The Laminin-binding Activity of the $\alpha 7$ Integrin Receptor Is Defined by Developmentally Regulated Splicing in the Extracellular Domain

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The expression pattern of the laminin-binding $\alpha 7\beta 1$ integrin is developmentally regulated in skeletal, cardiac, and smooth muscle. The X1/X2 alternative splicing in the extracellular domain of $\alpha 7$ is found in the variable region between conserved α -chain homology repeat domains III and IV, a site implicated in ligand binding. To assess differences in X1/X2 isoform activity, we generated MCF-7 cell lines transfected with $\alpha 7$ -X1/X2 cDNAs. Transfectants expressing the $\alpha 7$ -X2 variant adhered rapidly to laminin 1, whereas those expressing $\alpha 7$ -X1 failed to attach. That $\alpha 7$ -X1 exists in an inactive state was established in assays using an activating $\beta 1$ antibody that induced X1-dependent cell adhesion and spreading. Furthermore, the activation of $\alpha 7$ -X1 was cell type specific, and when expressed in HT1080 cells, the integrin was converted into a fully functional receptor capable of promoting adhesion. Thus, the expression of the $\alpha 7$ -X1/X2 integrin is a novel mechanism that regulates receptor affinity states in a cell-specific context and may modulate integrin-dependent events during muscle development and repair.

INTRODUCTION

Integrins are members of a large family of cell surface receptors that mediate adhesive interactions with extracellular matrix macromolecules (Hynes, 1992). Numerous studies have indicated that integrins are dynamically regulated, existing in a relatively non-functional state in which they are incompetent to bind ligand or in a fully active ligand bound form (Diamond and Springer, 1994; Humphries, 1996; Mould, 1996). Frequently, the ligand-binding regions of integrin subunits are cryptic and only become exposed when the subunit has been activated. A number of factors, originating from outside the cell or from the cell interior, can shift the inactive extracellular conformation of the integrin to an active one. For example, antibodies that stimulate integrin function have been shown to bind the extracellular domain of the $\beta 1$ subunit (Takada and Puzon, 1993; Diamond and Springer, 1994; Humphries, 1996; Mould, 1996). These

activating antibodies bind numerous epitopes, located primarily in the N-terminal region and cysteine-rich region of the $\beta 1$ subunit, that either induce or stabilize the integrin complex in an active conformation that is competent to bind ligand (Arroyo *et al.*, 1992; Takada and Puzon, 1993; Bazzoni *et al.*, 1995). It is also well established that divalent cations can alter integrin-ligand interactions. For example, Mn^{2+} and Mg^{2+} can promote ligand binding, whereas Ca^{2+} acts as an inhibitor for a number of the integrin complexes (Humphries, 1996; Sanchez-Mateos *et al.*, 1996). Because integrin ligand recognition sites lie at or near the divalent cation binding regions, it has been proposed that divalent cations can directly induce a conformational change that exposes the ligand binding sites (Mould, 1996).

In contrast to external factors such as cations and stimulating antibodies, other effectors can activate integrin subunits intracellularly. For example, phorbol esters, by simulating the protein kinase C pathway, can increase the adhesiveness of several cell types through specific integrins (Shimizu *et al.*, 1990; Diamond and Springer, 1994). Finally, activation of cer-

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tain integrins is dependent on cell-type-specific regulation (Chan and Hemler, 1993) that may be due to posttranslational modifications, variations in the sequences of the α or β subunits, and/or the activation of different signal transduction molecules (Chan and Hemler, 1993; Hughes *et al.*, 1997).

Additional complexity of integrin regulation is exemplified by that fact that integrin subunits can be alternatively spliced. This alternative splicing has been shown to occur not only in a number of mammalian α and β subunits but also in *Drosophila* integrin receptors (Brown *et al.*, 1989; van Kuppevelt *et al.*, 1989; Suzuki and Naitoh, 1990; Cooper *et al.*, 1991; Hogervorst *et al.*, 1991; Tamura, *et al.*, 1991; Languino and Ruoslahti, 1992; Collo *et al.*, 1993; Song *et al.*, 1993; Ziober *et al.*, 1993; Djaffar *et al.*, 1994; Delwel *et al.*, 1995; Meredith *et al.*, 1995; Zhidkova *et al.*, 1995; Belkin *et al.*, 1996). Such splicing, which generally takes place in the cytoplasmic and the extracellular domains, can give rise to a number of subunit isoforms with diverse functional properties (Grinblat *et al.*, 1994; Fornaro *et al.*, 1995; Meredith *et al.*, 1995; Tennenbaum *et al.*, 1995; van der Flier *et al.*, 1995; Belkin, *et al.*, 1996; Roote and Zusman, 1996).

A laminin-binding integrin complex designated $\alpha 7 \beta 1$ has recently been identified and shown to be expressed in skeletal, cardiac, and smooth muscle (Kramer *et al.*, 1991; Song *et al.*, 1992; Collo *et al.*, 1993; Ziober *et al.*, 1993; Yao *et al.*, 1997). Investigating the function of this receptor has been complicated by the number of alternatively spliced isoforms of the $\alpha 7$ subunit (Song *et al.*, 1992; Collo *et al.*, 1993; Ziober *et al.*, 1993). In the cytoplasmic domain, two isoforms, A and B, are expressed in a developmentally specific manner in skeletal muscle. Although an $\alpha 7C$ variant has been reported in rat myoblasts, its role in development is unknown (Song *et al.*, 1993). Alternative splicing, in the extracellular domain of this subunit, also produces two muscle-specific developmentally regulated isoforms, designated X1 and X2 (Ziober, *et al.*, 1993). This extracellular alternative splicing 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 even ligand binding competence (Ziober *et al.*, 1993; Irie *et al.*, 1995; Kamata *et al.*, 1995; Mould, 1996). In this study we transfected $\alpha 7$ -X1 and $\alpha 7$ -X2 into MCF-7 cells to characterize the function of each isoform. $\alpha 7$ -X2 bound laminin readily, but $\alpha 7$ -X1 bound only when activated by the $\beta 1$ -activating monoclonal antibody (mAb)¹ TS2/16, indicating that alternative splicing regulates $\alpha 7 \beta 1$ ligand binding competence. Furthermore, we

show that activation of this isoform is regulated in a cell-specific manner.

MATERIALS AND METHODS

Cell Culture and Materials

The human breast carcinoma cell line MCF-7 was obtained from the American Type Culture Collection and maintained in DMEM H-16 with 10% fetal bovine serum. The human fibrosarcoma cell line HT1080 was obtained from American Type Culture Collection and maintained in DMEM plus 10% fetal bovine serum. Human plasma fibronectin was purchased from Collaborative Biomedical Products (Bedford, MA). Laminin 1 and the laminin E8 fragment were purified from mouse Engelbreth-Holm-Swarm tumor as described previously (Kramer *et al.*, 1991; Kramer, 1994). Human laminin 5 was kindly provided by Dr. Robert Burgeson (Cutaneous Biology Research Center, Boston, MA). Human type I collagen was obtained from Collagen Biomaterials (Palo Alto, CA).

Antibodies against integrin subunits included the rat anti-human $\beta 1$ mAb A2B2 and the rat anti-human $\alpha 5$ mAb B2G2, kindly provided by Dr. Caroline Damsky (University of California, San Francisco, CA); mouse anti-human $\alpha 2$ mAb VM1, kindly provided by Dr. Vera Morhenn (SRI International, Menlo Park, CA); the $\beta 1$ -activating antibody TS2/16, kindly provided by Dr. Martin Hemler (Harvard Medical School, Boston, MA), and LM230, an anti-human αv mAb, kindly provided by Dr. Dean Sheppard (San Francisco General Hospital, San Francisco, CA). Anti-human $\alpha 3$ mAb P1B5 was purchased from Life Technologies (Gaithersburg, MD); rat anti-human $\alpha 6$ mAb GoH3 was purchased from AMAC (Westbrook, ME). The rabbit polyclonal antibody 1211 was prepared in this laboratory against peptide sequences specific to the $\alpha 7B$ cytoplasmic region (GTIQRSNWGN SQWEGSDAH; Yao *et al.*, 1996b). Rat anti-mouse $\alpha 7$ mAbs CA5, CY4, and CY8 were generated in this laboratory as described previously (Yao *et al.*, 1996a). Goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) and ECL kit were purchased from Amersham (Arlington Heights, IL).

Transfection

The construction of $\alpha 7$ -X2B cDNA has been described (Yao *et al.*, 1996b). Primers (5' primer, 5'-CTCGACAGGAAGTGGACCAG-3'; 3' primer, 5'-TGATCCCAAACATGGAATCAG-3') that flank the X1/X2 splice site were used to amplify a 430-bp fragment from C2C12 myoblast mRNA by using reverse transcription-coupled polymerase chain reaction (PCR) and Vent polymerase (with proof-reading activity, New England Biolabs, Beverly, MA). This fragment was digested with *EspI* and *ApaI* and ligated into the corresponding sites of the $\alpha 7B$ cDNA. Positive clones containing the X1 insert were identified by PCR using primers specific for X1 and repeat region IV (5' primer, 5'-GCCAGGGTGGAGCTCTG-3'; 3' primer, 5'-CTATCCTTGCGCAGAATGAC-3'). The X1 PCR inserts were confirmed by double-stranded DNA sequencing.

Transfection of MCF-7 cells was performed by the calcium phosphate precipitation method (Mammalian Transfection kit, Stratagene, La Jolla, CA). MCF-7 cells at 30% confluency were transfected with 25–30 μ g of DNA/10-cm plate. Cells were selected in growth medium containing 500 μ g/ml G418. Individual clones or cell lines were isolated by using cloning rings or by fluorescence-activated cell sorting (FACS). Expression of $\alpha 7$ in positive clones was verified by Western blot analysis using polyclonal antibody 1211 (Yao *et al.*, 1996b).

Western Blot

Transfected cell lines and parental cells were solubilized with SDS-solubilization buffer (50 mM Tris, pH 7.5, 0.5% Triton X-100, 1 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM N-ethylmaleimide). Equal amounts of protein were separated by

¹ Abbreviations used: mAb, monoclonal antibody; OPG, octyl β -D-glycopyranoside.

SDS-PAGE on 7.5% gels under nonreducing conditions, transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and then incubated with $\alpha 7$ polyclonal antibody 1211 followed by goat anti-rabbit IgG-HRP. Migration of the $\alpha 7$ subunit was determined by using an enhanced chemiluminescence (ECL) detection system (Amersham).

Flow Cytometry

After detachment with 2 mM EDTA, single-cell suspensions of 10^6 cells/ml were incubated with optimal concentrations of primary antibodies in wash buffer (2% normal goat serum in phosphate-buffered saline [PBS]) for 1 h on ice. Cells were washed three times and incubated with secondary fluorescein-labeled antibodies for 30 min on ice. After washing three times again, the cells were stained with propidium iodide (1 μ g/ml) to identify nonviable cells. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson). Control samples consisted of cells with or without secondary antibody binding. Nonviable cells stained with propidium iodide were eliminated from the analysis. FACS-isolated cell lines were sorted two or three separate times.

Immunoprecipitation of Surface Biotin-labeled Cells

Cells were washed twice with PBS and then labeled with sulfo-succinimidyl-6(biotinamide)-hexanoate (NHS-LC)-biotin (Pierce, Rockford, IL), at 1 mg/ml, in PBS at 4°C for 90 min. To stop labeling, cells were washed twice with 50 mM glycine blocking buffer followed by a 10-min incubation in same buffer. Cells were lysed in PBS with 0.1 M Tris, pH 7.5, 2% Nonidet P-40, 2 mM PMSF, and 1 mM N-ethylmaleimide and then precleared with protein A beads. Cell lysates were mixed by rotation with primary antibody and protein A beads for ≥ 3 h. Beads were washed with 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P-40, and 0.1% bovine serum albumin (BSA) three times and heated at 100°C in SDS sample buffer for 5 min. The samples were reduced with 2-mercaptoethanol, separated by SDS-PAGE on 7.5% gels, and detected by streptavidin-HRP and ECL.

Cell Adhesion Assay

Microtiter plates (96-well Immulon plates, Dynatech, Chantilly, VA) were coated with matrix proteins or antibodies at the indicated concentrations in PBS for 1 h at 37°C in a humidified atmosphere. Plates were washed with PBS and incubated with medium containing 0.1% BSA for 60 min in a CO_2 incubator to block nonspecific adhesion. Single-cell suspensions were prepared in DMEM containing 0.1% BSA at 4×10^5 cells/ml, added in triplicate to 96-well plates, and then incubated for 30–60 min at 37°C. Nonadherent cells were removed by shaking on a titer-plate shaker (Lab-Line Instruments, Melrose Park, IL) and washed with PBS. Cells were fixed with 1% formaldehyde, stained with 1% crystal violet, and solubilized in 2% SDS; absorbance was then read at 562 nm. Cells bound to collagen (10 μ g/ml) on a separate plate were used to represent 100% attachment. Background cell adhesion to 1% BSA-coated wells was subtracted from all readings. The effect of specific blocking antibodies was tested by preincubating the cells with the indicated dilutions of purified antibodies on ice for 30 min prior to the assay.

Laminin-Sepharose Affinity Chromatography

Binding of the $\alpha 7$ integrin on laminin E8 was performed as detailed by Kramer (1994). The E8 fragment of laminin 1 was coupled to CNBr-activated Sepharose to yield 0.5–1.0 mg/ml of packed gel. Cells were washed twice with PBS and then labeled with NHS-LC-biotin as described above. Labeled cells were then extracted with buffer containing 200 mM octyl β -D-glycopyranoside (OPG), in 50 mM Tris-HCl, pH 7.4, 1 mM $MnSO_4$, and 1 mM PMSF for 30–60 min at 4°C. The cell lysate was centrifuged first at $2000 \times g$ to remove nuclei and then at $20,000 \times g$. One milliliter of supernatant was

Figure 1. Structure of the $\alpha 7$ subunit. Diagram shows the relative positions of the identified splice sites in the extracellular domain (X1 and X2) and cytoplasmic domain (A/B/C). The seven homology repeat domains (I–VII solid boxes), the divalent cation-binding sites (open slots), the putative ligand-binding site (dotted line, residues 140–350), and the transmembrane domain (TM) are indicated.

mixed slowly by inversion with ~ 300 μ l of packed E8-Sepharose overnight. After application of the cell extracts, the column was carefully washed with three to five column volumes of OPG buffer. Finally, the bound $\alpha 7\beta 1$ complex was eluted with 10 mM EDTA followed by stabilization with excess Mg^{2+} . Fractions containing eluted integrin subunits were subjected to immunoprecipitation with the appropriate antibodies, separated in an SDS-PAGE gel under reduced and nonreduced conditions, and detected by ECL.

RESULTS

Generation of $\alpha 7$ -X1- and $\alpha 7$ -X2-expressing Cell Lines

We previously reported that the $\alpha 7$ integrin subunit is a laminin-binding integrin that is alternatively spliced in the extracellular domain, producing two isoforms, designated X1 and X2 (Ziober *et al.*, 1993). This alternative splicing occurs in the variable region between the III and IV homology repeat domains near the putative ligand-binding site (Ziober *et al.*, 1993; Irie *et al.*, 1995; Kamata *et al.*, 1995; Figure 1), which could define ligand specificity, affinity, or integrin activation state. To explore for functional differences between the alternatively spliced extracellular isoforms of $\alpha 7$, we constructed expression vectors for $\alpha 7$ -X1 and $\alpha 7$ -X2. The cDNAs were then stably transfected into human MCF-7 carcinoma cells, which normally adhere poorly to laminin (Yao *et al.*, 1996b).

After selection with G418, several $\alpha 7$ -X1- and $\alpha 7$ -X2-expressing cell lines were isolated either by FACS sorting or by cloning rings. Two $\alpha 7$ -X1-expressing cell lines, X1-A and X1-S3, and two $\alpha 7$ -X2 cell lines, X2-A and X2-6, were analyzed further. FACS analysis, using the anti- $\alpha 7$ CY-8 mAb, verified that all cell lines expressed equivalent levels of the integrin (Table 1). In addition, Western blotting confirmed that all $\alpha 7$ -X1 and $\alpha 7$ -X2 MCF-7 cell lines expressed similar levels of the $\alpha 7$ subunit, whereas the parental MCF-7 cells were negative for the receptor (Figure 2A). Interestingly, $\alpha 7$ -X1 displayed a slight decrease in gel mobility when compared with that of $\alpha 7$ -X2. Molecular mass calcu-

Figure 2. Expression of X1 and X2 transfectants in MCF-7 cells. MCF-7 cells were transfected with pRc/CMV- α 7-X1 and pRc/CMV- α 7-X2. (A) Equal quantities of cellular lysates from parental MCF-7 (lane 1), X2-6 (lane 2), X2-A (lane 3), X1-A (lane 4), and X1-S3 (lane 5) cells were processed for SDS-PAGE in a 7.5% polyacrylamide gel. After transfer, the nitrocellulose membrane was probed with anti- α 7 (1211) antiserum and the position of the α 7 subunit was determined by using ECL. (B) Surface-biotinylated parental MCF-7 cells (lane 1) and X1 (X1-A, lane 2) and X2 (X2-A, lane 3) transfectants were immunoprecipitated with anti- α 7 polyclonal antibody 1211. Immunoprecipitates were separated by SDS-PAGE on 7.5% gels under reducing conditions to resolve the α 7 subunit from comigrating β 1 and transferred to nitrocellulose membranes. When reduced, the α 7 subunit separates into its heavy and light chains (Yao *et al.*, 1996b). The heavy α 7 chain is not strongly biotinylated (Yao *et al.*, 1996b). Proteins were visualized by incubation with streptavidin-HRP and then detected by ECL. The positions of the α 7 and β 1 subunits are indicated.

lations indicated that this size difference cannot be due to the four additional amino acids encoded by exon X1 as compared with exon X2 (Ziober *et al.*, 1993). This variation in electrophoretic mobility reflects an $\sim$ 3300-kDa change in molecular mass between α 7-X1 and α 7-X2, suggesting that there is a conformational difference between the two isoforms. When samples were subjected to SDS-PAGE under reducing conditions, no differences in electrophoretic motility were detected, suggesting that the two α 7-X1 and α 7-X2 isoforms exist in two conformations that depend on intact disulfide bonds (our unpublished observations). Finally, immunoprecipitation of cell-surface-biotinylated cells verified that both isoforms were expressed at the cell surface and paired with the β 1 integrin subunit (Figure 2B).

Table 1. Flow cytometry expression levels of α 7-X1 and α 7-X2 in MCF-7 cells

Cell line	Peak area ^a (arbitrary units)
X1-A	64.6 $\pm$ 7.2
X1-S3	65.2 $\pm$ 1.2
X2-A	65.8 $\pm$ 3.7
X2-6	68.9 $\pm$ 2.4

^a Average $\pm$ SD of three experiments.

Differential Adhesion of α 7-X1 and α 7-X2 to Laminin

We compared the ligand binding and specificity of the α 7-X1 and α 7-X2 isoforms by testing the transfectants in standard adhesion assays using different concentrations of laminin 1 and other ligands. We have shown previously that parental MCF-7 cells express endogenous, potential laminin 1 receptors that are functionally inactive (Yao *et al.*, 1996b). On laminin 1, α 7-X2 transfectants attached with high efficiency; adhesion was dependent on the ligand coating concentration (Figure 3A). In previous studies we have shown by using α 7-blocking mAbs, that this binding to laminin 1 is specific for α 7-X2-expressing MCF-7 cells (Yao *et al.*, 1996b). In contrast, the α 7-X1 cell lines, like the parental MCF-7 cells, adhered poorly, even at the highest ligand concentrations. When laminin 1 was replaced with purified laminin 5, both X1 and X2 isoforms failed to bind (our unpublished observations).

The lack of adhesion of the α 7-X1 cell lines to laminin 1 suggested that the transfectants might have a general defect in their ability to adhere. To evaluate this possibility, we tested the capacity of the X1 transfectants to bind collagen I. Both X1 and X2 cell lines and parental MCF-7 cells showed similar binding to collagen I, and as expected, antibodies to the α 2 collagen receptor completely blocked this adhesion (Figure 3B). Binding to collagen IV showed similar results (our unpublished results).

We also evaluated whether α 7-X1 or α 7-X2 could bind fibronectin, because it was reported that the α 7 subunit binds to this ligand (Gu *et al.*, 1994). However, both X1 and X2 transfectants bound equally to fibronectin (Figure 4). Blocking antibodies to α 5 and α v dramatically decreased the ability of the X1 and X2 clones to bind fibronectin, and the combination of α 5 and α v blocking antibodies totally inhibited binding to fibronectin for all three cell lines. The parental MCF-7 cells adhered to fibronectin with somewhat higher efficiency than the α 7 transfectants, which presumably reflects an α 7-induced decrease in the fibronectin receptor expression. We have shown a similar down-regulation of α 3 β 1 receptor expression in MCF-7 cells transfected with α 7 (Yao *et al.*, 1996b). Thus, these results show that neither the X1 nor the X2 isoform of the α 7 subunit can function as a fibronectin receptor, but cells transfected with either isoform can bind collagen I and IV, indicating that the X1-transfected cells do not have a defect in adherence even though X1 does not bind laminin 1.

α 7-X1 Integrin Competency for Adhesion and Spreading

The failure of the X1 isoform to bind laminin suggested that this α 7 isoform may be functionally defective. To test for this possibility, we artificially cross-

Figure 3. $\alpha 7$ -X1 cell lines fail to bind laminin 1. (A) Dose-response curves of cell adhesion to laminin 1. Parental MCF-7, $\alpha 7$ -X1, and $\alpha 7$ -X2 cells (2×10^4 cells/well) were tested for adhesion to increasing concentrations of laminin 1 as described in MATERIALS AND METHODS. $\alpha 7$ -X2 clones showed a dose-dependent adhesion to increasing concentrations of laminin 1. $\alpha 7$ -X1 and parental cells bound poorly to laminin at all concentrations. (B) Adhesion of parental MCF-7, $\alpha 7$ -X1, and $\alpha 7$ -X2 cells to collagen I. Parental MCF-7, X1, and X2 cells (2×10^4 cells/well) were added to collagen-I-coated plates ($5 \mu\text{g/ml}$). Anti- $\alpha 2$ mAb (VM1) was preincubated with cells at $10 \mu\text{g/ml}$. Cells bound to collagen type I (at $100 \mu\text{g/ml}$) were used to indicate 100% adhesion. Adherence of cells in 1% BSA-coated wells was treated as background binding and subtracted. Data are presented as percentages of the total cells added to each well. (A and B) Values are the mean of triplicate wells; bars indicate the SD.

linked the $\alpha 7$ -X1 receptor by using an anti- $\alpha 7$ mAb capture assay. In this assay, mAbs directed against the extracellular domain of the $\alpha 7$ subunit were immobilized on culture dishes (see MATERIALS AND METHODS). Transfectants expressing either $\alpha 7$ isoform were allowed to bind the immobilized mAbs. Both $\alpha 7$ -X1 and $\alpha 7$ -X2 cell lines attached with equal efficiency to the bound anti- $\alpha 7$ mAb (Figure 5A); when anti- $\beta 1$ mAb was used, both transfectants attached somewhat more efficiently (our unpublished results). Importantly, both X1 and X2 transfectants were induced to spread within 60 min on the immobilized mAbs (Figure 5, B and C). These results show that both X2 and X1, when cross-linked by anti- $\alpha 7$ mAbs, induce an adhering and spreading response typical of functional integrins that requires assembly of the cytoskeleton. In addition, this suggests that although the $\alpha 7$ -X1 integrin is unable to engage laminin, it can still function normally in postligand-binding events.

Solubilized $\alpha 7$ -X1 Integrin Fails to Bind Ligand

To determine whether laminin binding by the two isoforms is regulated by their cellular environment,

transfectants were processed for ligand affinity chromatography. Receptors were solubilized in detergent and applied to E8-laminin-Sepharose. The presence of $\alpha 7$ in the unbound flow-through fractions and EDTA-eluted fractions was assessed by immunoprecipitation with an $\alpha 7$ mAb. Nearly all of the $\alpha 7$ -X2 bound to the E8-Sepharose column and was eluted with EDTA (Figure 6). In contrast, all of the $\alpha 7$ -X1 was found in the flow-through and was not detected in the EDTA-eluted fractions (Figure 6). Such a result is consistent with the X1 isoform being functionally unable to engage its ligand even when freed from potential cellular regulatory elements.

The $\alpha 7$ -X1 Isoform Can Exist in a Ligand-binding Incompetent State

The antibody-capture adhesion assay indicated that the $\alpha 7$ -X1 isoform can function as a bridge between the cytoskeleton and the extracellular matrix. However, it fails to explain why this isoform is deficient in binding its ligand. It is well known that many integrins can alternate between activated and inactive states (reviewed in Hemler *et al.*, 1994; Humphries, 1996; Mould, 1996). This reversible transition to the

Figure 4. Adhesion of parental MCF-7, α 7-X1, and α 7-X2 cells to fibronectin. Parental MCF-7, X1, and X2 cells (2×10^4 cells/well) were added to fibronectin coated plates ($10 \mu\text{g/ml}$) as described under MATERIALS AND METHODS. Anti- α 5 mAb (P1D6) and anti- α v mAb (LM230) were preincubated with cells at $10 \mu\text{g/ml}$. Cell adhesion was determined as detailed in Figure 3.

active state is associated with enhanced ligand-binding affinity and is believed to depend on conformational changes that can be influenced by several factors including stimulatory or activating anti-integrin antibodies and divalent cations (Humphries, 1996). To

test for the possibility that the inability of α 7-X1 to bind ligand is a result of the receptor's activation state, we performed adhesion assays to laminin 1 in the presence or absence of β 1 activating antibodies. Addition of the activating anti- β 1 mAb TS2/16 substantially increased the adhesion to laminin 1 for α 7-X1, α 7-X2, and parental MCF-7 cell lines (Figure 7). The most dramatic increase occurred in the α 7-X1 clone, which went from minimal adhesion in the absence of activating antibody to nearly the same level as the α 7-X2 cell line in the presence of mAb TS2/16.

To identify the integrin complexes responsible for α 7-X1's increased adhesion, we repeated the adhesion assays with TS2/16 and a combination of integrin-blocking antibodies. A mixture of blocking antibodies to integrin subunits α 2, α 3, and α 6 completely inhibited TS2/16-activated MCF-7 parental cells from binding to laminin 1 (Figure 7). In contrast, these same blocking antibodies only slightly reduced the TS2/16-activated α 7-X1 and α 7-X2 cell adhesion to laminin, suggesting that this adhesion was mediated by the α 7 subunit. When blocking antibodies to α 7 were added to the anti- α 2, - α 3, and - α 6 mAb mixture, adhesion to laminin 1 was completely inhibited for the α 7-X1 and α 7-X2 cell lines (Figure 7). Similar results were seen when TS2/16 was replaced with the 8A2 activating mAb but not with 9EG7 or 15/7 mAbs (our unpublished results). Results again were similar when these experiments were repeated with the laminin E8 fragment, which contains the α 7 binding site (our unpublished results). Interestingly, TS2/16-activated α 7-X2

Figure 5. α 7-X1 expressing cells adhere and spread on anti- α 7. (A) Microtiter plates (96-well) were coated with anti- α 7 mAb at $15 \mu\text{g/ml}$ in PBS. Plates were washed with PBS and incubated with DMEM containing 0.1% BSA to block nonspecific adhesion. Single cell suspensions were prepared in DMEM with 0.1% BSA; 2×10^4 cells were added to each well in triplicate in 96-well plates and incubated for 60 min at 37°C . Adherence of cells in 1% BSA-coated wells was treated as background binding and subtracted. Data are presented as relative adhesion based on optical density (OD) units. Values are the mean of triplicate wells; bars indicate the SD. Both α 7-X1 (B) and α 7-X2 (C) cells have attached and spread on immobilized anti- α 7 mAbs. After 1 h cells were fixed with 1% formaldehyde and stained with 1% crystal violet.

Figure 6. Detergent-solubilized $\alpha 7$ -X2 is able to bind immobilized E8 fragment of laminin 1. Cell-surface biotin-labeled $\alpha 7$ -X1- and $\alpha 7$ -X2-transfected MCF-7 cells were lysed in OPG and processed for ligand affinity chromatography on laminin E8-Sepharose columns. The unbound flow-through material was collected, and after extensive washing of the column, bound integrin was eluted with EDTA (see MATERIALS AND METHODS). The material present in the crude flow-through fraction (lane 1) and the first two fractions eluted with EDTA (lanes 2 and 3) were processed for immunoprecipitation with anti- $\alpha 7$ antibody (1211) and analyzed by SDS-PAGE under nonreduced (top) or reduced conditions (bottom). The positions of the $\alpha 7$ and $\beta 1$ subunits are indicated. Note that the $\alpha 7$ subunit separates into its heavy and light chains after reduction (Yao *et al.*, 1996b).

cells in the presence of anti- $\alpha 2$, - $\alpha 3$, and - $\alpha 6$ adhered at nearly the same level as the untreated $\alpha 7$ -X2 transfectants. Thus, these results clearly demonstrate that the failure of the $\alpha 7$ -X1 isoform to bind laminin 1 is linked to the activation state of the subunit, which in turn is regulated by the swapping of X2 and X1 inserts.

Mn²⁺ Fails to Activate $\alpha 7$ -X1 Adhesion

Ligand binding by integrins is dependent on divalent cations (Sanchez-Mateos *et al.*, 1996). Work by a num-

Figure 7. Stimulation of $\alpha 7$ -X1 laminin binding activity by mAb TS2/16. (A) Parental MCF-7, X1, and X2 cells (2×10^4 cells) were added to laminin 1-coated wells (15 $\mu\text{g}/\text{ml}$) as described under MATERIALS AND METHODS. Where indicated, cells were preincubated with TS2/16 (1.5 $\mu\text{g}/\text{ml}$) and combinations of blocking antibodies $\alpha 2$ (VM1), $\alpha 3$ (P1B5), $\alpha 6$ (GoH3), and $\alpha 7$ (CY8), all at 10 $\mu\text{g}/\text{ml}$. Cell adhesion was determined as detailed in Figure 3.

ber of investigators has shown that Mn^{2+} , and to a lesser extent Mg^{2+} , can enhance ligand binding by switching the integrin to an activated state via a mechanism believed to be similar to that produced by activating $\beta 1$ antibodies (Humphries, 1996; Mould, 1996). When Mn^{2+} replaced $\text{Ca}^{2+}/\text{Mg}^{2+}$ as the divalent cation in adhesion assays, the parental, X1, and X2 cell lines all displayed an increase in adhesion to laminin (Figure 8). However, the Mn^{2+} -induced increase in adhesion seen for X1 was inhibited by a mixture of blocking antibodies to integrins $\alpha 2$, $\alpha 3$, and $\alpha 6$, indicating that $\alpha 7$ -X1 is resistant to Mn^{2+} -induced activation. As with the TS2/16-treated cells, Mn^{2+} had only a slight stimulating effect on laminin 1 binding for $\alpha 7$ -X2, suggesting that the X2 isoform is already fully activated. These results are consistent with those of the E8-Sepharose chromatography assays, which used detergent-solubilized $\alpha 7$ receptor in the presence of Mn^{2+} .

Regulation of $\alpha 7$ -X1 Ligand Competency Is Cell Type Specific

It is now appreciated that individual integrins can display altered levels of activation depending on the

Figure 8. $\alpha 7$ -X1 is not cation dependent for laminin 1 adhesion. Parental MCF-7, X1, and X2 cells (2×10^4 cells) were added to laminin 1-coated plates (15 $\mu\text{g}/\text{ml}$) as described under MATERIALS AND METHODS. Where indicated, cells were preincubated with Mn^{2+} (0.1 mM) and combinations of blocking antibodies $\alpha 2$ (VM1), $\alpha 3$ (PIB5), $\alpha 6$ (GoH3), and $\alpha 7$ (CY8), as in Figure 7. Cell adhesion was determined as detailed in Figure 3.

cell type (Gehlsen *et al.*, 1989; Chan and Hemler, 1993). When we expressed the $\alpha 7$ -X1 or $\alpha 7$ -X2 variant integrins in MCF-7 cells, the two receptors showed differential binding activity for laminin 1. MCF-7 cells appear to be in the class of host cells that confer a poor level of integrin activation, as indicated by the fact that endogenously expressed $\alpha 2$ and $\alpha 6$ as well as $\alpha 7$ -X1 integrins in MCF-7 cells are not capable of binding laminin 1, yet can be fully activated by mAb TS2/16 (Figure 7; Yao *et al.*, 1996b). For an activation-permissive cell, we chose the HT1080 human fibrosarcoma cells. This cell line expresses a fully functional $\alpha 6\beta 1$ integrin, and adhesion to laminin 1 can be blocked by mAb to $\alpha 6$ (GoH3; Lin *et al.*, 1993; Matter and Laurie, 1994). For analysis of the cell-type-dependent activation of $\alpha 7$, HT1080 cells were transfected with the $\alpha 7$ -X1 or $\alpha 7$ -X2 isoform, and high-expressing subpopulations of each transfectant were enriched for $\alpha 7$ by flow cytometry after an initial selection with G418. Both the X1- and X2-transfected cell lines as well as the parent HT1080 cells showed strong adhesion to laminin (Figure 9). However, after treatment with antibodies to $\alpha 6$, the binding of the parental cells was greatly reduced and was completely inhibited in the presence of mAbs to $\alpha 2$, $\alpha 3$, and $\alpha 6$. Although the X1 and X2 transfectants also showed partial blocking of adhesion to laminin in the presence of this mixture of mAbs,

Figure 9. Adhesion of HT1080 parental and HT1080 $\alpha 7$ -X1 and $\alpha 7$ -X2 cell lines to laminin 1. Parental HT1080 cells were transfected with $\alpha 7$ -X1 and $\alpha 7$ -X2 as described in MATERIALS AND METHODS. HT1080, HT1080X1, and HT1080X2 cell lines (2×10^4 cells) were added to laminin 1-coated plates (15 $\mu\text{g}/\text{ml}$) as described under MATERIALS AND METHODS. Where indicated, cells were preincubated with combinations of blocking antibodies $\alpha 2$ (VM1), $\alpha 3$ (PIB5), $\alpha 6$ (GoH3), and $\alpha 7$ (CY8), as in Figure 7. Cell adhesion was determined as detailed in Figure 3.

substantial binding was still evident, but this binding was abolished when $\alpha 7$ mAb was included. This indicates that in contrast to $\alpha 7$ -X1 expression in MCF-7 cells, the integrin's expression in HT1080 cells leads to activation levels that are nearly equivalent to those of the $\alpha 7$ -X2 variant.

DISCUSSION

In this study we investigated possible functional differences between X1 and X2, two variants of the $\alpha 7$ integrin subunit that are generated by alternative mRNA splicing in the extracellular domain of the receptor. Our findings show that the alternatively spliced X1 sequence modifies the activation state of the integrin. Whereas the $\alpha 7$ -X2 isoform is constitutively active in both cell types, the X1 variant is inactive in MCF-7 cells and fully active in HT1080 cells. This is the first example of cell-type regulation of integrin function that is controlled by alternative splicing in the extracellular subunit domain.

Several α integrin subunits can be alternatively spliced in the extracellular domain between the III and IV N-terminal repeat domains, a region critically important for ligand binding (Ziober *et al.*, 1993; Irie *et al.*, 1995; Kamata *et al.*, 1995; Mould, 1996). Alternative

splicing in *Drosophila* integrin α PS2 has been shown to alter the binding efficiency of the receptor to the RGD-dependent ligands fibronectin and vitronectin (Brown *et al.*, 1989; Zavortink *et al.*, 1993; Roote and Zusman, 1996). The $\alpha 6$ subunit is also alternatively spliced; however, unlike $\alpha 7$, alternative splicing in $\alpha 6$ results in either exon X1 or a combination of both exons X1 and X2 being spliced in. In contrast to $\alpha 7$, the X1 and X1X2 isoforms of $\alpha 6$ showed no difference in ligand affinity, specificity, or integrin activation. Furthermore, $\alpha 6$ -X1X2 is found as a rare mRNA and is probably not physiologically relevant, whereas expression of the $\alpha 6$ -X2 isoform has not been detected (Delwel *et al.*, 1995). Loss of $\alpha 6$ -X2 expression probably reflects an evolutionary change in the primordial $\alpha 7$ -like gene that is believed to have given rise to $\alpha 6$. It has been suggested that the $\alpha 3$ subunit is also alternatively spliced extracellularly, but the X1 isoform for this subunit has yet to be described (Ziober *et al.*, 1993). In contrast to $\alpha 6$, both $\alpha 7$ alternatively spliced isoforms X1 and X2 are expressed (Ziober *et al.*, 1993; Wang *et al.*, 1995). More importantly, this alternative splicing, as shown herein, regulates the $\alpha 7$ receptor's ability to bind ligand.

Since $\alpha 7$ -X1 lacks detectable laminin 1 binding activity, we postulated that the $\alpha 7$ -X1 variant is expressed in an inactive but presumably reversible conformation, an event that can occur in other integrins. Furthermore, it is now appreciated that individual integrins can display altered levels of activation that are dependent on cell-type specificity (Hemler *et al.*, 1994). When we expressed the $\alpha 7$ -X1 or $\alpha 7$ -X2 variant integrins in MCF-7 cells, they showed differential binding activity for laminin 1 (Figure 3A). Endogenously expressed $\alpha 2$ and $\alpha 6$ integrins are not capable of binding laminin 1, indicating that MCF-7 cells appear to be a class of host cells that confers a poor level of integrin activation (Yao *et al.*, 1996b). These two integrins also behave similarly when transfected into the nonpermissive K562 cells (Chan and Hemler, 1993; Delwel *et al.*, 1993, 1995; Kawaguchi and Hemler, 1993). In contrast, the $\alpha 3$ subunit (the X2 form), when transfected into K562 cells, is constitutively active for binding laminin 5, as is endogenous $\alpha 3$ in MCF-7 cells (Weitzman *et al.*, 1993; Delwel *et al.*, 1994). This indicates that the $\alpha 3$ integrin is constitutively active even in cells such as K562 and MCF-7. Thus we consider the $\alpha 3$ integrin to be similar to $\alpha 7$ -X2 with regard to its activation state and its sequence homology at the III/IV variable region. In contrast, we suggest that the normally expressed form of $\alpha 6$, the $\alpha 6$ -X1 homologue, is cell type specific for activation because of its high homology to $\alpha 7$ -X1. HT1080 human fibrosarcoma cells appear to be an X1-activation-permissive cell line because they express constitutively activated $\alpha 6\beta 1$. Thus, $\alpha 7$ -X1 and $\alpha 7$ -X2, when transfected into HT1080 cells, were both active, indicating that activation of the

X1 isoform, either for $\alpha 6$ or $\alpha 7$, depends on cell-type specificity (Figure 9).

The functional state of an integrin complex is dependent on the conformation of its extracellular domain. A number of factors have been shown to modulate the activity of this extracellular conformation (Humphries, 1996; Mould, 1996). For example, integrins are sensitive to divalent cation occupancy, and it is well established that cations can regulate integrin activity (Humphries, 1996). In particular, low concentrations of Mn^{2+} have been shown to induce a high ligand affinity state in integrins (Schwartz *et al.*, 1995). In our studies with the MCF-7 transfectants, Mn^{2+} was able to activate only the $\alpha 2$ and $\alpha 3$ integrins and not the $\alpha 7$ -X1 isoform. However, Mn^{2+} did not appear to elevate X2 activity, suggesting that Ca^{2+}/Mg^{2+} are sufficient for a fully functional $\alpha 7$. This suggests that insertion of the X1 exon appears to alter the conformation of the subunit (which probably accounts for the ~3300-kDa molecular mass change in X1 as compared with X2, Figure 2). Such a conformational change may possibly affect cation binding. Similar studies with $\alpha 2$ showed that cations could not convert the VLA-2 form-O or form-C into a more active form but activating antibodies could (Chan and Hemler, 1993).

In general, activating antibodies appear to increase the ligand binding competency of integrin molecules. We have shown in this study that the X1 isoform is competent to bind laminin 1 only when activated by mAbs TS2/16 or 8A2 (Figure 7; our unpublished results). TS2/16 and 8A2 bind to the same epitope and activate the $\beta 1$ integrin subunit by altering its conformation (Faull *et al.*, 1993; Humphries, 1996). In contrast, 9EG7 was not able to induce an active conformation in the X1 isoform (our unpublished results). This antibody recognizes an epitope, distinct from that of TS2/16 and 8A2, that is present when $\beta 1$ is activated by Mn^{2+} (Bazzoni *et al.*, 1995; Humphries, 1996). 9EG7 is believed not to define an activation epitope but a ligand-induced binding site that can be induced by cations like Mn^{2+} (Bazzoni *et al.*, 1995). Perhaps the failure of 9EG7 to activate the $\alpha 7$ -X1 isoform is also due to this isoform's inability to be activated by Mn^{2+} . Antibody 15/7 also failed to activate $\alpha 7$ -X1; it too is considered a Mn^{2+} -inducible anti- $\beta 1$ antibody (Picker *et al.*, 1993; Puzon-McLaughlin *et al.*, 1996). These results and the failure of X1 to bind laminin 1 in the presence of Mn^{2+} support the notion that the X1 segment is able to alter the conformation of the subunit and inhibit cation binding and ligand adherence.

Comparison of the X1 and X2 alternative splice domains indicates a completely divergent amino acid sequence (Ziober *et al.*, 1993). Recent observations have indicated that critical residues for ligand-binding activity are clustered in a predicted β -turn of the C-terminal portion of the third repeat domain of inte-

Figure 10. Amino acid sequence and secondary structure of the $\alpha 7$ -X1 and X2 alternatively spliced domains. The amino acid sequences for X1 and X2 domains were analyzed by using the MacVector program. β -sheet conformations are boxed and β -turn or random coil regions are indicated. Both X1 and X2 have extensive β -sheet structures; however, X1 has a long random coil motif absent in the X2 that could act as a hinge.

grins $\alpha 4$, $\alpha 5$, and αIIb (Irie *et al.*, 1995; Kamata *et al.*, 1995). This site (GAPGTYNWKG) is conserved in all α -chains, including $\alpha 7$, and is immediately proximal to the $\alpha 7$ X1/X2 domains. This points to the possibility that the region defined by X1/X2, which directly borders the third repeat domain, may be important in regulating integrin activation. The β -turn at this site has been predicted by using several secondary structure prediction methods and from the alignment of the seven repeat domains from 16 different integrin sequences (Tuckwell *et al.*, 1994). Comparison of the $\alpha 7$ -X1 and $\alpha 7$ -X2 sequences shows a significant structural difference in the C-terminal half of the variable region (Figure 10). Although the X2 region seems to consist of multiple β -sheet structures that apparently align with β -sheet segments in the adjacent IV repeat domain, the X1 region contains a random coil structure of at least 10 residues that begin in the C-terminal half of this variable region. This coil motif in X1 may provide a "hinge-like" structure that may control conformational changes in this putative ligand-binding region of the $\alpha 7$ subunit (Figure 10). Such regulation may ultimately affect ligand-binding competency by turning on or off the subunit's ability to bind to divalent cations. Finally, as shown in this report, this apparent hinge-like structure can be modulated by $\beta 1$ -activating antibodies such as TS2/16.

How is the activation level of the $\alpha 7$ variants regulated? The observation that $\beta 1$ activation mAbs can convert the $\alpha 7$ -X1 isoform to the "on" state suggests that ligand-binding activity can be controlled by alterations in the partner subunit conformation. Affinity modulation by inside-out signaling via transmembrane conformational transitions has been observed previously (reviewed in Schwartz *et al.*, 1995). Since the $\alpha 7$ subunit expression is limited to a few highly differentiated tissues and the expression of the X1/X2 isoforms is developmentally regulated, it is possible that the functionality of this integrin needs to be controlled in a tissue-specific manner. Previous work has shown that $\alpha 7\beta 1$ is expressed during myoblast differentiation and at the myotendinous and neuromuscular junctions in adult skeletal muscle (Song *et al.*, 1992; Ziober *et al.*, 1993; Martin *et al.*, 1996). Both isoforms are expressed in myoblasts, but only X2 is detectable

in mature muscle, implying that the constitutively active form of the receptor is present at permanent myotendinous junctions and neuromuscular sites (Song *et al.*, 1992; Ziober *et al.*, 1993; Crawley *et al.*, 1996; Martin *et al.*, 1996). We propose that the regulatable X1 isoform is important during dynamic adhesion situations related to muscle development (motility, fusion, remodeling, repair, and matrix assembly) and that the X2 variant performs more stable adhesion functions (costamers and myotendinous junctions). The function of the two isoforms in skeletal myoblast/myotube adhesion is currently under investigation. Finally, the ras/raf/MAPK signaling pathway has lately been implicated in regulating the integrin affinity state (Hughes *et al.*, 1997). Whether this pathway plays an "inside-out" role in regulating the X1 isoform or even in cell-specific activation of this isoform remains to be determined.

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REFERENCES

- Arroyo, A.G., Sanchez-Madrid, P., Campamero, M.R., Martin-Padura, I., Dejana, E., and Sanchez-Madrid, F. (1992). Regulation of the VLA integrin-ligand interactions through the $\beta 1$ subunit. *J. Cell Biol.* 117, 659–670.
- Bazzoni, G., Shih, D.T., Buck, C.A., and Hemler, M.E. (1995). Monoclonal antibody 9EG7 defines a novel $\beta 1$ integrin epitope induced by soluble ligand and manganese, but inhibited by calcium. *J. Biol. Chem.* 270, 25570–25577.
- Belkin, A.M., Zhidkova, N.I., Balzac, F., Altruda, F., Tomatis, D., Maier, A., Tarone, G., Koteliensky, V.E., and Burridge, K. (1996). Beta 1D integrin displaces the beta 1A isoform in striated muscles: localization at junctional structures and signaling potential in non-muscle cells. *J. Cell Biol.* 132, 211–226.
- Brown, N.H., King, D.L., Wilcox, M., and Kafatos, F.C. (1989). Developmentally regulated alternative splicing of *Drosophila* integrin PS2 alpha transcripts. *Cell* 59, 185–195.
- Chan, B.M.C., and Hemler, M.E. (1993). Multiple functional forms of the integrin VLA-2 can be derived from a single $\alpha 2$ cDNA clone: interconversion of forms induced by an anti- $\beta 1$ antibody. *J. Cell Biol.* 120, 537–543.

- Collo, G., Star, L., and Quaranta, V. (1993). A new isoform of the laminin receptor integrin $\alpha 7\beta 1$ is developmentally regulated in skeletal muscle. *J. Biol. Chem.* 268, 19019–19024.
- Cooper, H.M., Tamura, R.N., and Quaranta, V. (1991). The major laminin receptor of mouse embryonic stem cells is a novel isoform of the $\alpha 6\beta 1$ integrin. *J. Cell Biol.* 115, 843–850.
- Crawley, S.C., Kaufman, S.J., and Cooper, D.N.W. (1996). Comparison of rat myoblast receptors for laminin-1 and laminin-2–4. *Mol. Biol. Cell* 7(suppl), 67a. (Abstract).
- Delwel, G.O., *et al.* (1994). Distinct and overlapping ligand specificities of the $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins: recognition of laminin isoforms. *J. Biol. Chem.* 268, 25865–25875.
- Delwel, G.O., Hogervorst, F., Kuikman, I., Paulsson, M., Timpl, R., and Sonnenberg, A. (1993). Expression and function of the cytoplasmic variants of the integrin $\alpha 6$ subunit in transfected K562 cells. Activation-dependent adhesion and interaction with isoforms of laminin. *J. Biol. Chem.* 268, 25865–25875.
- Delwel, G.O., Kuikman, I., and Sonnenberg, A. (1995). An alternatively spliced exon in the extracellular domain of the human $\alpha 6$ integrin subunit—functional analysis of the $\alpha 6$ integrin variants. *Cell Adh. Comm.* 3, 143–161.
- Diamond, M.S., and Springer, T.A. (1994). The dynamic regulation of integrin adhesiveness. *Curr. Biol.* 4, 506–517.
- Djaffar, I., Chen, Y.P., Creminon, C., Maclouf, J., Cieutat, A.M., Gayet, O., and Rosa, J.P. (1994). A new alternative transcript encodes a 60 kDa truncated form of integrin $\beta 3$. *Biochem. J.* 300, 69–74.
- Faull, R.J., Kovach, N.L., Harlan, J.M., and Ginsberg, M.H. (1993). Affinity modulation of integrin $\alpha 5\beta 1$: regulation of the functional response by soluble fibronectin. *J. Cell Biol.* 121, 155–162.
- Fornaro, M., Zheng, D.Q., and Languino, L.R. (1995). The novel structural motif Gln795–Gln802 in the integrin $\beta 1C$ cytoplasmic domain regulates cell proliferation. *J. Biol. Chem.* 270, 24666–24669.
- Gehlsen, K.R., Dickerson, K., Argraves, W.S., Engvall, E., and Ruoslahti, E. (1989). Subunit structure of laminin-binding integrin and localization of its binding site on laminin. *J. Biol. Chem.* 264, 19034–19038.
- Grinblat, Y., Zusman, S., Yee, G., Hynes, R.O., and Kafatos, F.C. (1994). Functions of the cytoplasmic domain of the $\beta 5$ integrin subunit during *Drosophila* development. *Development* 120, 91–102.
- Gu, M., Wang, W., Song, W.K., Cooper, D.N.W., and Kaufman, S.J. (1994). Selective modulation of the interaction of $\alpha 7\beta 1$ integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. *J. Cell. Sci.* 107, 175–181.
- Hemler, M.E., Weitzman, J.B., Pasqualini, R., Kawaguchi, S., Kassner, P.D., and Berdichevsky, F.B. (1994). Structure, biochemical properties, and biological functions of integrin cytoplasmic domains. In: *Integrins: The Biological Problems*, ed. Y. Takada, Boca Raton, FL: CRC Press, 1–35.
- Hogervorst, F., Kuikman, I., van Kessel, A.G., and Sonnenberg, A. (1991). Molecular cloning of the human $\alpha 6$ integrin subunit. Alternative splicing of $\alpha 6$ mRNA and chromosomal localization of the $\alpha 6$ and $\beta 4$ genes. *Eur. J. Biochem.* 199, 425–433.
- Hughes, P.E., Renshaw, M.W., Pfaff, M., Forsyth, J., Keivens, V.M., Schwartz, M.A., and Ginsberg, M.H. (1997). Suppression of integrin activation: a novel function of a ras/raf-initiated MAP kinase pathway. *Cell* 88, 521–530.
- Humphries, M.J. (1996). Integrin activation: the link between ligand binding and signal transduction. *Curr. Opin. Cell Biol.* 8, 632–640.
- Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69, 11–25.
- Irie, A., Kamata, T., Puzon-McLaughlin, W., and Takada, Y. (1995). Critical amino acid residues for ligand binding are clustered in a predicted β -turn of the third N-terminal repeat in the integrin $\alpha 4$ and $\alpha 5$ subunits. *EMBO J.* 14, 5550–5556.
- Kamata, T., Puzon, W., and Takada, Y. (1995). Identification of putative ligand-binding sites of the integrin $\alpha 4\beta 1$ (VLA-4, cd49d/CD29). *Biochem. J.* 305, 945–951.
- Kawaguchi, S., and Hemler, M.E. (1993). Role of the α subunit cytoplasmic domain in regulation of adhesive activity mediated by the integrin VLA-2. *J. Biol. Chem.* 268, 16279–16285.
- Kramer, R.H., Vu, M.P., Cheng, Y.F., Ramos, D.M., Timpl, R., and Waleh, N. (1991). The laminin-binding integrin $\alpha 7\beta 1$: functional characterization and expression in normal and malignant melanocytes. *Cell. Regul.* 2, 805–817.
- Kramer, R.H. (1994). Isolation and characterization of integrin laminin receptors. *Methods Enzymol.* 245, 129–147.
- Languino, L.R., and Ruoslahti, E. (1992). An alternative form of the integrin $\beta 1$ subunit with a variant cytoplasmic domain. *J. Biol. Chem.* 267, 7116–7120.
- Lin, C.S., Zhang, K., and Kramer, R. (1993). $\alpha 6$ integrin is up-regulated in step increments accompanying neoplastic transformation and tumorigenic conversion of human fibroblasts. *Cancer Res.* 53, 2950–2953.
- Martin, P.T., Kaufman, S.J., Kramer, R.H., and Sanes, J.R. (1996). Synaptic integrins in developing, adult, and mutant muscle: selective association of $\alpha 1$, $\alpha 7A$, and $\alpha 7B$ integrins with the neuromuscular junction. *Dev. Biol.* 174, 125–139.
- Matter, M.L., and Laurie, G.W. (1994). A novel laminin E8 cell adhesion site required for lung alveolar formation in vitro. *J. Cell Biol.* 124, 1083–1090.
- Meredith, J., Jr., Takada, Y., Fornaro, M., Languino, L.R., and Schwartz, M.A. (1995). Inhibition of cell cycle progression by the alternatively spliced integrin $\beta 1C$. *Science* 269, 1570–1572.
- Mould, P.A. (1996). Getting integrin into shape: recent insights into how integrin activity is regulated by conformational changes. *J. Cell Sci.* 109, 2613–2618.
- Picker, L.J., Treer, J.R., Nguyen, M., Terstappen, L.W., Hogg, N., and Yednock, T. (1993). Coordinate expression of $\beta 1$ and $\beta 2$ integrin “activation” epitopes during T cell responses in secondary lymphoid tissue. *Eur. J. Immunol.* 23, 2751–2757.
- Puzon-McLaughlin, W., Yednock, T.A., and Takada, Y. (1996). Regulation of conformation and ligand binding function of integrin $\alpha 5\beta 1$ by the $\beta 1$ cytoplasmic domain. *J. Biol. Chem.* 271, 16580–16585.
- Roote, C.E., and Zusman, S. (1996). Alternatively spliced forms of the *Drosophila* $\alpha ps1$ subunit if integrin are sufficient for viability and can replace the function of the $\alpha ps1$ subunit if integrin in the retina. *Development* 122, 1985–1994.
- Sanchez-Mateos, P., Cabanas, C., and Sanchez-Madrid, F. (1996). Regulation of integrin function. *Cancer Biol.* 7, 99–109.
- Schwartz, M.A., Schaller, M.D., and Ginsberg, M.H. (1995). Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.* 11, 549–599.
- Shimizu, Y., Van Seventer, G.A., Horgan, K.J., and Shaw, S. (1990). Regulated expression and function of three VLA ($\beta 1$) integrin receptors on T cells. *Nature* 345, 250–253.
- Song, W.K., Wang, W., Sato, H., Bielser, D.A., and Kaufman S.J. (1993). Expression of $\alpha 7$ integrin cytoplasmic domains during skeletal muscle development: alternate forms, conformational change, and homologies with serine/threonine kinases and tyrosine phosphatases. *J. Cell Sci.* 106, 1139–1152.

- Song, W.K., Wang, W., Foster, R.F., Bielser, D.A., and Kaufman, S.J. (1992). H36- α 7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. *J. Cell. Biol.* 117, 643–657.
- Suzuki, S., and Naitoh, Y. (1990). Amino acid sequence of a novel integrin beta 4 subunit and primary expression of the mRNA in epithelial cells. *EMBO J.* 9, 757–763.
- Takada, Y., and Puzon, W. (1993). Identification of a regulatory region of integrin β 1 subunit using activating and inhibiting antibodies. *J. Biol. Chem.* 268, 17597–17601.
- Tamura, R.N., Cooper, H.M., Collo, G., and Quaranta, V. (1991). Cell type-specific integrin variants with alternative alpha chain cytoplasmic domains. *Proc. Natl. Acad. Sci. USA* 88, 10183–10187.
- Tennenbaum, T., Belanger, A.J., Glick, A.B., Tamura, R., Quaranta, V., Yuspa, S.H. (1995). A splice variant of alpha 6 integrin is associated with malignant conversion in mouse skin tumorigenesis. *Proc. Natl. Acad. Sci. USA* 92, 7041–7045.
- Tuckwell, D.S., Humphries, M.J., and Brass, A. (1994). A secondary structure model of the integrin alpha subunit N-terminal domain based on analysis of multiple alignments. *Cell Adh. Comm.* 2, 385–402.
- van der Flier, A., Kuikman, I., Baudoin, C., van der Neut, R., and Sonnenberg, A. (1995) A novel beta 1 integrin isoform produced by alternative splicing: unique expression in cardiac and skeletal muscle. *FEBS Lett.* 369, 340–344.
- van Kuppevelt, T.H., Languino, L.R., Gailit, J.O., Suzuki, S., and Ruoslahti, E. (1989) An alternative cytoplasmic domain of the integrin beta 3 subunit. *Proc. Natl. Acad. Sci. USA* 86, 5415–5418.
- Wang, W., Wu, W., Desai, T., Ward, D.C., and Kaufman, S.J. (1995). Localization of the alpha 7 integrin gene (ITGA7) on human chromosome 12q13: clustering of integrin and Hox genes implies parallel evolution of these gene families. *Genomics* 26, 568–570.
- Weitzman, J.B., Pasqualini, R., Takada, Y., and Hemler, M. (1993). The function of distinct regulation of the integrin VLA-3 in cell adhesion, spreading, and homotypic cell aggregation. *J. Biol. Chem.* 268, 8651–8657.
- Yao, C.-C., Ziober, B.L., Sutherland, A.E., Mendricks, D.L., and Kramer, R.H. (1996a). Laminins promote the locomotion of skeletal myoblasts via the alpha 7 integrin receptor. *J. Cell Sci.* 109, 3139–3150.
- Yao, C.-C., Ziober, B.L., Squillace, R.M., and Kramer, R.H. (1996b). Alpha7 integrin mediates cell adhesion and migration on specific laminin isoforms. *J. Biol. Chem.* 271, 25598–25603.
- Yao, C.-C., Breuss, J., Pytela, R., and Kramer, R.H. (1997). Functional expression of the alpha 7 integrin receptor in differentiated smooth muscle cells. *J. Cell Sci.* 110, 1477–1487.
- Zavortink, M., Bunch, T.A., and Brower, D.L. (1993). Functional properties of alternatively spliced forms of the *Drosophila* PS2 integrin alpha subunit. *Cell Adh. Comm.* 1, 251–264.
- Zhidkova, N.I., Belkin, A.M., and Mayne, R. (1995). Novel isoform of beta 1 integrin expressed in skeletal and cardiac muscle. *Biochem. Biophys. Res. Commun.* 214, 279–285.
- Ziober, B.L., Vu, M.P., Waleh, N., Crawford, J., Lin, C.-S., and Kramer, R.H. (1993). Alternative extracellular and cytoplasmic domains of the integrin α 7 subunit are differentially expressed during development. *J. Biol. Chem.* 268, 26773–26783.