X2 (forward primer,ACCCTGCTGACCGGCTC; reverse primer, same as X1) and for total α7 (forward primer, AACCAATGGCTGGGAGTCAG; reverse primer, ATCCCGAGTCTCCAAAGCCT) were generated commercially (BioMolecular resource, UCSF). These primers were designed so that the melting temperature was 10^0 C lower than the specific probes and were optimized with regard to AmpliTaq Gold (ABI) and $MgCl_2$ concentration at 60^0 C.

Total RNA was extracted from C2C12 myoblasts (ATCC) as described previously. Isolated total RNA integrity was electrophoretically verified by ethidium bromide staining and the purity was checked by an average optical density (OD) OD_{260}/OD_{280} nm absorption ratio of 1.8. 5 μg of total RNA was reverse transcribed with 250 U MuLv reverse transcriptase (Gibco-BRL) in a volume of 100 μL, using 5 μM random hexamer primers (Gibco) according to following protocol: 25 ^{0}C 10 second, 48

^{0}C 40 second, 95 ^{0}C 5 second, with dNTP 1 mM, $MgCl_2$ 7.5 mM and RNAse inhibitor. cDNA of 25 ng and three 10 times serial dilutions were used to check the efficiency of probes for quantitative PCR. FAM(5'-) and TAMRA(3'-) tagged probes were commercially generated from ABI (Athens, GA). The probe specific for mouse α7X1 was: TCCCGGTCCCTGCCAACAGC. The probe for mouse α7X2 was: AGGACCAGCCGGAGACTTGACCTTG. The probe for total α7 was: CAAGATTGTTACGTGTGCACACCGATATG. For all of the quantitative PCR in this experiment, relative expression was obtained in comparison to mouse GUS gene expression, thus eliminating the operator loading error for the differences in the amount of cDNA added to each reaction. Real-time master mix was prepared as follows: $MgCl_2$ (7.5 mM), forward primer (480 nM), reverse primer (480 nM), probe (120 nM), dNTP (200 μM), 50 ng cDNA and 1.3 U AmpliTag Gold per 50 μL reaction in buffer A. Each sample was in triplicate. The machine and software for quantitative PCR was PRISM 7700 and Sequence Detection Software version 1.6 (ABI). A four step experimental run protocol was used: (i) initial denaturation program (10 min at 95^{0}C); (ii) amplification and quantification program repeated 40 times (15 second at 95^{0}C; 60 second at 60^{0}C); (iii) melting curve program (60-95^0 C with a heating rate of 1^{0}C per second and a continuous fluorescence measurement); (iv) cooling program down to 4^{0}C at the end of the 40th cycle. For the determination of relative expression from mRNA it is necessary to determine the threshold cycle (Ct) for each transcript. The Ct is defined as the point (in terms of cycle number) at which the fluorescence rises appreciably above the background fluorescence. Usually, this value is determined by the software automatically.

Growth curve analysis

The MCF-7 Tet-Off double transfectants were kept in the media containing doxycycline (10 ng/ml). The day before the start of growth curve analysis, these cells well seeded at 6000 cells per well in the 96-well tissue culture plates. For each cell line, a triplicate was kept in doxycycline while the other triplicate was grown in the absence of doxycycline. The medium was replenished every day. At a specific time point, the cells were gently washed and fixed with 0.5 % formaldehyde. At the end of the experiment, all sample plates were gently washed, stained with crystal violet, and solubilized in 2% SDS. Absorbance was read at 562 nm as described in adhesion assay.

Chapter Three

Characterization of β1D-expression cells

Generation of β1D-expression cells

To test the hypothesis that alternatively spliced amino acid sequences in the β1 cytoplasmic domain may regulate the activity of the α7β1 integrin, we transfected human MCF-7 cells with mouse α7X1 alone (X1/A-p cell line) or with both mouse α7X1 and mouse β1D cDNA (X1/D cell line). Following selection, the transfectants expressed high levels of α7X1 and β1D at their surface, as detected by flow cytometry using a mAb directed against α7 (CY8) or mouse β1 (MB1.2) (Table II). Besides the population of double transfectants, a series of clones were also isolated by serial dilution and by single cell FACS sort (designated as X1/D-clone number). We also acquired high α7X1-expressing cells by sorting the high expresser of α7X1 by FACS using the antibody against α7 in X1/A-p cells. The cells derived were designated as X1/A. We previously reported that MCF-7 cells expressing α7X2 with the endogenous human β1A partner adhered readily to laminin-1, so this cell line was included in this study and was designated as X2/A. MCF-7 cells transfected with mouse β1D or with β1A were generated for control purposes and were designated as β1D cells and β1A cells, respectively.

The expression of β1D specifically augments the expression of α7X1

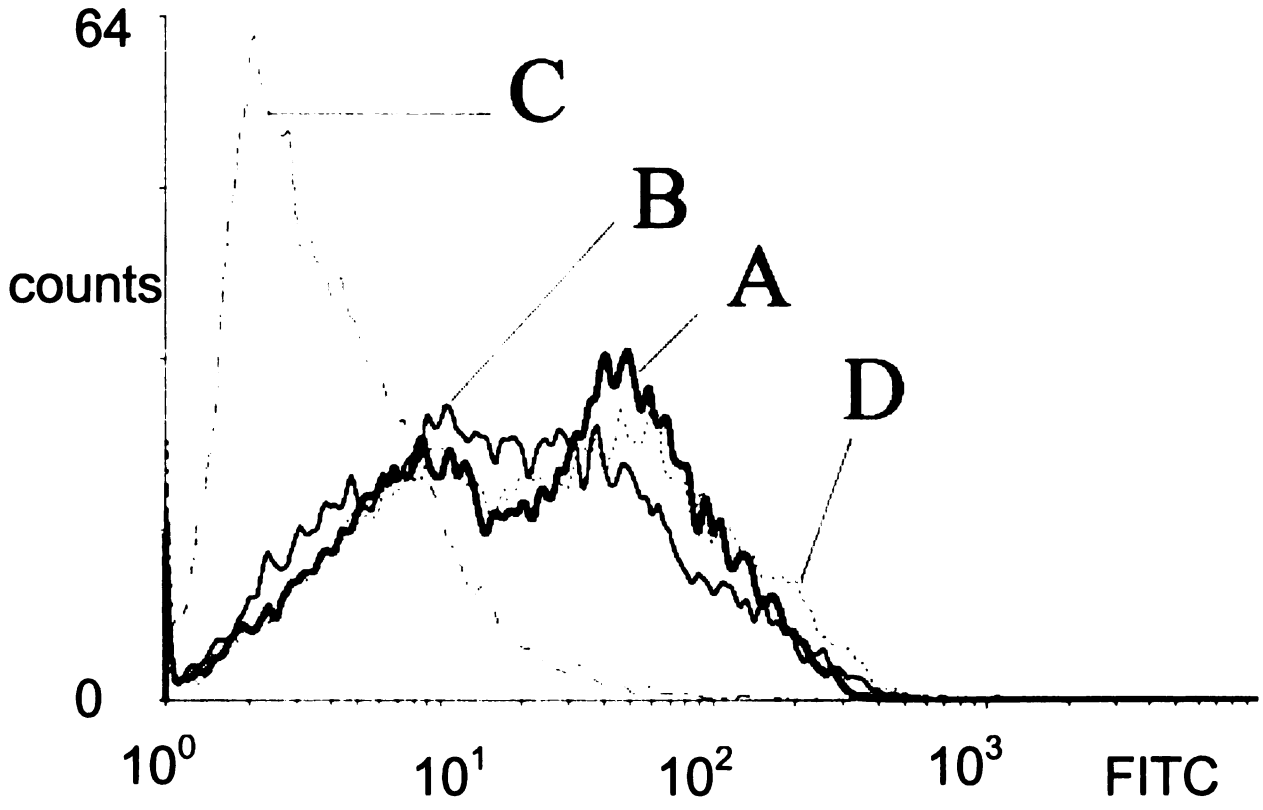
The surface expression profile of potential integrin receptors for laminins in MCF-7 cells and single or double transfectants is shown in table II. The expression of β1D usually augments the expression level of α7X1 as evidenced by FACS analysis. It is necessary to increase the expression level of α7X1 in the single transfectant so that the level of α7X1 in X1/A cells is comparable to that in X1/D cells. This is mandatory so that the adhesive properties can be compared. After enrichment of the expression of α7X1, the α7X1 level of X1/A cells was comparable to that of X1/D cell as evidenced by FACS scan (Figure 4). Other α subunits also varied; however, the variation was normally distributed and no specific trend was noticed (Appendix I). The morphology and growth rates were not changed noticeably.

The expression of β1D does not affect the general adhesive properties of α7X1

To test 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/D and X1/A cells showed a similar ligand dose-response pattern (Figure 5 A, B) over a range of ligand-coating concentrations. Thus, expression of β1D did not alter the adhesion of α7X1-expressing cells to these components. Previous reports have shown that in MCF-7 cells, adhesion to vitronectin and type I collagen was mediated primarily by αVβ1 (Maemura *et al.*, 1995) and α2β1 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. The same procedures were carried out in one of the clones, X1/D-27, and the results were the same (Figure 4 and Figure 6 A, B).

Figure 4. α7X1 expression profile in MCF-7 α7X1 and β1D transfectants



A. X1/A cells

B. X1/D cells

C. Control sample

D. X1/D-27 cells

Table II. Surface expression levels of α7/β1 in MCF-7 and transfectants

Cell line	Integrin pair*	Mean fluorescence intensity		
		α7[a]	Huβ1A[b]	Msβ1D[c]
MCF-7	— β1A	—	78	—
X1/A	Msα7X 1/β1A	43	86	—
X1/D	Msα7X 1/β1D	32	55	20
X2/A	Msα7X 2/β1A	40	58	—

*The parental MCF-7 cells and transfectants were processed for FACS analysis as described in Material and Methods section. The Huβ1A is the endogenous integrin partner.

[a]mAb CY8, rat anti-mouse α7.

[b]mAb AIIB2, rat anti-human β1.

[c]mAb MB1.2, rat anti-mouse β1.

Figure 5.

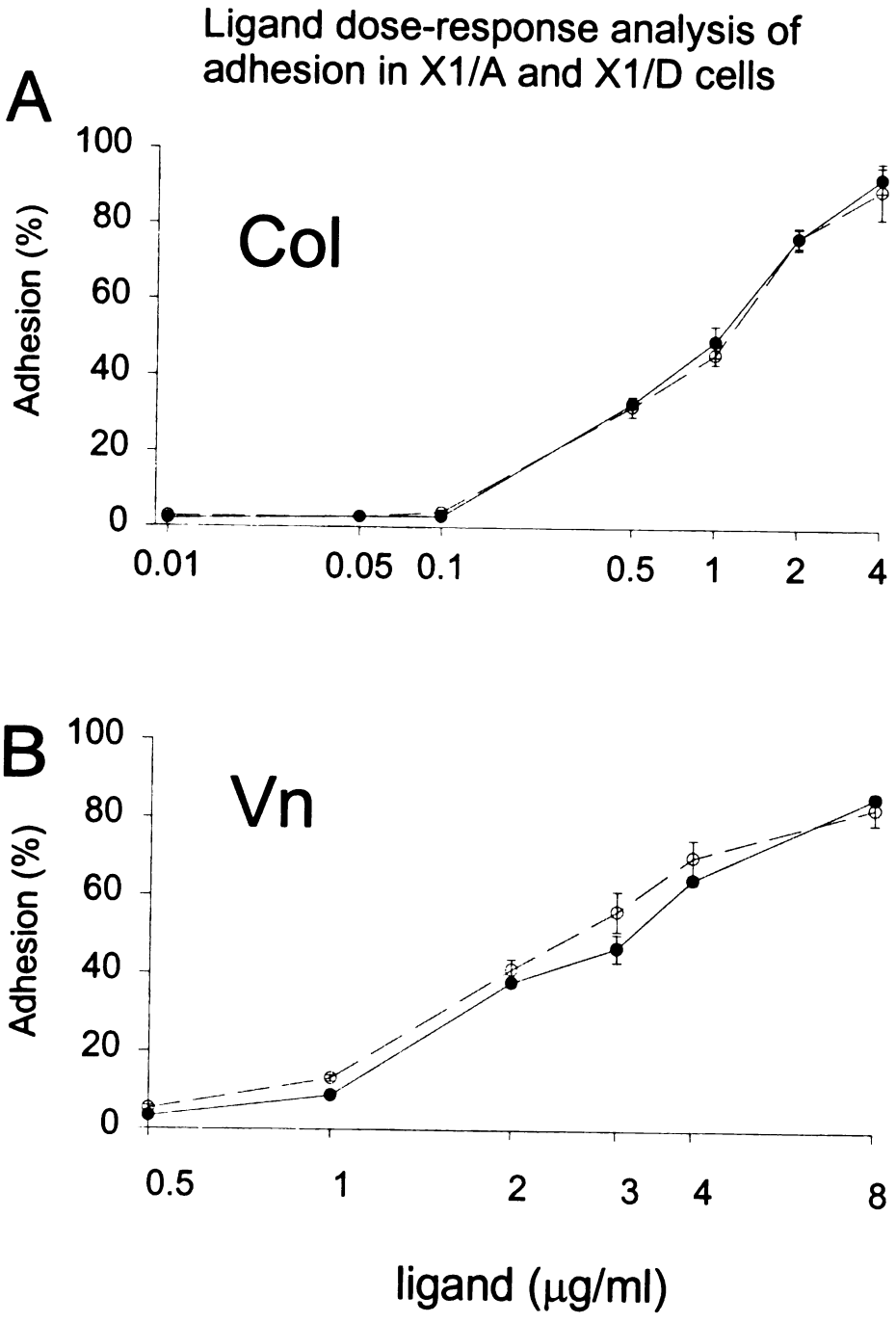
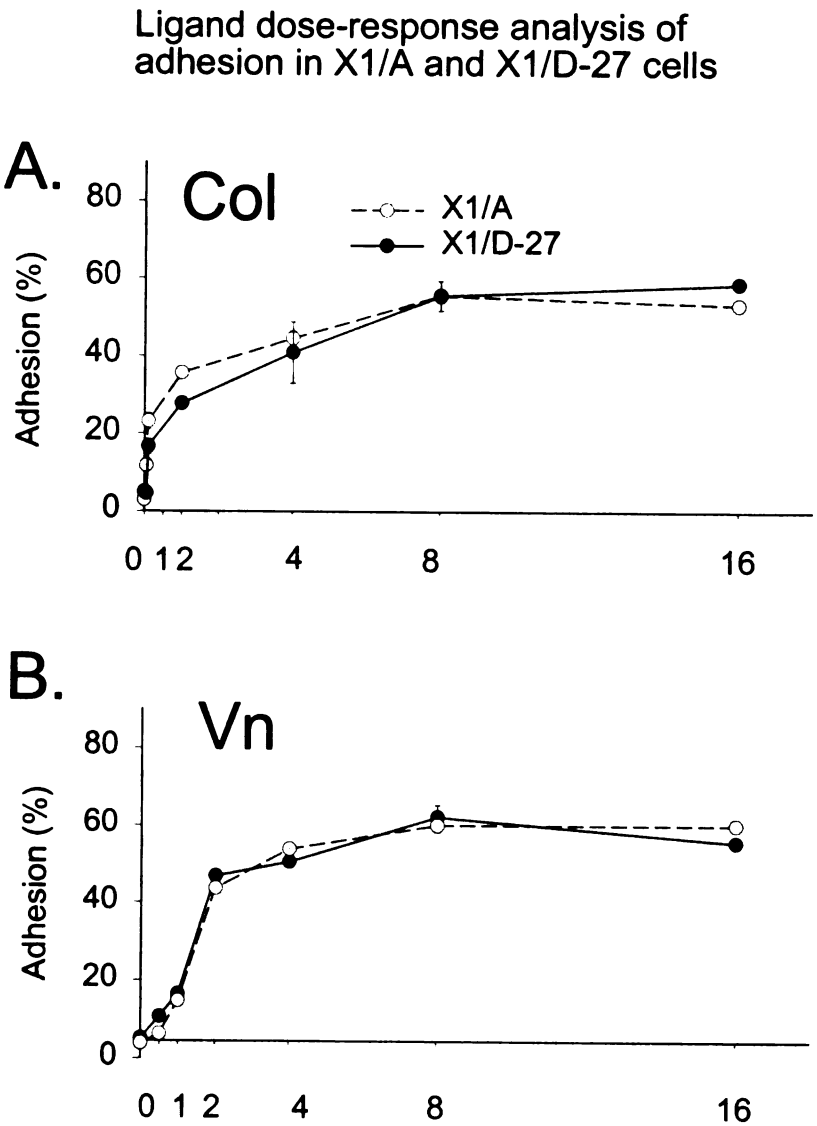

Ligand dose-response analysis of adhesion in X1/A and X1/D cells
A
Col
Adhesion (%)
100
80
60
40
20
0
0.01
0.05
0.1
0.5
1
2
4
B
Vn
Adhesion (%)
100
80
60
40
20
0
0.5
1
2
3
4
8
ligand (μg/ml)

Figure 6.


Ligand dose-response analysis of adhesion in X1/A and X1/D-27 cells
A.
Col
X1/A
X1/D-27
Adhesion (%)
0
20
40
60
80
0 1 2
4
8
16
B.
Vn
Adhesion (%)
0
20
40
60
80
0 1 2
4
8
16
Ligand (μg/ml)

There is no preference of α7X1 in coupling with human β1A or mouse β1D

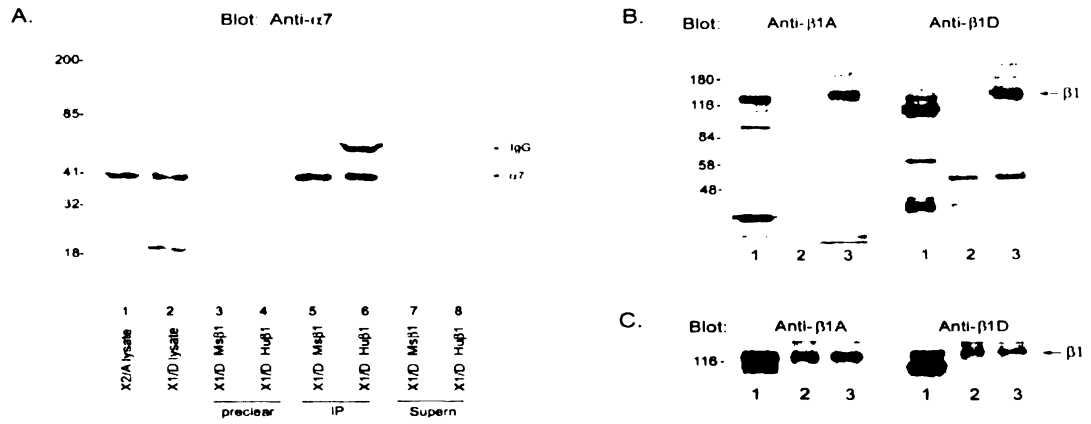
Analysis of Integrin Complexes in the β1D Transfectants

To determine what fraction of the α7 subunit was partnered with endogenous β1A or transfected β1D, two different strategies were used. In the first, lysates prepared from X1/D cells were exhaustively immunoprecipitated with a mAb specific to either human β1 or mouse β1, followed by immunoblotting for the partner α7 (Figure 7A). The results show that the fraction of α7 that heterodimerized with either the β1D (lane 5) or the β1A (lane 6) isoform was approximately equal. Thus, neither subunit appears to have any preferential affinity for α7 during integrin heterodimerization.

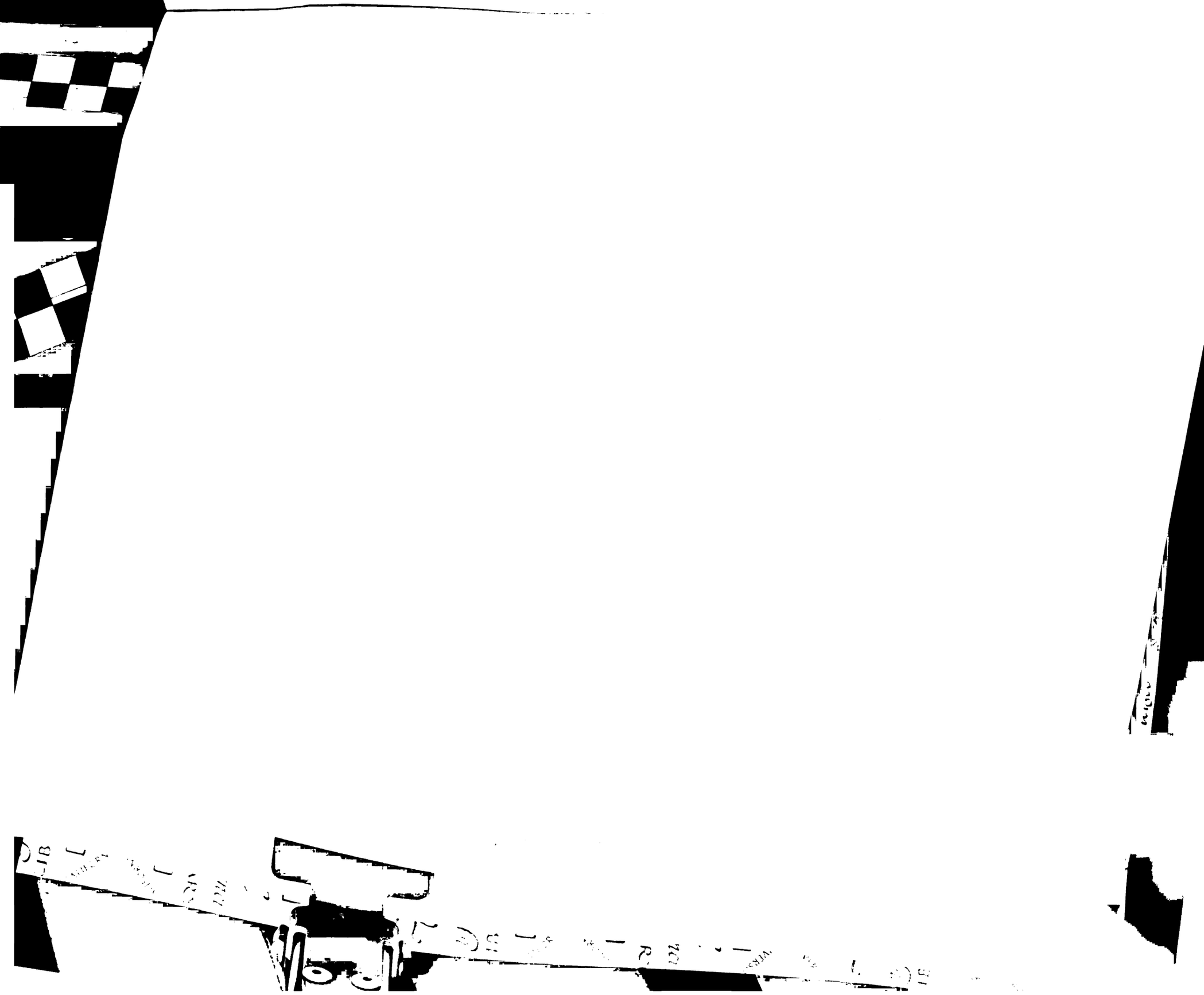
In the second approach to estimate the relative pairing of the α7 integrin subunit with endogenous β1A or transfected β1D, we used the X1/D-27 cell line which expresses high levels of both α7X1 and β1D (Figure 7B). Cells were first processed for immunoprecipitation with anti-α7 antibody, followed by immunoblotting analysis with an antibody specific for either the human β1(A isoform) or mouse β1(D isoform). Again, similar levels of β1A and β1D were detected in the immunoprecipitates (Figure 11B, lane 3). There appeared to be an abundance of β1D precursor (Figure 11B, lane 1; right panel) compared to the mature, fully processed form of the endogenous β1A subunit. In contrast, nearly all of the endogenous β1A subunit was present in the mature form (Figure 11B, lane 1; left panel). α2 and α3 subunits were associated with similar

proportions of β1A and β1D (Figure 7C). In other studies, we examined the amount of α2 and α3 subunits associated with β1A and β1D by immunoprecipitation of ^{35}S-methionine-labeled cells and similar results were observed. On the basis of these studies, we conclude that α7 as well as α2 and α3 subunits is able to complex with similar efficiencies to the β1A and β1D subunits.

Figure 7. Association of α7 with β1D.



(A) Immunodepletion of lysates with antibody against β1. To determine the relative association of α7 with human β1 or with mouse β1, X1/D double transfectant cells were processed for immunodepletion experiments using antibodies against the β1D (mouse) or β1A (human) subunit. Cell lysates were prepared from X2/A cells (lane 1) or X1/D cells (lane 2) as controls for α7 expression. X1/D cell lysate was subjected to a preclearing control (beads and secondary antibody only, lanes 3 and 4). X1/D cell lysate was exhaustively precipitated with mAb against mouse β1 (mAb MB1.2, lane 5) or against human β1 (mAb AIIB2, lane 6), and after depletion with mouse β1 (lane 7) or human β1 (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-α7 antibody and visualization by chemiluminescence. The position of the 38-kDa α7 light chain is



indicated. (B) Immunodepletion of lysates with antibody against α7. Cell lysate from X1/D-27 cells (a clone of X1/D cells) was immunoprecipitated with a mAb to α7. Immunoprecipitates were processed for immunoblotting under nonreducing conditions and were probed with rabbit antibodies specific to β1A (left) or β1D (right) and by chemiluminescence. Lane 1, whole-cell lysate; lane 2, control anti-rat IgG-beads; lane 3, anti-α7. The position of the mature β1 subunit is indicated. (C) Same procedures as in (B) except that the lysates (lane 1) were processed for immunoprecipitation with anti-α2 integrin (mAb VM1, lane 2) and anti-α3 integrin (mAb J143, lane 3) and blotted with antibodies against β1A (left) or β1D (right).

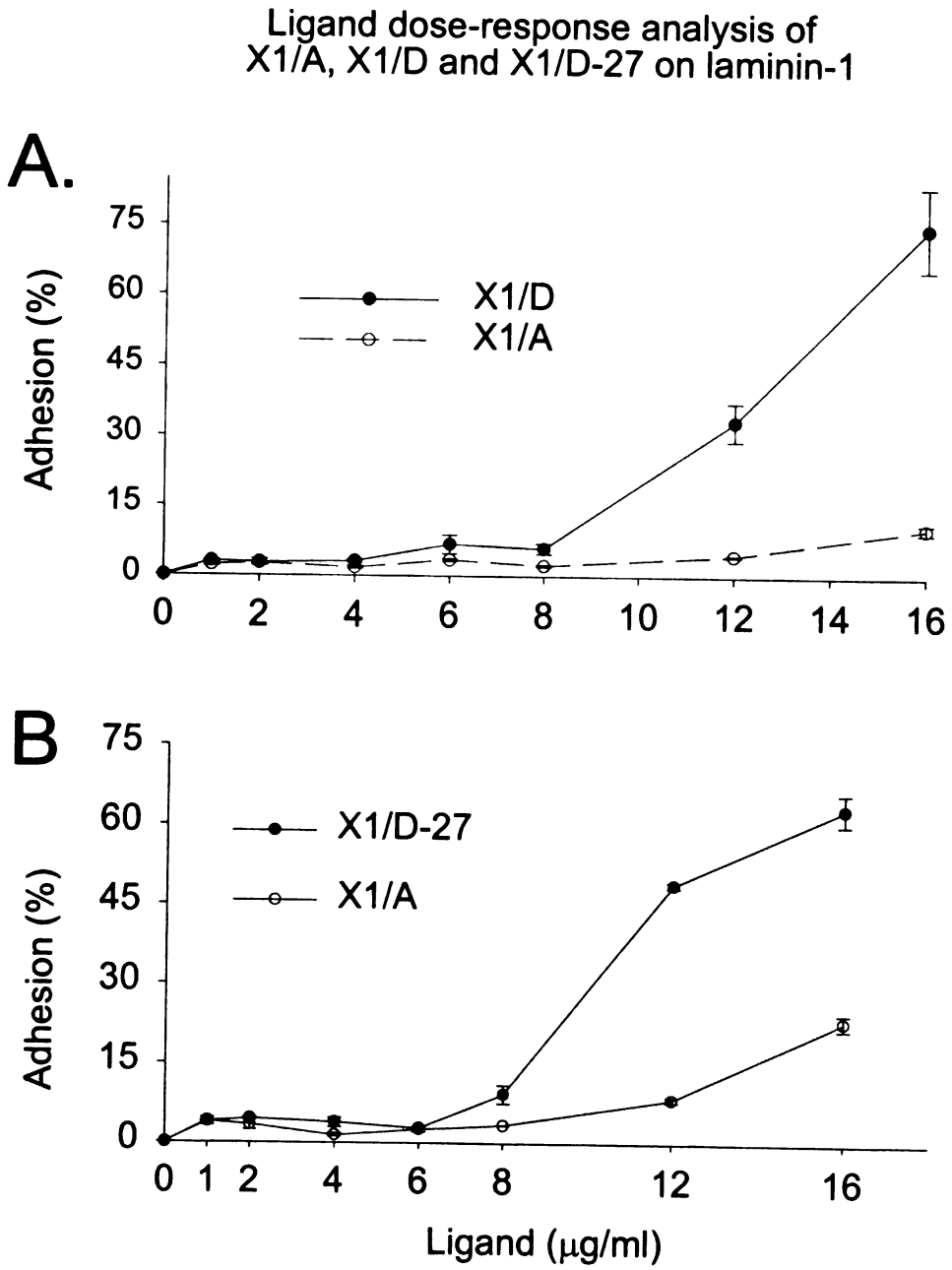
Chapter Four

The expression of β1D specifically enhanced the adhesion of α7X1 to laminin-1

The expression of β1D enhanced the adhesion of α7X1 to laminin-1

We previously reported that MCF-7 cells expressing α7X1 with the endogenous human β1A partner adhered poorly to laminin-1 (Ziober *et al.*, 1997). However, following transfection with the cDNA of the mouse β1D subunit, X1/D cells acquired the capacity to strongly bind to laminin-1 (Figure 8A). This increase in adhesion was evident at coating concentrations above 8 μg/ml. We also generated 20 single cell clones by limiting dilution and by single-cell sorting. A representative clone (X1/D-27) was chosen for the same assay, and the results were similar (Figure 8B). Since the stimulatory effect of β1D on adhesion was restricted to laminin-1 and not vitronectin or collagen I, this suggested that laminin-binding integrins such as α7X1, but not other potential β1 partner subunits, were involved. The poor adhesion of X1/A cells to laminin-1 was not related solely to the level of α7X1 expressed at the surface, since this cell line had a significantly higher expression level of α7X1 than did X1/D cells (Table II).

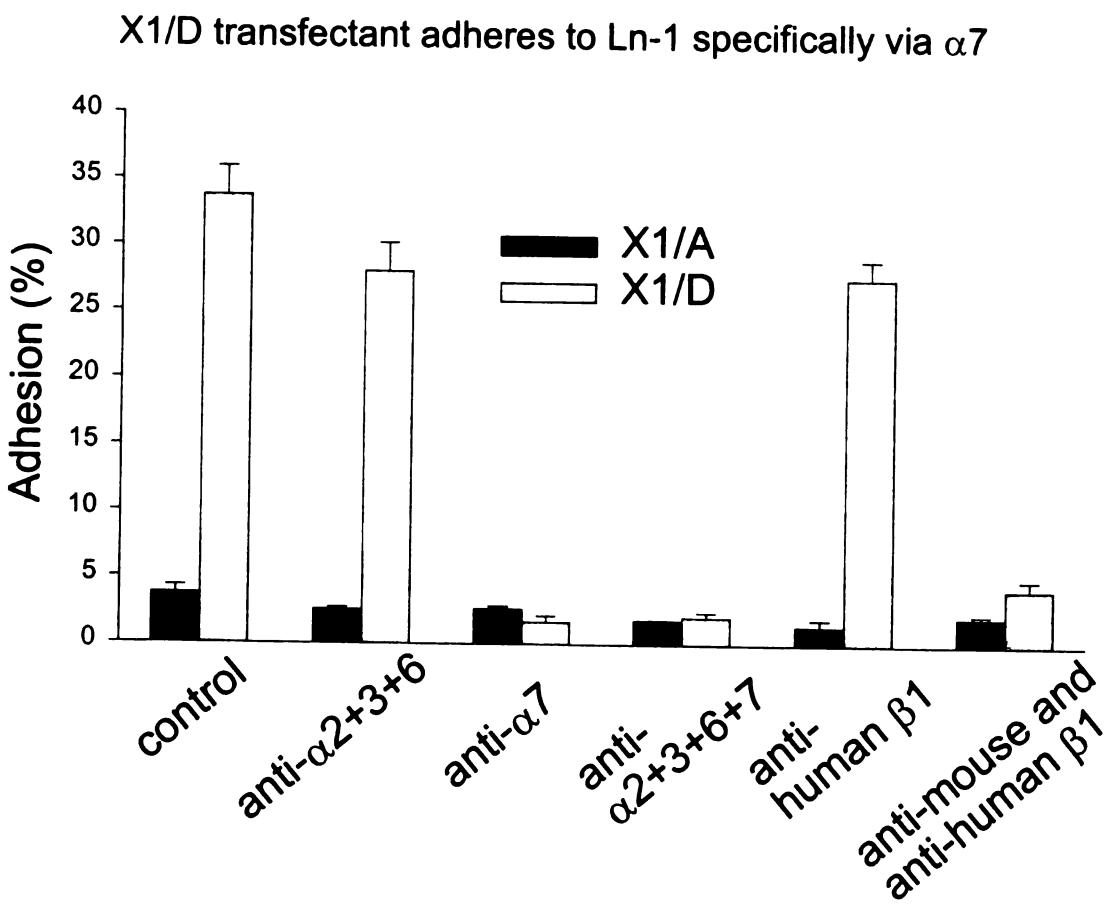
Figure 8.


Ligand dose-response analysis of
X1/A, X1/D and X1/D-27 on laminin-1
A.
Adhesion (%)
75
60
45
30
15
0
X1/D
X1/A
0
2
4
6
8
10
12
14
16
B
Adhesion (%)
75
60
45
30
15
0
X1/D-27
X1/A
0 1 2 4 6 8 12 16
Ligand (μg/ml)

Enhanced Adhesion of β1D Transfectants to Laminin-1 Is α7 Specific

MCF-7 expresses several integrin subunits that are potential receptors for laminin-binding (appendix I). To establish that the β1D-induced increase in laminin-1 adhesion was attributable specifically to α7X1, we performed standard adhesion assays with blocking mAbs against other expressed α subunits (Figure 9). The α7X1-expressing cells (X1/A) showed weak adhesion to laminin-1, and adhesion could be diminished further by treatment with a cocktail of blocking mAbs to α2, α3, and α6 integrins. In contrast, the X1/D cells expressing β1D showed enhanced adhesion to laminin-1, and most of the adhesion to laminin-1 was inhibited by a blocking mAb to α7. Since the β1D cDNA was from mouse, the human β1 functional perturbing antibody, AIIB2, did not inhibit the adhesion. The other antibody (Ha2/5) which blocks both human and mouse β1 almost completely inhibits the adhesion to laminin-1.

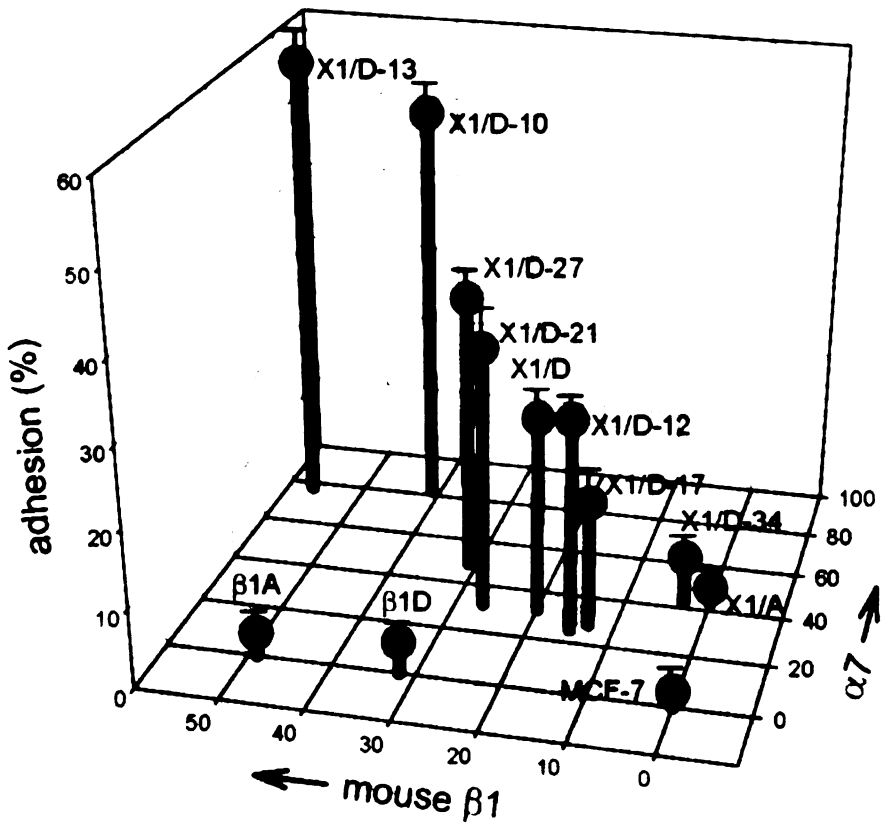
Figure. 9


X1/D transfectant adheres to Ln-1 specifically via α7
Adhesion (%)
40
35
30
25
20
15
10
5
0
X1/A
X1/D
control
anti-α2+3+6
anti-α7
anti-α2+3+6+7
anti-human β1
anti-mouse and anti-human β1

Adhesion to Laminin-1 Correlates with α7X1 and β1D Levels

To further define the relationship between β1D and α7 activity, we compared the relative adhesion to laminin-1 of a large series of α7/β1D-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 α7, but also with the relative level of expression of β1D (Figure 10). The parental cells with endogenous β1A (MCF-7) or cells transfected with α7X1 alone (X1/A) both showed poor adhesion to laminin-1. The α7X1 and β1D double transfectants (X1/D and clones 10, 12, 13, 17, 21, and 27) showed a strong correlation between levels of α7 and β1D expression. Thus, the expression of mouse β1D in MCF-7 cells transfected with α7X1 consistently enhanced the expression of α7 in this system. The level of α7 increased in parallel with that of the transfected mouse β1D and correlated with correspondingly greater adhesion to laminin-1. The surface expression level of relevant integrins was shown in appendix I. Furthermore, transfection of cells with mouse β1A or β1D in the absence of α7 to yield the β1A or β1D cell lines, did not result in enhanced adhesion to laminin-1. This result indicated that the overexpression of mouse β1A or β1D was unable to augment adhesion activity of endogenous α2, α3, or α6 integrins for laminin-1. In other studies, we generated human α7X1- and α7X2-expressing MCF-7 Tet-Off cells and found that these transfectants showed a similar adhesion profile to laminin-1, with α7X1 showing poor binding to laminin-1 and α7X2 showing strong binding (Figure 11).

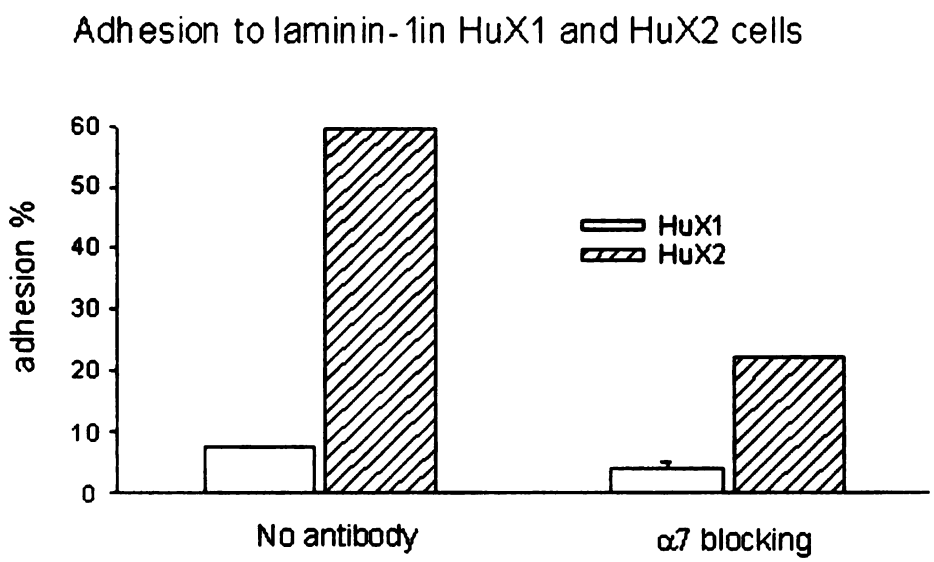
Figure 10. Adherence to laminin-1 correlates with the surface expression level of β1D



MCF-7 transfectants and clones derived from X1/D double transfectants was compared in standard adhesion assay as described in Figure 5. Parental cells (MCF-7), transfectants expressing α7X1 (X1/A), mouse β1A (β1A), or mouse β1D (β1D) all showed poor adhesion to laminin-1. The α7X1 and β1D double transfectants (X1/D and its derived cell clones) showed increased adhesion to laminin-1 that correlated closely with the relative level of β1D and α7X1 integrin expression.

Figure 11. Adhesion to laminin-1 in human X1 and human X2 cells

	α7 FITC level
HuX1	114
HuX2	80



Functionality of Human α7X1 in Cells Expressing Tetracycline-regulatable β1D

In a parallel approach to establish the role of integrin β1 cytoplasmic variants on the expression and function of human α7, we created stable cell lines constitutively expressing either the human α7X1 or α7X2 isoform and tetracycline-regulatable mouse β1A or β1D integrin. These cells lines were designated as HuX1/A, HuX1/D, HuX2/A, and HuX2/D.

Characterization of HuX1/A, HuX1/D, and HuX2/A cells

To regulate the expression of the mouse β1 integrin subunit, cells were cultured in medium containing increasing concentrations of doxycycline, and integrin expression was then measured by FACS using mAb to α7 integrin and mouse β1 integrin. Several clones of Tet-Off double transfectants were screened for doxycycline regulation of mouse β1A or β1D expression. Cells expressed high level of mouse β1 in the absence of doxycycline and low background in the presence of doxycycline were chosen for further study. Figures 12, 13, and 14 show the dose-response curve of doxycycline in modulating regulatable mouse β1 expression as assayed by FACS. In all three cells, the expression of human α7 was also modulated. If the concentration of doxycycline was further reduced, the increase in integrin expression eventually reached a plateau. Analysis of integrin profiles revealed that, besides α7 and β1D subunits, other integrins (human α2, α3, and β1) remained relatively stable as the regulatable mouse β1 was modulated.

Figure 12.

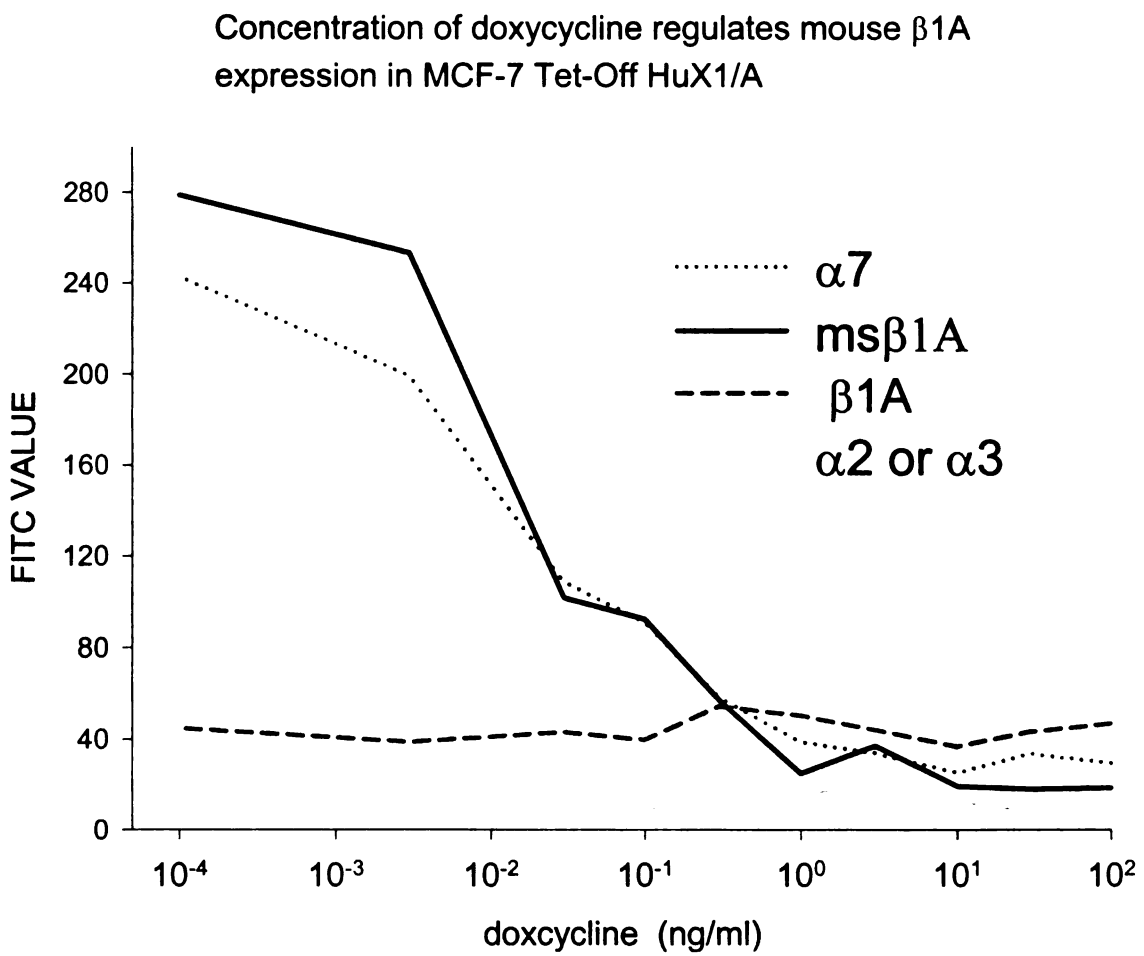

Concentration of doxycycline regulates mouse β1A expression in MCF-7 Tet-Off HuX1/A
FITC VALUE
280
240
200
160
120
80
40
0
10-4
10-3
10-2
10-1
100
101
102
doxcycline (ng/ml)
α7
msβ1A
β1A
α2 or α3

Figure 13.

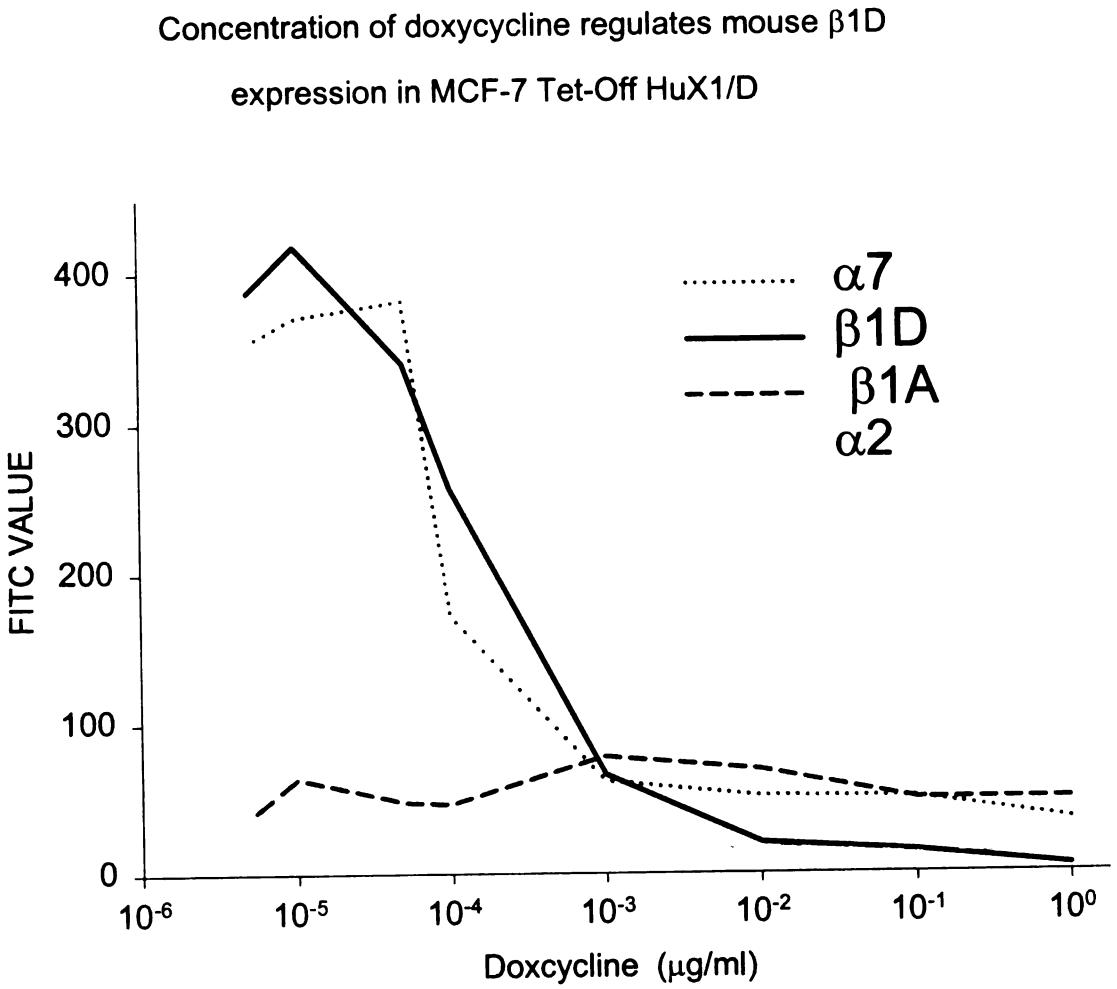
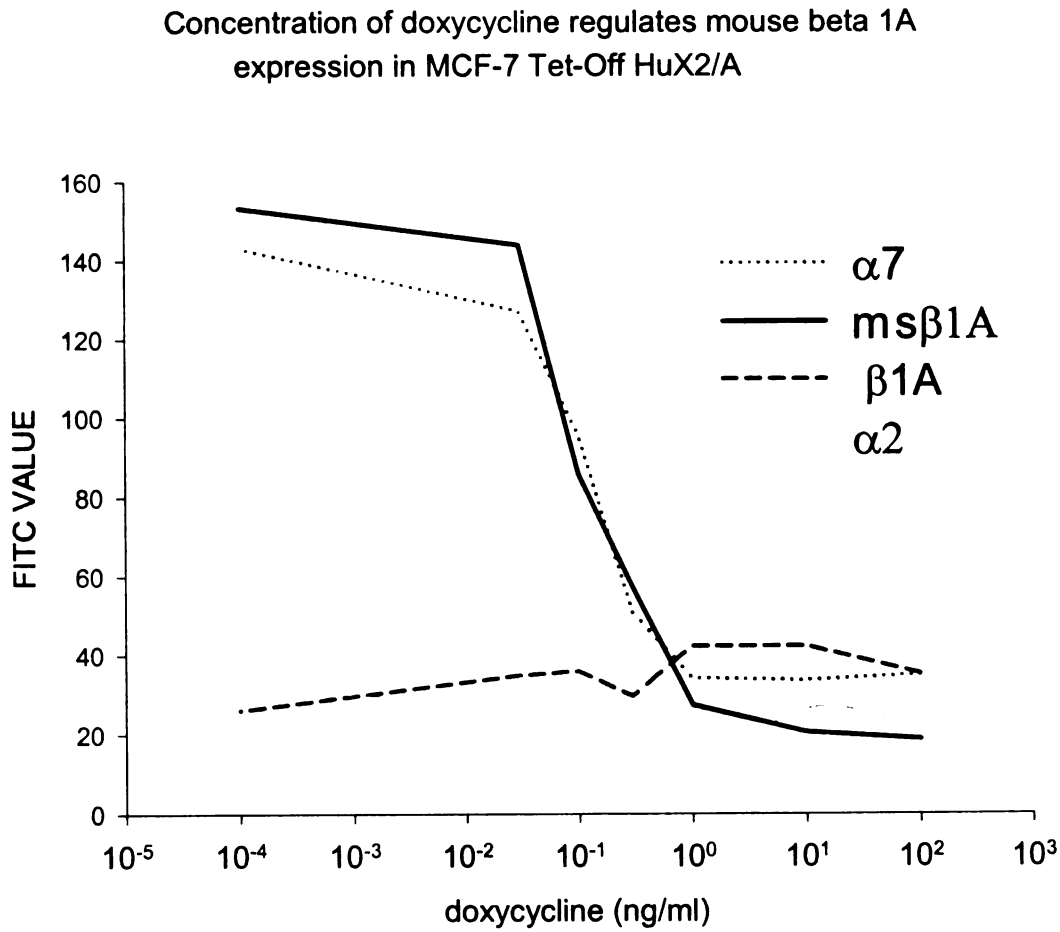

Concentration of doxycycline regulates mouse β1D
expression in MCF-7 Tet-Off HuX1/D
FITC VALUE
400
300
200
100
0
α7
β1D
β1A
α2
10-6
10-5
10-4
10-3
10-2
10-1
100
Doxcycline (μg/ml)

Figure 14.


Concentration of doxycycline regulates mouse beta 1A expression in MCF-7 Tet-Off HuX2/A
FITC VALUE
160
140
120
100
80
60
40
20
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
doxycycline (ng/ml)
α7
msβ1A
β1A
α2

The tet-regulatable expression of β1D but not β1A enhances the adhesion to laminin-1

Doxycycline suppressed the expression of mouse β1 integrin in a dose-dependent manner (Figure 15A) and at the lower range of doxycycline shown the integrin expression was inversely proportional to doxycycline concentration. 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 β1 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 β1D and 0.65 ng/ml for β1A. By modulating the levels of doxycycline, comparable levels of mouse β1 integrin expression were reached for both cell lines (Fig. 15A). Due to the difference in sensitivity to doxycycline repression, we chose to use 0.1 and 0.3 ng/ml of doxycycline for HuX1/A and HuX1/D respectively for adhesion assays on laminin-1. When cultured at this low concentration of doxycycline, the integrin profiles of these cells were quantified as detected by FACS and is summarized in Table III. Interestingly, in the Tet-Off cells, high levels of human α7 and the mouse β1 level are maintained as compared to that in the MCF-7 transfectants. The mouse β1 appears to be the dominant subunit compare to the endogenous human β1A. As the level of mouse β1A or β1D integrin was increased following induction, we observed a corresponding increase in α7 surface expression (Figure 15B). The levels of induced expression of α7 were 5 to 6 times that of the background expression at high doxycycline concentrations.

After the optimization of doxycycline concentration in regulating the surface expression level of mouse β1, we then analyzed the influence of α7X1 and β1 expression on the adhesion of doxycycline-regulatable cells to laminin-1 in standard adhesion assays (Figure 15C) at the doxycycline concentration chosen above (0.1 ng/ml and 0.3 ng/ml for HuX1/A and HuX1/D respectively). Mouse β1A-expressing cells showed poor adhesion at all levels of β1A integrin expression even though α7X1 was increased several fold over the range of doxycycline treatment (shown in Figure 15B). In contrast, induction of β1D at low doxycycline concentrations resulted in a strong adhesive response to laminin-1 that corresponded to elevated β1D and α7X1 expression levels. In fact, the elevation in adhesion to laminin-1 correlated closely to the increase in α7X1 expression.

Table III. Surface expression level of α7/β1 in MCF-7 Tet-Off double transfectants

Cell line	Integrin pair α7/β1	Mean fluorescence intensity*		
		Human α7[a]	Human β1A[b]	Mouse β1A/D[c]
HuX1/A	Human α7X1/mouse β1A[c]	125	45	130[d]
HuX1/D	Human α7X1/mouse β1D[c]	110	36	120[c]
HuX2/A	Human α7X2/mouse β1A[d]	86	40	116[d]

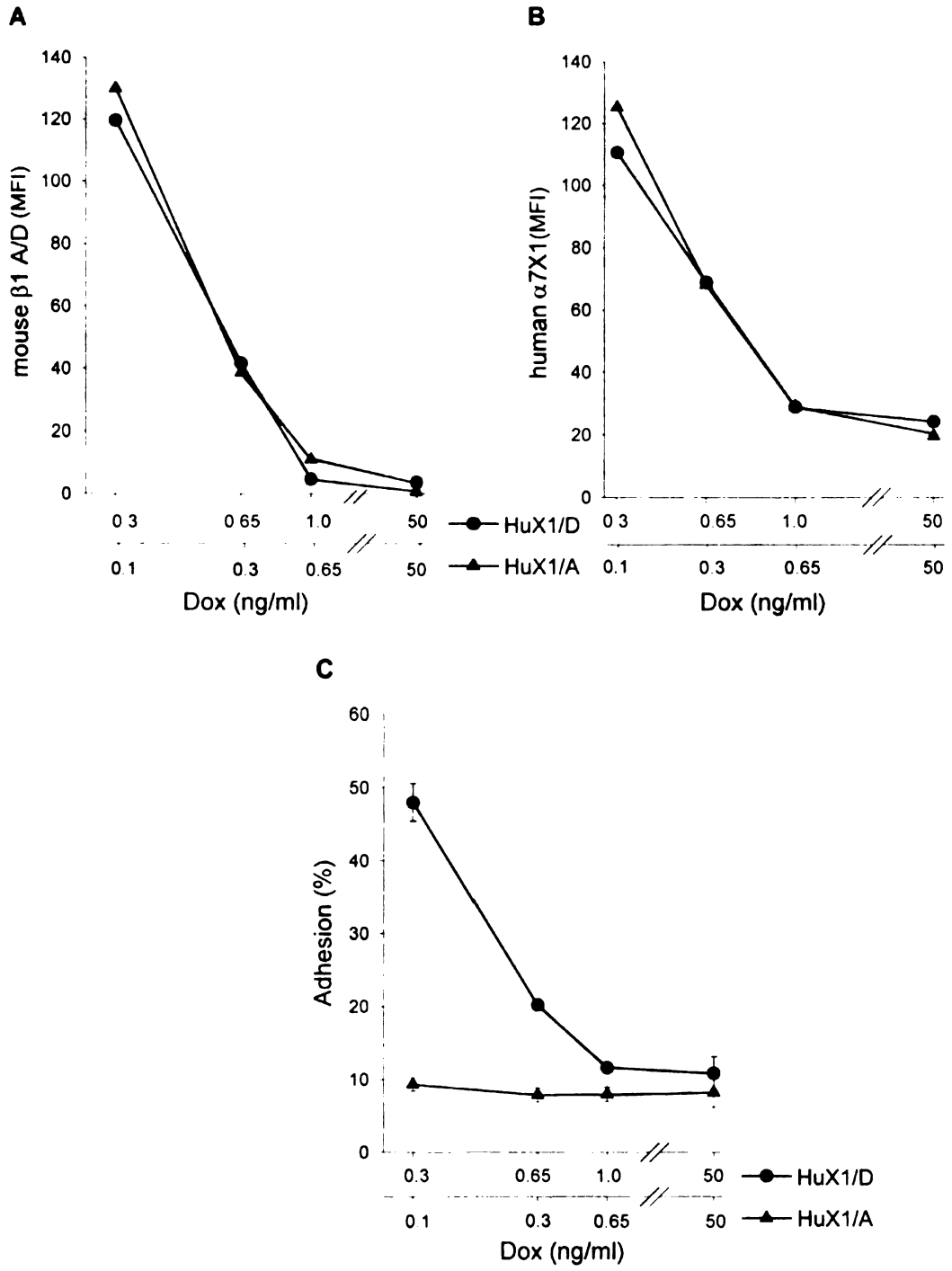
*The transfected cell lines were processed for FACS analysis using the indicated antibody as detailed in Material and Methods Section.

[a]mAb CY8, rat anti-mouse α7.

[b]mAb AIIB2, rat anti-human β1.

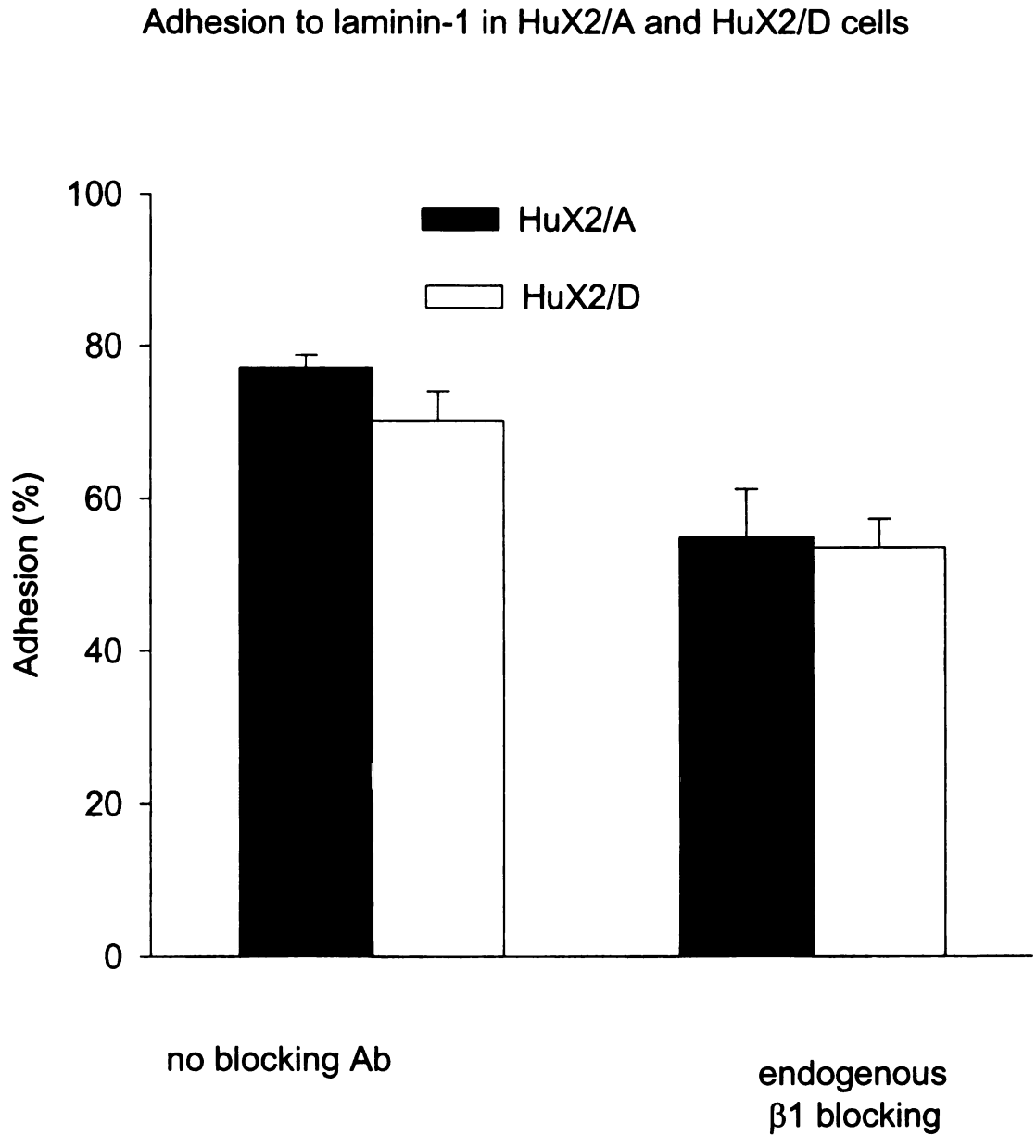
[c]mAb MB1.2, rat anti-mouse β1.

Figure 15. β1D expression promotes adhesion of human α7X1 Tet-Off cells to laminin-1



Previously we showed that α7X2β1 is fully functional to bind laminin-1 (Yao *et al.*, 1996a; Ziober *et al.*, 1997). Since it was shown that the β1D tail binds better to talin than β1 A tail does (Belkin *et al.*, 1997; Pfaff *et al.*, 1998), then we asked the question whether the expression of exogenous mouse β1D enhance the adhesion to laminin-1. To answer this question, we analyzed the adhesive efficiency of Tet-Off cells expressing α7X2/A and α7X2/D. After verifying the expression of α7X2 with FACS analysis by the mAb 9.1, these cells were induced to express either mouse β1A or mouse β1D by retroviral transduction. We found no significant difference in adhesion to laminin-1 between α7X2 cells expressing either β1A or β1D (Figure 16). Both double transfectants adhered strongly to laminin-1 (70 to 80%). The functional blocking antibody against human β1 did not reduce adhesion because the expression level of endogenous human β1A was much lower than that of mouse β1 (see appendix II). This result indicated that α7X2β1 is fully functional for adhesion to laminin-1, and that exogenous β1D did not enhance adhesion further.

Figure 16.


Adhesion to laminin-1 in HuX2/A and HuX2/D cells
100
80
60
40
20
0
Adhesion (%)
HuX2/A
HuX2/D
no blocking Ab
endogenous
β1 blocking

Expression of β1D Does Not Alter Adhesion of α7X1 to Laminin-2/4 or Laminin-10/11

The above results indicate that β1D was able to modify the binding of α7X1β1 to laminin-1. Recently, von der Mark and collaborators (von der Mark *et al.*, 2002) have reported that truncated forms of α7X1β1 and α7X2β1 bind differently to various laminin isoforms. We compared the relative adhesion efficiency of α7X1 expressing cells in the presence of β1A or β1D to available laminin isoforms. We first tested the adhesion of α7β1-expressing Tet-Off cells on preparations of human placental merosin. These cells have much higher expression level of α7 and the levels of α2, α3, and α6 are very low (see appendix II and the data in characterization of HuX1/A, HuX1D, and HuX2/A section). Previous analyses have indicated that these preparations contain primarily human laminin-4 (α2-β2γ1, S-merosin) with lesser amounts of laminin-2 (α2-β1γ1, 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 17A). Interestingly, cells expressing α7X1/β1A (HuX1/A) or α7X1/β1D (HuX1/D) adhered poorly. In contrast, cells expressing α7X2/β1A (HuX2/A) showed strong attachment. Adhesion by HuX2/A cells to laminin-2/4 was not sensitive to a mixture of mAbs to α2, α3, and α6 integrins, but adhesion was completely blocked by anti-α7 mAb 9.1 (Figure 17B).

In a similar fashion, we examined the adhesive capacity of these cell lines to laminin-10/11 (Figure 18A). In contrast to adhesion on laminin-2/4 substrates, HuX2/A adhered poorly to laminin-10/11, whereas HuX1/A and HuX1/D both attached well.

Treatment of cells with anti-human α7 blocking mAb effectively inhibited adhesion confirming that this receptor mediates binding (Figure 18B). Taken together, these results show that replacing β1A with β1D confers activity of α7X1 for laminin-1 but does not alter binding of the double transfectants to laminin-2/4 or laminin-10/11 substrates.

Figure 17.

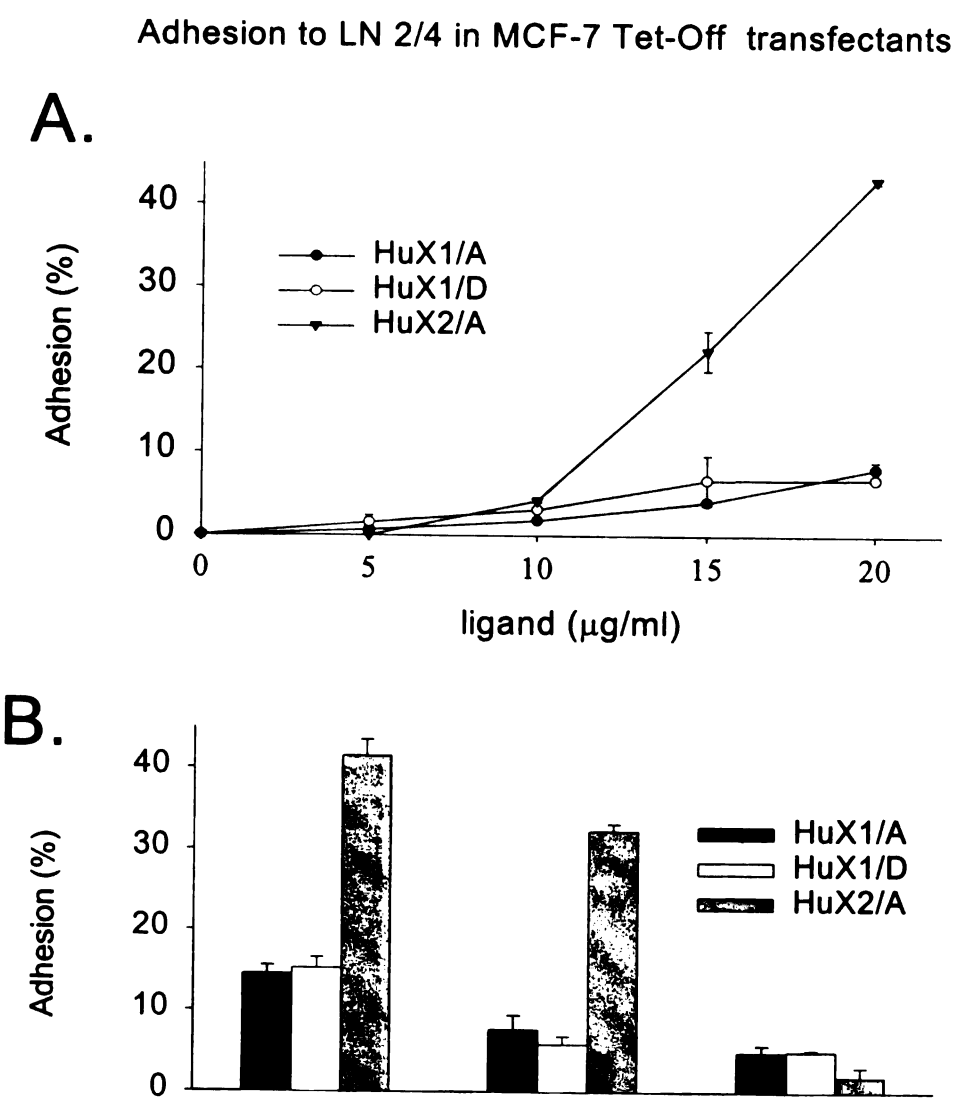
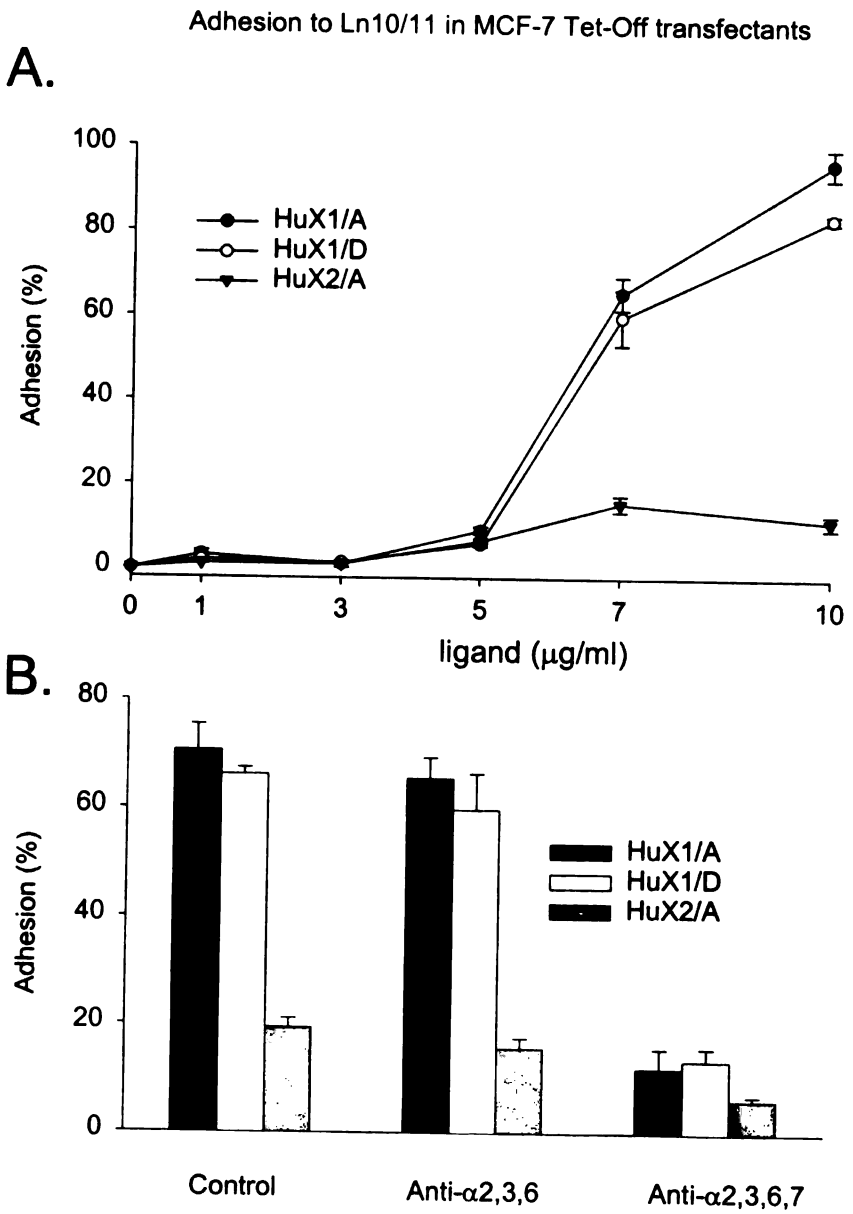

Adhesion to LN 2/4 in MCF-7 Tet-Off transfectants
A.
Adhesion (%)
40
30
20
10
0
HuX1/A
HuX1/D
HuX2/A
0
5
10
15
20
ligand (μg/ml)
B.
Adhesion (%)
40
30
20
10
0
HuX1/A
HuX1/D
HuX2/A

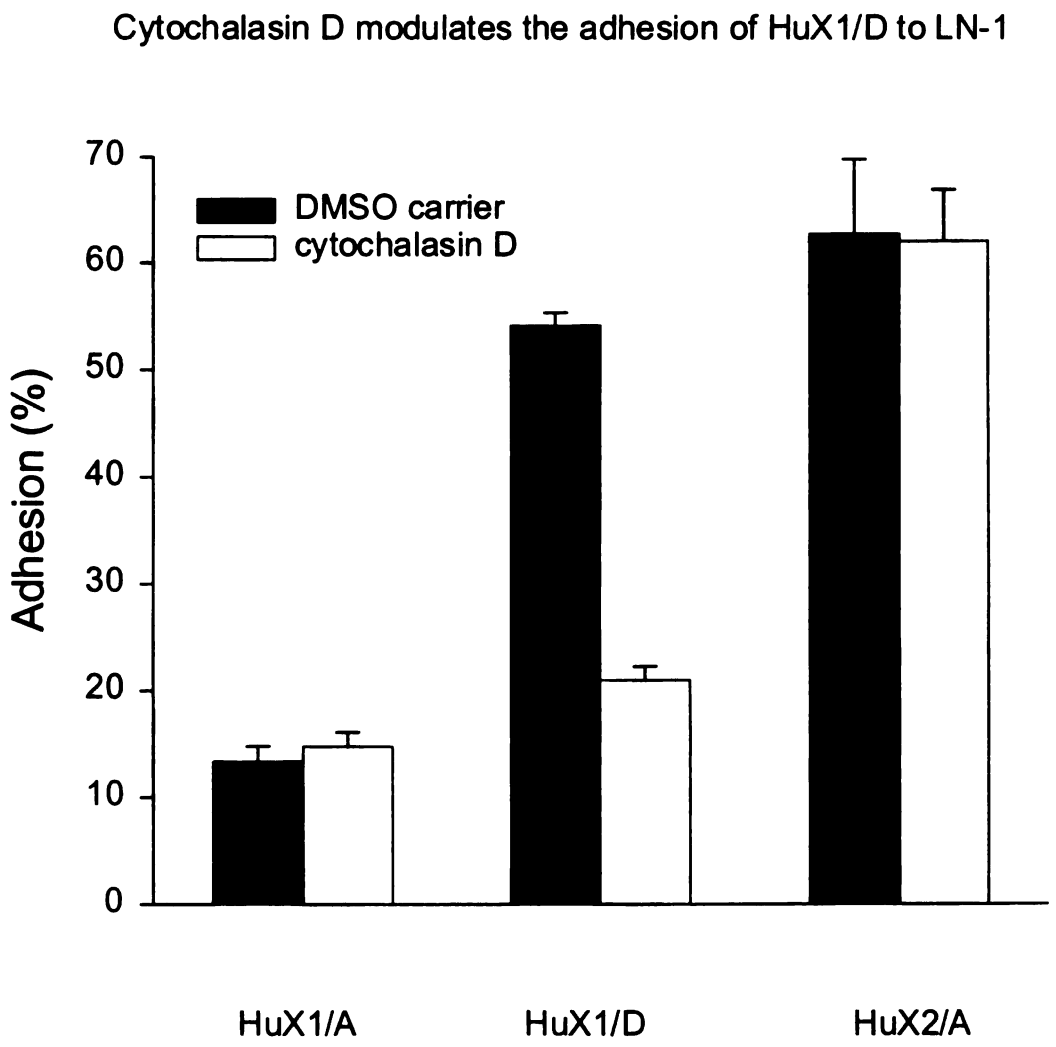
Figure 18.


Adhesion to Ln10/11 in MCF-7 Tet-Off transfectants
A.
100
80
60
40
20
0
Adhesion (%)
HuX1/A
HuX1/D
HuX2/A
0
1
3
5
7
10
ligand (μg/ml)
B.
80
60
40
20
0
Adhesion (%)
HuX1/A
HuX1/D
HuX2/A
Control
Anti-α2,3,6
Anti-α2,3,6,7

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 β1 cytoplasmic domain (Liddington and Ginsberg, 2002). Furthermore, it has been shown that the β1D tail binds talin better than the β1A cytoplasmic domain does (Belkin *et al.*, 1997; Pfaff *et al.*, 1998). To test the potential importance of cytoskeleton linkage to the adhesive activity of α7X1 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 μM cytochalasin D did not affect the adhesion of the cell lines to type I collagen (Figure 19). Similarly, adhesion of HuX1/A and HuX2/A to laminin-1 was unaffected by cytochalasin D. However, the presence of the drug significant reduced HuX1/D cell adhesion to laminin 1 by nearly 70%. This result suggests that the formation of polymerized actin during the adhesive process was necessary for the β1D cytoplasmic domain to confer enhanced binding of α7X1β1 to laminin-1.

Figure 19.


Cytochalasin D modulates the adhesion of HuX1/D to LN-1
Adhesion (%)
70
60
50
40
30
20
10
0
DMSO carrier
cytochalasin D
HuX1/A
HuX1/D
HuX2/A

Chapter 5

The potential role of β1D in growth arrest is not demonstrated when it is expressed in MCF-7 Tet-Off cells

The use of doxycycline regulatable expression system

The onset of β1D expression during myodifferentiation coincides with the timing of myoblast withdrawal from the cell cycle. It has been shown that the expression of the exogenous β1D integrin in C2C12 myoblasts and NIH 3T3 or REF 52 fibroblasts inhibits cell proliferation (Belkin and Retta, 1998) by nuclear injection of β1D cDNA. This kind of experiment is technique sensitive and the conclusion is drawn from a limited number of experiment cells. To avoid the drawback of this kind of technique, we used the regulatable β1D in MCF-7 cells to explore the role of β1D in growth inhibition. The MCF-7 Tet-Off cells were kept in the media containing doxycycline to suppress the expression of mouse β1 for 48 hours after the transfection of pRev-TRE β1A or β1D (see material and methods section). The expression vector carries hygromycin selection marker upstream of the tetracycline responsive element; thus the transfectants can be selected for stable transfectants by hygromycin even though the regulatable mouse β1 is suppressed by doxycycline. The mouse β1A or β1D were never induced before the growth curve analysis was carried out.

β1A- and β1D- expressing cells have the same growth rate

Upon the removal of doxycycline, the level of surface expression of tet-regulatable β1 in MCF-7 Tet-Off cells starts to increase and reaches its maximum level in four days as detected by FACS scan (Figure 20). In the first 24 hours, there was a decent amount of mouse β1 expressed on cell surface. We then examined the growth curves of HuX1/A and HuX1/D cells for 8 days. Although the medium was replenished every day, we included the parental cells for growth curve analysis as a control. Each cell line was grown under doxycycline and hygromycin before experimental analysis. On day zero, the cells were counted and seeded in two series, one still cultured in doxycycline, the other in doxycycline-free media. At each time point, the cells were fixed and stained for OD. Surprisingly, we found that there were no differences in the rates of growth between doxycycline and doxycycline-free cultures in all three cell lines (Figure 21). Regardless of the expression of mouse β1A or β1D, the cells grew exponentially until day 6. After day 6, cells growth reached a plateau. The presence of 10 ng/ml of doxycycline did not alter the growth rate of parental cells nor did it alter the growth rate of transfectants. These results indicated that the low concentration of doxycycline has no toxic effect to MCF-7 cells. According to the doxycycline titration data, 10 ng/ml is more than enough to suppress the expression of regulatable β1. Since there was no difference in growth rate in all groups, we concluded that expression of β1D apparently did not alter the cell cycle progression.

Figure 20.

Kinetics of surface expression of β1 upon induction in MCF-7 Tet-Off cells

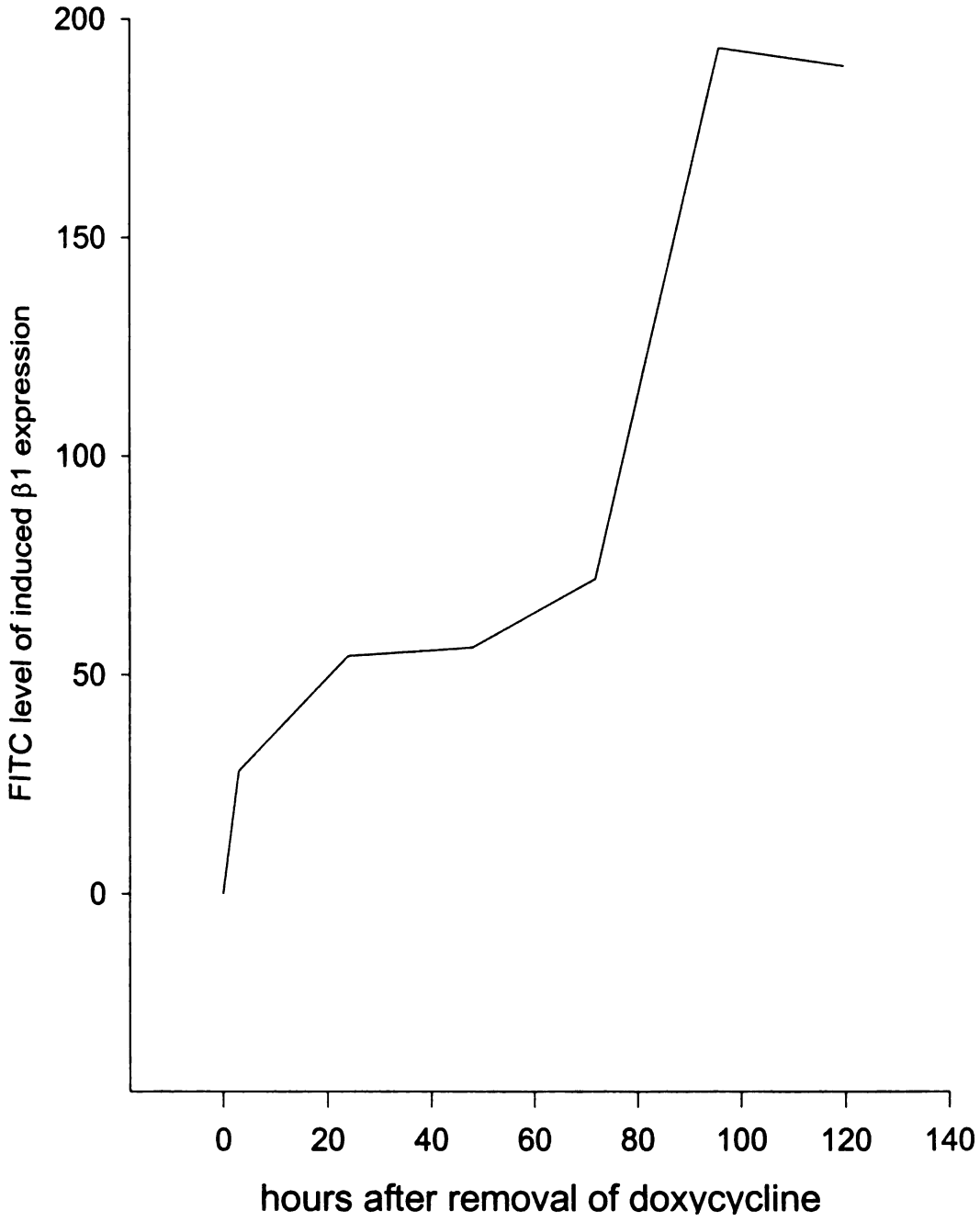
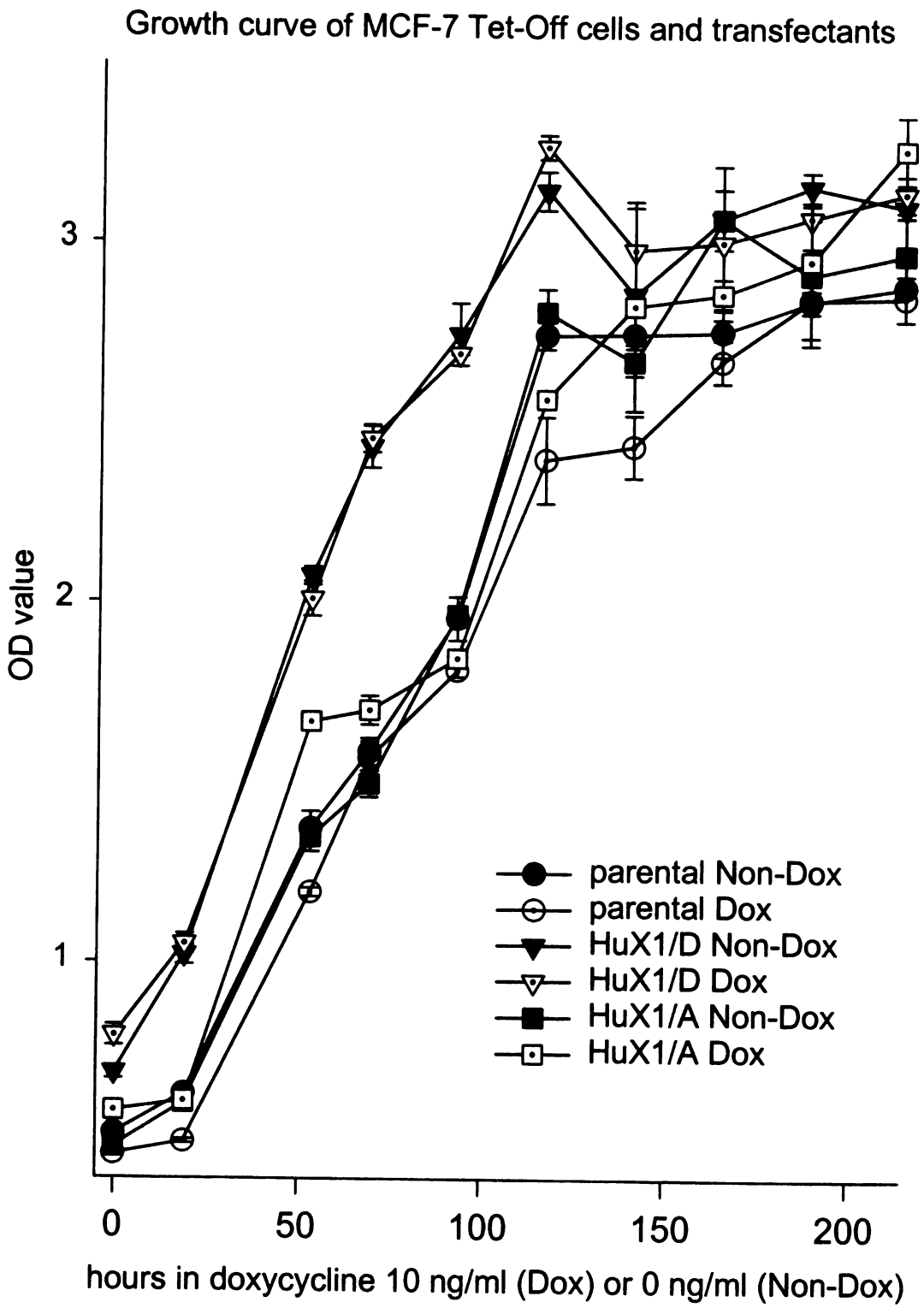


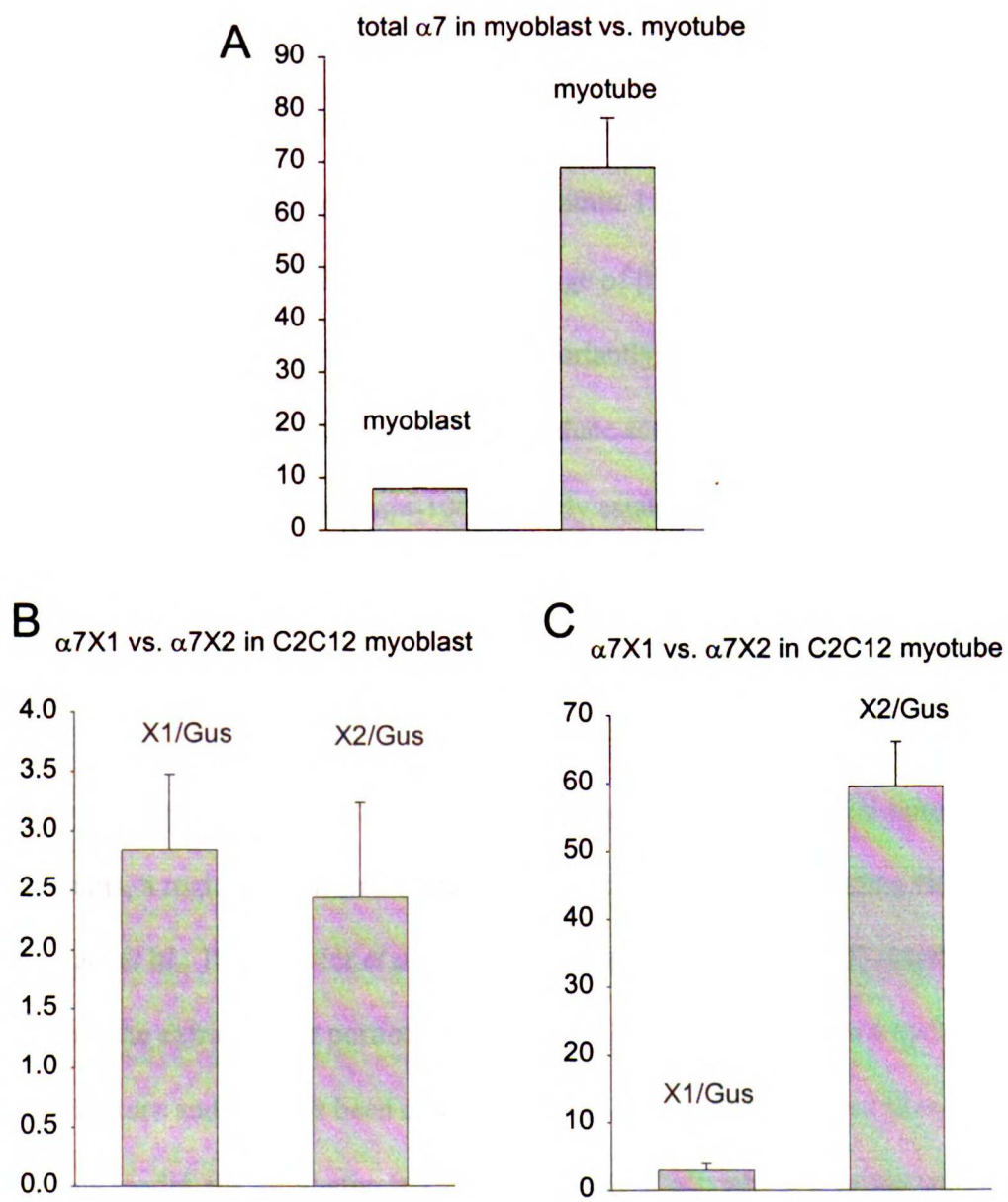
Figure 21.


Growth curve of MCF-7 Tet-Off cells and transfectants
OD value
1
2
3
0
50
100
150
200
hours in doxycycline 10 ng/ml (Dox) or 0 ng/ml (Non-Dox)
parental Non-Dox
parental Dox
HuX1/D Non-Dox
HuX1/D Dox
HuX1/A Non-Dox
HuX1/A Dox

It has been shown in many reports that α7X1 and α7X2 are developmentally regulated. Since antibodies specific to α7X1 or α7X2 is not available, all the reports are based on low cycle PCR. Basically, it is a semi-quantitative analysis of mRNA. The validity of the result depends on how the experimental conditions are chosen and may vary from experiment to experiment. To get reproducible data about the expression profiles of α7X1 and α7X2 mRNA during myodifferentiation, we use quantitative PCR to compare the mRNA levels of total α7, α7X1, and α7X2 in C2C12 myoblasts and myotubes which were derived by in vitro myodifferentiation (Blau *et al.*, 1985). Basically, the myoblasts were cultured in 2% horse serum in DMEM for 6 days and the mRNA of differentiated myotubes was collected. The probes designed as reporter were verified for efficiency before utilization. The levels of mRNA of total α7, α7X1, and α7X2 were normalized reference to an internal house keeping gene (GUS). The results (Figure 22) were similar to what have been reported in low-cycle PCR. In myoblasts, the mRNA level of α7X1 and that of α7X2 were comparable. After myodifferentiation, the total α7 mRNA was markedly increased in myotubes and this increase was mainly attributed to the increase in α7X2.

Figure 22.

Quantitative PCR of α7X1 and α7X2 in myoblasts and myotubes



Chapter 6

Conclusion and discussion

We demonstrate that when the α7X1 alternatively spliced integrin subunit heterodimerizes with the muscle β1D subunit, the ligand specificity is altered. When α7X1 is coupled to β1A it is unable to bind to laminin-1 but can efficiently mediate adhesion to laminin-10/11. Following the exchange of β1A for β1D, the receptor can then also promote cell binding to laminin-1. Importantly, these results show that the replacement of β1A with β1D does not simply induce activation of the receptor since the capacity of α7X1β1A to bind laminin-10/11 clearly establishes that it is already activated. Furthermore, these data appear to represent the first example of direct modulation of ligand specificity of an integrin by the associated β1 cytoplasmic domain.

The alternative splicing of α7X1/X2 occurs between the III and IV homology repeat domains, a region implicated in ligand specificity, affinity, or binding (Irie *et al.*, 1995; Kamata *et al.*, 1995; Tozer *et al.*, 1999). Springer (Springer, 1997) originally proposed that the extracellular portion of integrin α chain is organized in a cyclic-β propeller structure and this has been confirmed by X-ray crystallography (Xiong *et al.*, 2002). In this model, the alternatively spliced segment of α7, 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 α7X1, when bound to the β1D variant, forms an active laminin-1 ligand-binding pocket that resembles that formed by the constitutively active α7X2/β1A. However, recent work (von der Mark *et al.*, 2002) indicates that a double truncated and soluble form of α7X1β1 complex lacking the cytoplasmic domains is unable to bind to laminin-1 in the presence of Mn^{+2}.

Exogenous β1D expression in MCF-7 cells did not enhance cell adhesion to laminin-1 in the absence of α7 and, in the presence of α7X1 and β1D, the enhancement of adhesion was mainly attributed to α7 and not to the other moderately expressed α2, α3, or α6 integrins that could potentially bind to laminin-1. Since there is no preference in the coupling of these α subunits with β1A or β1D ((Belkin *et al.*, 1997; Pfaff *et al.*, 1998); this study), our results indicate that β1D-induced functional augmentation in MCF-7 cells is specific for the α7X1 integrin. MCF-7 cells have β1 integrin receptors for other ligands, including α2β1 for collagen I (Jones *et al.*, 1995; Maemura *et al.*, 1995) and αvβ1 for vitronectin (Wong *et al.*, 1998). Adhesion to these two ligands was not altered by the expression of β1D. In addition, the forced expression of β1D in fully functional α7X2 cells did not further enhance the adhesion. These results suggest that the β1D partner subunit can selectively alter α7X1 functionality.

It is well documented that affinity modulation can occur by inside-out signaling via transmembrane conformational transitions (Faull *et al.*, 1994; Schwartz *et al.*, 1995; Hughes and Pfaff, 1998; Longhurst and Jennings, 1998; Liddington and Ginsberg, 2002).

Previous studies using cells transfected with β1D indicated that the subunit induced a number of cellular alterations that included decreased spreading and migration, and increased binding of fibronectin and incorporation into matrix (Belkin *et al.*, 1997). These results are consistent with stabilized integrin-cytoskeleton interaction that appears to be mediated by increased binding of β1D with talin and elevated levels of integrin activation. In the context of the current studies, α7X1 appears 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 (Ziober *et al.*, 1997). Formation of the α7/β1D complex appears to induce a similar conversion from an intermediate level of activation to a fully active state. It is possible that association with β1D modulates the ligand-binding affinity of α7 for the laminin-1 ligand rather than altering its activation level. If this was the case, then α7X1/β1A would assume an intermediate affinity state and formation of α7X1/β1D generates a high affinity state. There is precedence for intermediate affinity states (Takagi et al., 2002). Alternatively, β1D-induced activation of α7X1 may be related to post-receptor occupancy events such as receptor clustering and avidity regulation, although as Springer (Shimaoka et al., 2002) has indicated the data supporting this form of integrin activity modulation remains controversial. Furthermore, the cellular environment can also modulate integrin function (Zhang *et al.*, 1996; Hughes *et al.*, 1997). Thus, in contrast to MCF-7 cells, when HT-1080 cells were transfected with α7 splice variants, both X1 and X2 isoforms were fully active for binding to laminin-1 (Ziober *et al.*, 1997), indicating that factors related to cell-type specificity are important in regulating integrin function. Additional studies are needed to

further define the specific mechanism responsible for β1D-induced functional modulation of α7X1.

Importantly, differences in the interaction of β1 alternatively spliced cytoplasmic tails with the cytoskeleton have been reported. The muscle-specific β1D splice variant has been shown to bind talin with higher affinity than β1A (Belkin and Retta, 1998; Pfaff *et al.*, 1998). Conversely, the β1A tail has been reported to bind filamin and α-actinin more strongly than does β1D (Belkin *et al.*, 1997; Pfaff *et al.*, 1998). Although the β1D cytoplasmic domain-talin interaction may occur, this potential effect does not explain how β1D induces binding to laminin-1 by α7X1 or why adhesion to other laminin isoforms by these receptors is unchanged. Also the α2 integrin subunit was shown to associate with β1D, yet adherence to collagen I was unaltered. However, the experiments revealing that treatment of HuX1/D cells with cytochalasin D decreased binding to laminin-1, suggests a mechanism by which a conformational shift is transmitted to the extracellular domain following association of the β1D tail with the cytoskeleton.

We investigated the mechanism underlying functional regulation of the alternatively spliced X1 and X2 variants by its partner β1 integrin. The physiological importance of the α7 and β1 splice variants is related to the varied roles of α7β1 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 of α7 switches from the β1A to the β1D cytoplasmic form. Our results indicate that coupling of the α7X1 variant with the β1D isoform modifies the receptor's ligand binding specificity and forms a more promiscuous receptor that can mediate adhesion to laminin-1 substrates.

The mechanism by which the β1D cytoplasmic domain can lead to enhanced binding of α7X1 expressing cells to laminin-1 may be related to induced changes in integrin conformation. For example, it possible that a conformational shift in the ligand-binding pocket could account for the modified ligand specificity we observed. It is interesting that α7X1 when partnered to either β1A or β1D binds efficiently to laminin-10/11 yet exhibits activity to laminin-1 only when coupled to β1D. Thus α7X1 show an intrinsic binding activity to laminin 10/11 but cannot bind laminin-1 unless it is coupled to β1D. In contrast, α7X2 shows strong activity toward laminin-1 and laminin-2/4 but not to laminin-10/11. These results suggest a complex regulation of ligand specificity that must relate to structural components not only on the integrin receptor but also on the laminin isoforms as well.

The exchange of β1A with β1D may lead to enhanced ligand-binding affinity of α7β1 integrin. Most likely the conversion of α7X1 to a permissive laminin-1-binding state by β1D is a consequence of the ability of the cytoplasmic domain of β1D to transmit conformational changes to the α7 subunit that then regulate ligand specificity and/or affinity. Since α7X1 when coupled with β1D did not alter its poor binding to laminin

2/4, this implies that the increase in activity toward laminin-1 is specific and presumably represents a subtle change in how the integrin regulates its ligand specificity. Clearly additional studies are needed to evaluate these possibilities.

The onset of β1D expression during myodifferentiation coincides with the timing of myoblast withdrawal from the cell cycle. It has been shown that the expression of the exogenous β1D integrin in C2C12 myoblasts and NIH 3T3 or REF 52 fibroblasts inhibited cell proliferation (Belkin and Retta, 1998) by nuclear injection of the β1D cDNA. In contrast, our results showed that the parental cells and the transfectants have the same growth rate regardless of the expression of β1D. Although we monitored cell surface expression level as a indicator of β1D level inside the cell, it is unlikely that β1D reaches the cell surface without exerting its effect on growth rate if it has any. MCF-7 is a tumor cell so it is possible that this cell can overcome the growth inhibitory effect. Usually this kind of adaptation would take some time to develop; however, our experiment was done in only a few days and these cells has never been induced to express β1D before the experimental procedures. Moreover, Baudoin *et al.* knockout β1A and knockin in β1D exon (Baudoin *et al.*, 1998) and found the embryos were viable till 10.5 dpc. The embryonic fibroblasts can be cultured as cell line. Unless there was some compensation mechanism, the animal should not be viable at 10 dpc if β1D inhibits growth.

α7 and β1D are expressed during myoblast differentiation and are located at the

sarcolemma, MTJs, and NMJs in adult skeletal muscle (Bao *et al.*, 1993; Ziober *et al.*, 1993; Belkin *et al.*, 1996; Martin *et al.*, 1996). In mouse skeletal muscle, α7 is expressed as early as E10.5 days of development, whereas the β1D variant appears in the late stage embryo. Furthermore, there is a concurrent expression of both α7 and β1D during the first formation of sarcomeres and membrane infoldings at the MTJ (van der Flier *et al.*, 1997). In addition, when β1D is detected in the mouse embryos at E17.5, it is enriched in MTJ along with the α7 subunit. Conversely, laminin-1 is expressed during embryonic and fetal stages. Interestingly, laminin-1 is concentrated at the MTJ of human skeletal muscle and persists until birth (Gullberg *et al.*, 1999). Because the expression of the X1/X2 isoforms is developmentally regulated, we hypothesize that the functionality of α7 is controlled in a tissue-specific manner by the coexpression of β1D. Both X1 and X2 isoforms are expressed in myoblasts, but X2 is more abundant in mature muscle, implying that this form of the receptor is present at permanent anchorage sites. It has been reported that in the hind limb following early muscle differentiation, α7X1 shows a relative increase in expression but that postnatally the X2/X1 ratio also increases (Hodges *et al.*, 1997). We propose 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 assembles) (Ziober *et al.*, 1997). This is consistent with the dynamics of the expression of α7 and β1 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 receptor and potential ligand is

coordinately coupled.

In summary, we have demonstrated that the cytoplasmic domain of the β1 integrin can modulate the functionality and ligand specificity of the α7β1 receptor. We show that when coupled to the β1D partner, α7X1 splice variant shows a broader spectrum of specificity and can efficiently bind laminin-1. The formation α7X1/β1D 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 α7X1 along with β1D could facilitate the organization and stability of the MTJ during the early stages of muscle fiber maturation.

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Appendix I. Surface expression level of integrins in MCF-7 and transfectants

Cell line	Integrin pair*	Mean fluorescence intensity					
		α2[a]	α3[b]	α6[c]	α7[d]	Endogenous β1A[e]	Mouse β1A/D[f]
MCF-7	--/β1A	30	39	3	-	78	-
X1/A	α7X1/β1A	25	27	6	43	86	-
X1/D	α7X1/β1D	41	23	13	32	55	20
X2/A	α7X2/β1A	56	64	7	40	58	-
X1/D-10	α7X1/β1D	29	27	10	34	30	41
X1/D-12	α7X1/β1D	18	8	9	25	14	15
X1/D-13	α7X1/β1D	28	17	7	78	55	57
X1/D-17	α7X1/β1D	21	17	12	28	31	15
X1/D-21	α7X1/β1D	19	18	20	32	21	27
X1/D-27	α7X1/β1D	28	18	5	49	47	31
X1/D-34	α7X1/β1D	23	15	6	43	19	3
β1A	--/β1A	52	31	2	--	27	49
β1D	--/β1D	14	11	2	--	10	32

*The parental and transfectants were processed for FACS using the indicated antibody as detailed in Material and Methods Section. The level of expression is expressed as the mean of 10,000 cells scanned excluding non-vital cells.

[a]mAb VM1, mouse anti-α2

[b]mAb VM2, mouse anti-α3

[c]mAb GoH3, rat anti-α6

[d]CY8, rat anti-mouse α7.

[e]mAb AIIB2, rat anti-human β1.

[f]mAb MB1.2, rat anti-mouse β1.

Appendix II. Surface expression level of integrins in MCF-7 Tet-Off cells and transfectants

Cell line	Integrin pair*	Mean fluorescence intensity					
		α2[a]	α3[b]	α6[c]	α7[d]	Endogenous β1A[e]	Mouse β1A/D[f]
Tet-Off	--/β1A	16	8	0	-	17	-
HuX1/A	α7X1/β1A	4	3	0	294	24	291
HuX1/D	α7X1/β1D	6	7	0	315	39	317
HuX2/A	α7X2/β1A	10	5	0	374	25	299
HuX2/D	α7X1/β1D	15	11	1	312	44	277

*The parental and transfectants were processed for FACS using the indicated antibody as detailed in Material and Methods Section. The level of expression is expressed as the mean of 10,000 cells scanned excluding non-vital cells.

[a]mAb VM1, mouse anti-α2

[b]mAb VM2, mouse anti-α3

[c]mAb GoH3, rat anti-α6

[d]mAb 9.1, mouse anti-human α7.

[e]mAb AIIB2, rat anti-human β1.

[f]mAb MB1.2, rat anti-mouse β1.