

Developmental Regulation and Functional Significance of Alternative Splicing of NCAM and $\alpha 7\beta 1$ Integrin in Skeletal Muscle

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Abstract

Alternative splicing of cell adhesion molecules is extensive in developing skeletal muscle and regulated at distinct periods of myogenic differentiation. Here, we discuss our current understanding of the functional properties of the alternatively spliced domains in the NCAMs and $\alpha 7\beta 1$ integrins that are involved in myogenesis and muscle integrity.

Key words: NCAM, alternative splicing, integrin, $\alpha 7\beta 1$, myogenesis, muscle development, muscle integrity.

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During embryonic development precursor cells of skeletal muscle migrate from somites to sites of further development, aggregate and fuse to form myotubes, express and assemble the proteins that form the myofibrillar contractile apparatus and specialized basal lamina, become innervated with motor neurons, and form myotendinous junctions (for review see [31]). These morphological and biochemical events depend in part on the interactions of cell adhesion molecules with ligands in the extracellular matrix and on the surface of other cells [9, 25, 37, 49, 72]. The cadherins, NCAMs, and integrins comprise the major classes of adhesion molecules in myogenic cells. Functional diversity within these molecules can be generated by (1) the expression of alternatively spliced isoforms from a single gene, as seen in NCAMs, (2) expression of isoforms from separate genes, as exhibited by the cadherins and laminins, or (3) in the case of the integrins, by a combination of both processes. The significance of the alternatively spliced domains to cell adhesion and myogenesis is the focus of this review.

Skeletal muscle development in the limb results from two waves of cell differentiation that is often referred to as primary and secondary myogenesis [31, 61]. Primary myoblasts migrate and fuse with one another to form multinucleate syncytia. These primary myotubes continue their differentiation, expressing muscle-specific proteins and accumulating a basal lamina rich in laminin that ensheaths each myofiber. Primary myotubes serve as scaffolds for the adhesion, alignment, and fusion of secondary myoblasts. The formation of secondary myotubes follows primary myogenesis, and these secondary fibers eventually give rise to the bulk of adult muscle. During myofiber

maturation, the contractile apparatus assembles and myotubes split from one another and accumulate their own surrounding basal lamina. Neuronal connectivity and directed force generation are established by innervation with motor axons and formation of myotendinous junctions.

Alternative Splicing of NCAM During Myogenesis

Neural cell adhesion molecules (NCAMs) comprise a family of cell adhesion receptors that belongs to the immunoglobulin gene superfamily. NCAMs mediate calcium-independent homophilic associations [64]. NCAM is important for the aggregation of myoblasts preceding fusion [43], the interaction of primary myofibers and secondary myoblasts [27], and the interaction of muscle fibers and motor neurons during synaptogenesis [5, 7, 14, 21, 62, 73]. Functional diversity of NCAMs during myogenesis is the consequence of expression of alternatively spliced isoforms and posttranslational modifications [18, 25, 33, 51, 63, 65].

Dividing myoblasts *in vitro* and primary myoblasts *in vivo* primarily synthesize the 140 kD transmembrane isoform of NCAM (referred to in some papers as 145 kD NCAM). As differentiation proceeds, the glycosphosphatidyl inositol (GPI)-linked 125 kD and 155 kD isoforms are expressed, as is a minor 180 kD transmembrane isoform [51, 59]. C2 myoblasts transfected to express the 140 kD or 180 kD isoforms, or a mixture of both NCAMs behave similarly: they become postmitotic more quickly and fuse earlier than control myoblasts [17, 57]. During myogenic differentiation *in vitro* and secondary myogenesis *in vivo*, an alternatively spliced, muscle-specific 125 kD isoform accumulates. This NCAM isoform lacks the trans-

membrane and cytoplasmic carboxyl terminus of the 140 kD and 180 kD isoforms but does contain an extracellular insertion of 37 amino acids called the muscle-specific domain [18, 73, 77]. This isoform also is inserted into the membrane via a GPI anchor and it is modified by polysialic acid (PSA). Transfection of C2 myoblasts to express the 125 kD NCAM isoform enhances myoblast fusion, but less effectively than the 140 kD version. A 120 kD mutant of the 125 kD isoform that lacks the MSD domain has no effect on C2 myoblast fusion [57].

Expression of the 140 kD isoform of NCAM predominates during primary myogenesis *in vivo* and this form is not modified by PSA [26]. During secondary myogenesis, modification of NCAM by PSA parallels the increase in the GPI-anchored 125 kD and 155 kD isoforms [26]. During this period the 140 kD form lacks PSA and is localized at cell-cell contacts. The heavily PSA modified 125 kD isoform is localized to the noninteracting surfaces of myotubes [26]. The 37 amino acid muscle-specific domain is abundant in serine, threonine, and proline, and this domain is the site of addition of O-linked polysialic acid [33]. The addition of N- and O-linked polysialic acid to NCAM is developmentally regulated during myogenesis and correlates with the switch in alternatively spliced NCAM isoforms [26]. The negative charge and hydration of the molecule that results from polysialylation contributes to the anti-adhesive property of PSA-NCAM [80, 81]. Although PSA modified 125 kD NCAM may have anti-adhesive properties, it is required for fusion as removal of the this GPI anchored isoform by phosphoinositol phospholipase C inhibits myoblast fusion [42].

The progression in expression of alternatively spliced forms of NCAM and the subsequent increase in expression of PSA is blocked if secondary myogenesis is inhibited by d-tubocurarine [26], or if contractile activity is inhibited by calcium channel blockers such as verapamil [59]. These results suggest that the extent of secondary myogenesis and/or contractile activity underlies the developmental switch from the 140 kD to the 125 kD NCAM isoforms. Also, blockage of myotube separation subsequent to PSA removal suggests that PSA is actively involved in breaking cell-cell interactions during fiber separation [26, 80, 81].

In adult muscle fibers NCAM expression diminishes and is restricted to the base of the neuromuscular junction [14, 15]. Expression of NCAM returns extrasynaptically upon denervation or paralysis and continues until synaptic regeneration is complete. This indicates that the expression of NCAM in muscle is regulated by the muscle's state of innervation and that NCAM plays a role in modulating the interactions that lead to synaptogenesis [14, 15]. The level of expression and the cytoplasmic domains of NCAM are major determinants of motor neuron migration [21, 22]. 3T3 fibroblasts transfected to express the 140 kD, or 125 kD alternatively spliced isoforms of NCAM, or the 120 kD mutant that lacks the MSD domain, can all promote neurite outgrowth from cocultured cerebellar neurons. This effect is dependent on the level of NCAM expression [20, 21].

The consequences of alternative splicing on NCAM function are summarized:

- (1) Induction of terminal differentiation of C2 myoblasts by the 140 kD and 180 kD isoforms of NCAM suggests that the transmembrane and/or cytoplasmic domains of NCAM negatively regulates myoblast proliferation. No selective function of their respective long or short cytoplasmic domains appears to be required for myoblast fusion *in vitro* [57].
- (2) Alternative splicing during muscle differentiation results in a change from a transmembrane to a GPI anchored form, and the insertion of the muscle-specific domain and its polysialylation [57, 77]. The GPI anchored isoforms may provide recognition between adjacent cells, whereas transmembrane NCAM isoforms, and their associated signaling pathways, may be required for cells to respond to positional information [78].
- (3) Following myoblast fusion and differentiation, GPI anchored isoforms of NCAM that are PSA modified function to separate maturing myofibers [26].
- (4) The 125 and 140 kD isoforms, and the 120 kD MSD deletion mutant, promote neurite growth during synaptogenesis. The 140 kD isoform promotes neurite outgrowth via G-protein coupled L- and N-type calcium channels [19, 21, 22]. Thus, alternative splicing modifies NCAM structure and generates alternative functional domains. These specialized domains selectively and specifically promote myoblast adhesion and fusion, myofiber maturation, neurite outgrowth during synaptogenesis, and synaptic maintenance [15].

Alternative Splicing of the $\alpha 7 \beta 1$ Integrin During Myogenesis

Integrins are transmembrane, heterodimeric receptors composed of α and β subunits. Through interactions with the extracellular matrix and other cell surface molecules, the integrins mediate cell adhesion, proliferation, and migration [10, 37]. Developing skeletal muscle expresses the $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 9$, and αv integrin chains and all of these isoforms heterodimerize with the $\beta 1$ subunit (reviewed in [49]). Although all of these integrins also are expressed in other tissues, their expression in skeletal muscle is developmentally regulated and they assume a wide variety of functions at different stages of development and in the adult [4, 6, 41, 44, 60, 68, 69, 76, 82].

The α integrin chain is thought to be largely responsible for ligand binding specificity. The β subunit can associate with the actin cytoskeleton via cytoskeletal proteins such as α -actinin and talin [34, 54, 55] and it participates in signal transduction [1, 10, 38]. Thus, integrins serve as specific links between the extracellular matrix and cell cytoskeleton. Through these associations structural integrity and signal transduction between cells and their environment are maintained. For example, binding of the $\alpha 7 \beta 1$ integrin to the extracellular matrix promotes an association of the complex with the cell cytoskeleton and a conforma-

tional change in the α chain cytoplasmic domain, thereby initiating signal transduction within the cell [68].

The $\alpha 7 \beta 1$ integrin is a laminin receptor and it is the focus of our research. Its expression in the developing limb is highly regulated during rat skeletal muscle development [28]. The $\alpha 7$ chain is first detected in the sarcolemma of primary myotubes and adherent secondary myoblasts. At this same time a basal lamina rich in laminin accumulates around the primary myofibers and myoblasts. The amount of this integrin is increased in the myofibers as they develop and this increase is dependent on differentiation [28, 39, 69]. The $\alpha 7 \beta 1$ integrin persists in adult muscle where it is localized to the myotendinous and neuromuscular junctions and to non-junctional sites [2, 46, 69]. The presence of the $\alpha 7 \beta 1$ integrin on proliferating, mobile myoblasts as well as at discrete junctional sites on adult muscle, suggests that it has numerous and distinct roles. As will be discussed, the $\alpha 7 \beta 1$ integrin is important for myoblast proliferation and mobility, thereby supporting the expansion of the population of precursor cells and their migration to the sites of secondary fiber formation. In contrast, in adult muscle the integrin appears to promote and stabilize cohesiveness between fibers and the integrity of junctional sites. We believe that these diverse functions result from the structural diversity of the $\alpha 7 \beta 1$ integrin and its ligand, laminin.

During muscle development $\alpha 7$ chains with three very different cytoplasmic domains ($\alpha 7A$, $\alpha 7B$, and $\alpha 7C$), and $\beta 1$ chains with various cytoplasmic domains ($\beta 1A$, $\beta 1B$, and $\beta 1D$), are synthesized as a consequence of developmentally regulated alternative splicing [4, 11, 68, 76, 82, 83]. $\alpha 7B \beta 1A$ is the sole isoform expressed in secondary myoblasts, and it functions in laminin-dependent myoblast migration [13]. Monoclonal antibodies specific for the $\alpha 7$ subunit block the adhesion and migration of L8E63 myoblasts on laminin but not fibronectin. Likewise, cells transfected to express this integrin adhere and spread more on laminin (Huang and Kaufman, unpublished results) and exhibit increased mobility [23]. These results explain earlier reports that laminin selectively promotes myoblast migration [29, 53] and proliferation [27]. Differentiation results in the accumulation of the alternatively spliced $\alpha 7A$ cytoplasmic domain isoform [11, 68, 83]. The $\alpha 7A$ isoform is generated by alternative RNA splicing and it is unique to skeletal muscle. Formation of $\alpha 7A$ is differentiation dependent; developmentally defective *fu-1* myoblasts do not synthesize this isoform, nor do nonmyogenic tissues or cell lines [68].

The $\alpha 7$ subunit is the predominant α integrin chain detected by immunofluorescence on adult mouse skeletal muscle [46]. The $\alpha 7B$ isoform is initially expressed on the surfaces of both migrating secondary myoblasts and differentiated myofibers. As myofiber and extracellular matrix development proceed, $\alpha 7B$ localization is restricted to the neuromuscular (NMJ) and myotendinous junctions (MTJ)

[3, 46]. $\alpha 7A$ also localizes to the NMJ and MTJ and as it is synthesized relatively late, it does not participate in the migratory events associated with early myogenesis. A third cytoplasmic isoform, $\alpha 7C$ [68], is present on differentiated myofibers at the NMJ but not at the MTJ. On adult fibers $\alpha 7C$ is the only isoform localized to extrasynaptic regions [46] where it presumably serves to "glue" fibers together and thereby participate in the concerted generation of force.

The composition of the basal lamina also is modified during muscle development, as illustrated by the expression and specific localization of different laminin isoforms. Laminin-1 (LAM-1, $\alpha 1 \beta 1 g 1$) is the dominant form early during development. As development proceeds, merosin (LAM-2, $\alpha 2 \beta 1 g 1$) accumulates around fibers and predominates. A specialized basal lamina localizes at the neuromuscular junction and contains s-laminin (LAM-3, $\alpha 1 \beta 2 g 1$) and s-merosin (LAM-4, $\alpha 2 \beta 2 g 1$) [24, 36, 45, 66]. The $\beta 2$ laminin chain is the product of a unique gene and it is important for the development of the neuromuscular junction. $\beta 2$ laminin contains specific sequences that target its localization to the NMJ [47]. The $\beta 2$ chain is thought to be part of the stop signal that localizes motoneurons to the NMJ as $\beta 2$ laminin inhibits neurite growth, a process promoted by the $\beta 1$ chain [35, 58, 74]. Mutant mice that lack $\beta 2$ laminin have abnormal NMJs [52].

Specific laminins and $\alpha 7 \beta 1$ isoforms appear to participate in the formation and/or stability of muscle junctions [2, 46]. Immunofluorescence analysis of skeletal muscle from $\beta 2$ laminin *-/-* mice demonstrates a selective absence of $\alpha 7B$ from the NMJ and a marked reduction in $\alpha 7A$ and $\alpha 7B$ at the MTJ [46]. Some sites in $\beta 2$ *-/-* mice that normally would have localized $\beta 2$ laminin chain compensate for the $\beta 2$ laminin deficiency by utilizing the $\beta 1$ chain of laminin. For example, at the NMJ $\beta 1$ laminin replaces the missing $\beta 2$ laminin and this likely accounts for the localized retention of $\alpha 7A$ and $\alpha 7C$. In contrast, $\beta 1$ laminin does not replace the $\beta 2$ chain at the MTJ and the amounts of both $\alpha 7A$ and $\alpha 7B$ are reduced at these sites. These results suggest that the $\beta 2$ laminin chain promotes localization/retention of $\alpha 7B$ at junctional sites. The absence of $\alpha 7B$ at non-junctional sites of normal adult muscle (where $\beta 1$ laminin is localized), and its absence at the NMJ of $\beta 2$ *-/-* mice that has replaced $\beta 2$ with $\beta 1$, suggests that $\beta 1$ laminin may inhibit localization of $\alpha 7B$ integrin [46]. The exclusion of $\alpha 7B$ and $\alpha 7A$ from non-junctional sites where LAM-2 ($\alpha 2 \beta 1 g 1$) is found, and their binding to LAM-1 ($\alpha 1 \beta 1 g 1$) (Gu and Kaufman, unpublished results) suggests that both the α and β laminin chains may influence integrin binding and localization. Localized synthesis of the different $\alpha 7$ chain isoforms also could account for their residence *in situ*. In any event, the developmental transition from $\beta 1$ to $\beta 2$ laminins and their localization on skeletal muscle does appear to regulate localization of the $\alpha 7 \beta 1$ isoforms [46].

Molecules that interact with the $\alpha 7$ cytoplasmic domains

have not yet been identified. However, the $\alpha 7$ cytoplasmic domains do have several motifs that may underlie such associations [68]. For example, the cytoplasmic domain of $\alpha 7$ B contains the AVKIL(P)R motif that is also present in the catalytic site of serine/threonine protein kinases as well as a region homologous with the ATP binding consensus sequence LGXXGXXGXV in these protein kinases. The $\alpha 7$ B cytoplasmic domain also contains the amino acid sequence TVPQYHAVKILREDR that is 80% homologous to a non-catalytic region in receptor-like protein tyrosine phosphatases, and three DXHP repeats, each separated by four amino acid residues. The functional significance of these DXHP repeats is uncertain and this motif does not exist in other protein sequences in current public databases. The sequence QQFKEEK in the $\alpha 7$ B cytoplasmic domain is a potential actin binding site homologous to that in villin, suggesting that the $\alpha 7$ B cytoplasmic domain may interact with the cytoskeleton. The GGP/XGXXGW sequence in the $\alpha 7$ A cytoplasmic domain is homologous with the ATP binding sites in serine/threonine kinases and two diacylglycerol kinases [67]. These results suggest the $\alpha 7$ A and $\alpha 7$ B cytoplasmic domains contain distinct signal transduction capacities. In the context of their junctional localization these domains may serve to propagate signals at these structures that are required for junctional connectivity, stability and fiber maintenance. The motifs in the $\alpha 7$ B cytoplasmic domain also may function in integrin mediated cell adhesion and mobility. A common tyrosine residue, eleven amino acids C-terminal to the conserved GFFK/XR/X sequence is present in all three $\alpha 7$ cytoplasmic domains and is a potential site of phosphorylation. This conserved placement of tyrosine in the context of the GFFK/XR/X region suggests that a spatially restricted, phosphorylation-dependent molecular association may exist in this region.

To search for additional alternatively spliced domains of the $\alpha 7$ chain, we used sets of paired primers to amplify RNA encoding segments of the extracellular domain. This led to the detection of a pair of alternative sequences and these same sequences were subsequently reported as $\alpha 7$ X1 and X2 ([83], Figure 1A). The $\alpha 7$ X2 isoform is the predominant form in adult skeletal muscle, suggesting alternative splicing of the $\alpha 7$ extracellular domain also is developmentally regulated during myogenesis. We therefore asked whether the splicing that underlies the alternative $\alpha 7$ integrin extracellular domains was muscle-specific and/or dependent on terminal differentiation.

RT-PCR analysis of RNA from L8E63 myoblasts and myotubes shows that the ratio of $\alpha 7$ X2/ $\alpha 7$ X1 RNA changes during differentiation from 10:1 to 5:1, respectively (Figure 2A). The ratio of $\alpha 7$ X2/ $\alpha 7$ X1 transcripts in fu-1 cells, a developmentally defective clone of L8E63 myoblasts, was never more than 2:1 and this ratio did not fluctuate during extended culture (Figure 2A). RT-PCR analysis of RNA from cultures of newborn myoblasts

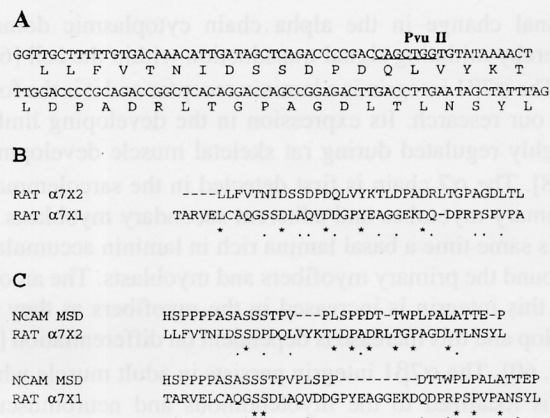


Figure 1. A: Sequence analysis of rat $\alpha 7$ X2 cDNA. Rat $\alpha 7$ X2 deduced amino acid sequence. The rat $\alpha 7$ X2 peptide sequence is identical to mouse and human $\alpha 7$ X2 [3]. The underlined sequence in the $\alpha 7$ X2 cDNA is the unique Pvu II site used to distinguish $\alpha 7$ X1 and $\alpha 7$ X2 RT-PCR products. B: Alignment of the alternatively spliced $\alpha 7$ X1 and $\alpha 7$ X2 peptide sequences. C: Alignment of the muscle-specific domain (MSD) peptide sequence of NCAM with the $\alpha 7$ X1 and $\alpha 7$ X2 peptide sequences. Alignments were performed using the Clustal V program. (*) conserved amino acid substitutions. (·) semiconserved amino acid substitutions.

demonstrates a similar decrease in the ratio of $\alpha 7$ X2/ $\alpha 7$ X1 upon differentiation, as detected in the myogenic cell line (Figure 2B). In contrast, a progressive increase in the ratios of $\alpha 7$ X2/ $\alpha 7$ X1 transcripts was found in 2 week, 4 week and adult hindlimb RNA (Figure 2D), suggesting that the $\alpha 7$ X1 isoform functions predominantly during differentiation. Once fibers are formed, $\alpha 7$ X2 becomes the dominant functional form, as also reported by Ziober et al. [83]. Our continued detection of $\alpha 7$ X1 in adult skeletal muscle is likely due to the increased sensitivity of detection using $\alpha 32$ P-dCTP incorporation as compared to ethidium bromide stained agarose gels [83]. Just as the localization of the alternative $\alpha 7$ chain cytoplasmic domain forms is altered in the $\beta 2$ laminin $-/-$ mutant mice [46], different isoforms of laminin also may influence the relative amounts of $\alpha 7$ X2 and $\alpha 7$ X1 seen *in vitro* and *in vivo*.

The dissimilar ratios of $\alpha 7$ X2/ $\alpha 7$ X1 transcripts in fu-1 and L8E63 myoblasts may be the consequence of different myogenic capacities. Likewise, L8E63 myoblasts can fuse, biochemically differentiate, and synthesize the muscle-specific $\alpha 7$ A isoform, whereas the developmentally defective fu-1 cells do not fuse or biochemically differentiate, are transformed and tumorigenic [39, 40], and synthesize only $\alpha 7$ B [68]. Thus, myogenic capacity appears to influence the expression of $\alpha 7$ X2 and $\alpha 7$ X1, and, it is essential to generate the alternative $\alpha 7$ A and $\alpha 7$ C cytoplasmic do-

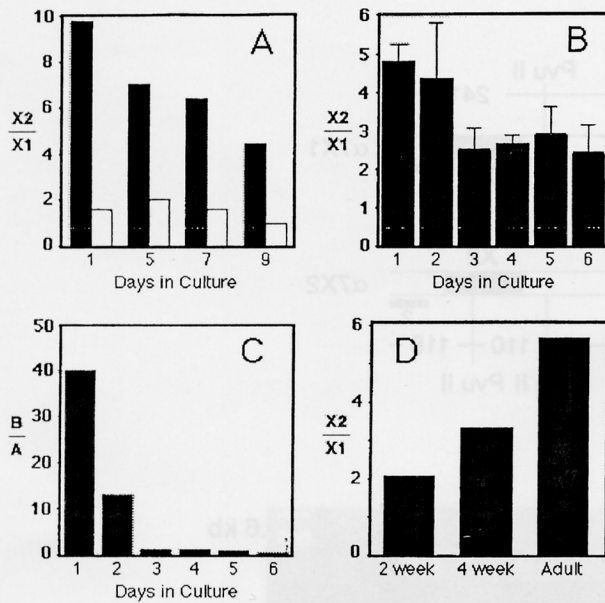


Figure 2: RT-PCR analysis of the alternatively spliced RNAs of the $\alpha 7$ integrin. A: Ratios of $\alpha 7 X 2 / \alpha 7 X 1$ RNAs detected in myogenic L8E63 (■) and developmental defective *fu-1* (□) myoblasts. B: Ratios of $\alpha 7 X 2 / \alpha 7 X 1$ RNAs detected in newborn rat primary cultures of thigh muscle tissue grown from 1 to 6 days. C: Ratios of $\alpha 7 B / \alpha 7 A$ cytoplasmic domain RNAs detected in newborn rat primary cultures of thigh muscle tissue grown from 1 to 6 days. D: Ratios of $\alpha 7 X 2 / \alpha 7 X 1$ RNAs detected in 2 week, 4 week, and adult rat thigh muscle tissue.

main. Although alternative splicing of the $\alpha 7$ extracellular domain is not muscle-specific, alternative splicing in muscle does favor expression of the $\alpha 7 X 2$ form.

RT-PCR analysis indicates that where detected, the $\alpha 7 X 1$ or $\alpha 7 X 2$ forms are preferentially expressed in different cell types. For example, rat embryo fibroblasts express both $\alpha 7 X 2$ and $\alpha 7 X 1$, and $\alpha 7 X 2$ predominates in these fibroblasts, whereas only $\alpha 7 X 2$ was detected in brain, and $\alpha 7 X 1$ predominates in stomach and lung RNA (Figure 3B). Thus both $\alpha 7 X 1$ and $\alpha 7 X 2$ RNAs are expressed in non-myogenic cells. In contrast, the $\alpha 7 B$ cytoplasmic domain is the only form detected in myoblasts and non-myogenic cells, and the $\alpha 7 A$ and $\alpha 7 C$ variants are exclusive to differentiated skeletal muscle. Thus, the splicing mechanism that gives rise to these alternative extracellular and cytoplasmic domains appears to act selectively on different portions of the same transcripts, in different cells. Alternatively, two different splice mechanisms may act on different portions of the same transcripts and these may be selectively and uniquely regulated in skeletal muscle.

It was suggested that the $\alpha 7 X 1$ and $\alpha 7 X 2$ domains may recognize different ligands in the extracellular matrix [83]. Unfortunately, the lack of purified preparations of the

various laminin isoforms has precluded discerning whether the $\alpha 7 X 1$ or $\alpha 7 X 2$ extracellular domains favor binding to different laminin isoforms. Any selective localization of the $\alpha 7 X 1$ and $\alpha 7 X 2$ isoforms also has not yet been demonstrated. In contrast, CHO $\beta 1 A$ cells transfected to express $\alpha 7 A X 1$, $\alpha 7 B X 1$, $\alpha 7 A X 2$, or $\alpha 7 B X 2$ attach and spread faster on laminin than nontransfected cells, but no differences in adhesion rate or morphology were detected between isoforms (Huang and Kaufman, manuscript in preparation). Likewise, no differences in binding to particular laminin isoforms were detected using cells expressing the $\alpha 6 X 1$ and $\alpha 6 X 2$ isoforms, the closest relatives of $\alpha 7 X 1$ and $\alpha 7 X 2$ [16].

The muscle-specific domain (MSD) of NCAM that is expressed in differentiating myotubes is the site of O-linked polysialylation [77]. As this domain in NCAM is similar in size and sequence to the $\alpha 7 X 1$ and $\alpha 7 X 2$ domains, this region in the $\alpha 7$ chain also may be glycosylated (Figure 1C). Developmentally regulated glycosylation of $\alpha 7 X 1$ and $\alpha 7 X 2$, as in NCAM, may modulate the function of the $\alpha 7$ extracellular domains. Selective site-specific glycosylation of the $\alpha 7 X 1$ and $\alpha 7 X 2$ domains could explain the interaction of the integrin with the muscle lectin L-14, a lactose-binding protein that is secreted during terminal differentiation [12]. L-14 can prevent the interaction of laminin and the $\alpha 7 \beta 1$ integrin and dissociate them, thereby modulating the interaction of myoblasts and matrix that precedes fusion [30]. As L-14 is a homodimer that can bind to both the integrin and to laminin, its abundance on adult muscle fibers may serve to strengthen the association of the integrin and matrix, and contribute to fiber integrity. The associations of L-14 with the various $\alpha 7 \beta 1$ isoforms, at different times and in different concentrations, may have distinct consequences. The extracellular portion of the $\alpha 7$ chain also is ADP-ribosylated upon myogenic differentiation [84, 85]. The functional significance of this modification remains unclear: it does not affect $\alpha 7 \beta 1$ binding to immobilized laminin (Gu and Kaufman, unpublished data) or the association of the $\alpha 7$ and $\beta 1$ chains [84, 85].

In addition to the diversity in potential ligand-receptor interactions that results from distinct forms of the $\alpha 7$ chain and laminin isoforms, $\beta 1$ integrin subunits with alternative cytoplasmic domains also are expressed in a developmentally regulated manner during myogenesis ([4, 76, 82], Figure 4). Myoblasts initially express the $\beta 1 A$ cytoplasmic domain isoform that is common to most other cell types. As differentiation proceeds, a muscle-specific, alternatively spliced cytoplasmic domain isoform, $\beta 1 D$, accumulates and eventually replaces the $\beta 1 A$ protein in fibers. Immunofluorescence staining of adult skeletal muscle shows that $\beta 1 D$ is present at myotendinous and neuromuscular junctions, and at costameres [4]. In cardiac muscle $\beta 1 D$ localizes to the intercalated discs, regions of cell-cell adhesion in cardiomyocytes, further indicating that the alternatively spliced $\beta 1 D$ subunit is important for stabiliz-

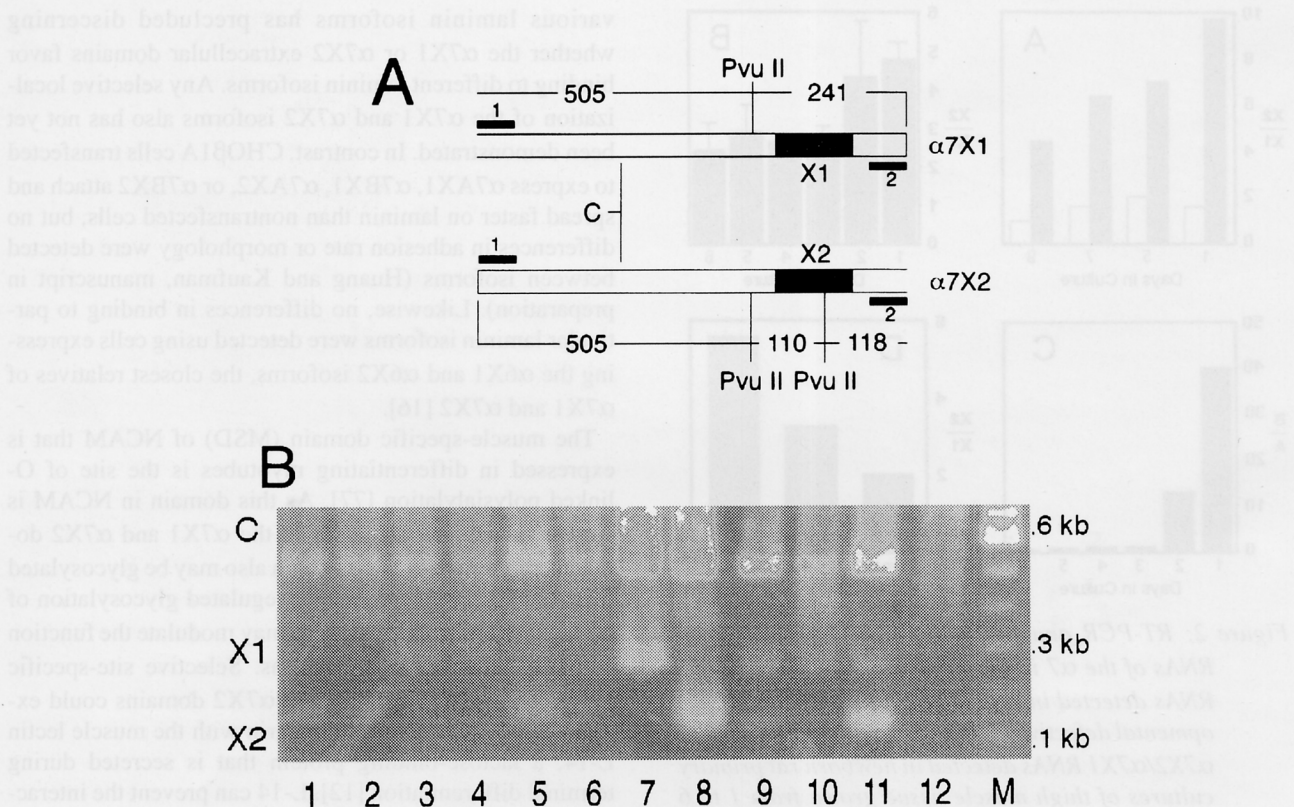


Figure 3. A: Strategy to distinguish $\alpha 7X1$ and $\alpha 7X2$ RT-PCR products. Common primers, 1 and 2, were used to amplify $\alpha 7X1$ and $\alpha 7X2$ RNAs in the same reaction mixtures. RT-PCR products were digested with *Pvu II* endonuclease, run on a 1.5% agarose gel, and stained with ethidium bromide to separate the $\alpha 7X1$ (X1) and $\alpha 7X2$ (X2) sequences from the common (C) sequence. B: RT-PCR analysis of RNA from: (1) Rat-1 fibroblasts, (2) adult rat thigh muscle, (3) stomach, (4) lung, (5) brain, (6) liver, (7) $\alpha 7X1$ cDNA, (8) $\alpha 7X2$ cDNA, (9) day 2 normal rat kidney (NRK) cells, (10) day 8 NRK cells, (11) Fischer embryonic fibroblasts, (12) day 6 10T 1/2 fibroblasts, (M) Molecular weight markers.

ing regions subject to mechanical stress. The $\beta 1D$ chain heterodimerizes with the $\alpha 7A$ and $\alpha 7B$ integrin subunits in skeletal muscle [4]. It is not yet known if $\alpha 7C$ associates with $\beta 1A$ and/or $\beta 1D$.

The alternative $\beta 1A$ and $\beta 1D$ domains both have two NPXY motifs that are believed to regulate binding affinity of the integrin [4, 56, 76, 82]. The $\beta 1A$ and $\beta 1D$ isoforms appear to function similarly in integrin mediated signal transduction, and localization of $\beta 1D$ and $\beta 1A$ at focal adhesions, tyrosine phosphorylation of pp¹²⁵FAK (focal adhesion kinase), and activation of mitogen-activated protein (MAP) kinases are indistinguishable between these isoforms [4]. An intriguing possibility for the function of the $\beta 1D$ subunit is that it may form stronger linkages between the cytoskeleton and extracellular matrix than its $\beta 1A$ counterpart [4, 82]. This could contribute to fiber and junctional integrity during muscle activity. The replacement of $\beta 1A$ with $\beta 1D$ during myogenesis also may modulate assembly of the contractile apparatus [76].

Another $\beta 1$ cytoplasmic domain isoform, $\beta 1B$, is expressed at a low level in skeletal muscle [2]. Upon transfection into cultured cells, $\beta 1B$ behaves as a dominant negative integrin that is unable to trigger tyrosine phosphorylation of pp¹²⁵FAK, or support adhesion or migration, apparently due to its inability to interact with the cytoskeleton [2] and lack of NPXY sequences. Transient or low expression of the $\beta 1B$ isoform may have a regulatory role during myogenesis. $\beta 1B$ heterodimerized with various α integrin subunits may bind ligands but its capacity for signal transduction and association with the cytoskeleton appears reduced. As a consequence, this isoform could modulate the transition of myoblasts from an integrin-dependent proliferative, migratory state to the quiescent, post-mitotic state that precedes fusion. In doing so, it also may promote the formation of satellite cells, and maintain them as a residual population of quiescent precursors that can later become activated and used to repair muscle.

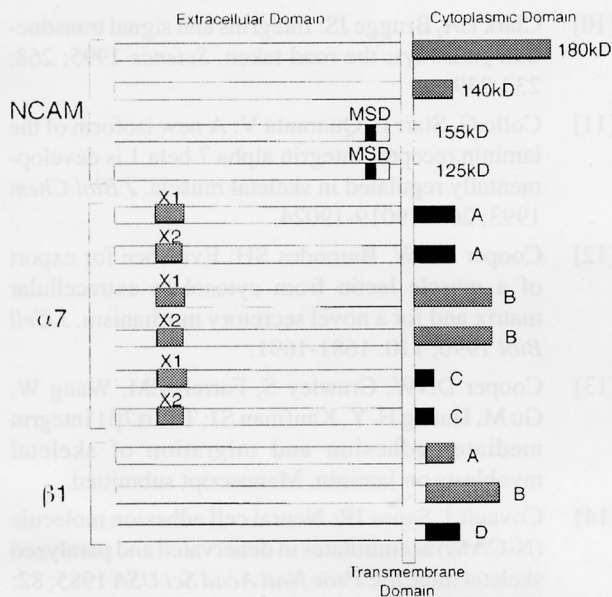


Figure 4. Alternative isoforms of cell adhesion molecules involved in myogenesis. In skeletal muscle, alternative splicing generates four major isoforms of NCAM, six isoforms of the $\alpha 7$ integrin chain and three isoforms of the $\beta 1$ integrin chain. Splicing of the extracellular muscle specific domain (MSD) of NCAM and the cytoplasmic domains of $\alpha 7A$, $\alpha 7C$, and $\beta 1D$ integrin are muscle-specific.

In addition to the internal myogenic regulation of alternative splicing of $\alpha 7$ RNA, laminin appears to regulate $\alpha 7$ gene expression. This adds an additional level of complexity to the synthesis of specific $\alpha 7$ integrin chain isoforms. In the *mdx* mouse and in humans with Duchenne muscular dystrophy (DMD), the lack of dystrophin and failure to assemble dystrophin associated proteins, results in muscle myopathy [8, 48, 50, 71]. Normally, the dystrophin-dystroglycan complex links laminin in the matrix with the cell cytoskeleton, and this association is essential for maintaining the structural integrity of muscle. In the absence of this transmembrane complex the amount of laminin remains unchanged. In contrast, expression of the $\alpha 7 \beta 1$ integrin increases in these patients and in *mdx* mice. We suggest that the increase in unbound laminin in the *mdx* mouse and in DMD patients results in the upregulation of integrin expression. Analysis of RNA from *mdx* skeletal muscle indicates this regulation occurs at the level of gene transcription (Kaufman et al., manuscript in preparation). The enhancement of the $\alpha 7 \beta 1$ transmembrane linkage between laminin and the cytoskeleton in myofibers may compensate in part for the lack of the dystrophin-mediated linkage system and attenuate the course of myopathy.

In the *dy* mouse and in children with congenital $\alpha 2$ laminin muscular dystrophy, the lack of the $\alpha 2$ laminin chain results in a severe degenerative myopathy [32, 70, 75, 79]. In these cases, the absence of the laminin isoforms

LAM-2 and LAM-4 is accompanied by a major reduction in the amount of the $\alpha 7 \beta 1$ integrin. In the mouse, where this has been studied, this appears to be transcriptionally regulated: in the absence of laminin, $\alpha 7$ gene transcription is inhibited (Kaufman et al., manuscript in preparation).

In summary, alternative splicing of the $\alpha 7$ integrin during myogenesis results in the formation of six $\alpha 7$ chain isoforms and each may be modified posttranslationally. At least three isoforms of the $\beta 1$ chain also are generated in a developmentally regulated fashion. The additional diversity in the isoforms of laminin that accompanies myogenesis, combined with the structural diversity of the integrin, can account for the diverse roles of this matrix protein and its receptor in muscle development and fiber function. The temporal expression and localization of particular $\alpha 7 \beta 1$ isoforms indicates that the regulation of $\alpha 7 \beta 1$ expression and localization are complex. Defining the mechanisms by which selective splicing of different portions of $\alpha 7$ transcripts occurs, and those that underlie isoform-specific junctional and non-junctional localization in fibers remain interesting problems to resolve. The pathologies associated with changes in laminin-dependent expression and localization of the $\alpha 7 \beta 1$ integrin that are seen in $\beta 2$ laminin $-/-$ mice, in *mdx* mice and Duchenne dystrophy, and in *dy* mice and $\alpha 2$ laminin congenital muscular dystrophy tell us that the transmembrane linkage between the matrix and cytoskeleton that is mediated by the $\alpha 7 \beta 1$ family of receptors is significant to muscle integrity. Based on these results, it would not be surprising to find that some form of muscular dystrophy is directly associated with a defect in this integrin.

One final question to consider is why alternative splicing plays such a significant role in myogenesis. (1) Alternative splicing allows for the efficient utilization of genetic information. Evolving new domains in a molecule generates functional diversity while retaining other properties. For example, the alternative $\alpha 7$ cytoplasmic domains can transmit very different signals in response to common extracellular cues. Likewise, the alternative $\beta 1$ cytoplasmic domains may regulate the affinity of associations both inside and outside the cell, thereby determining the capacity of the integrin to support either migration or structural integrity. (2) Alternative splicing bypasses the need to activate new genes. This may be of particular significance if the structure of chromatin in differentiated myofibers does not support the activation of quiescent genes as readily as chromatin in undifferentiated, proliferating cells. As this relative "dormancy" of chromatin may be important to maintain the stability of the differentiated state, alternative splicing may serve to provide functional diversity in the absence of activation of quiescent genes. (3) Localized alternative splicing may produce specific isoforms that are designed to function in regional domains of the myofiber. The local synthesis of different isoforms, for example at the neuromuscular and myotendinous junctions, would obviate the need for different isoforms to sort out and be

transported to these specialized sites in muscle fibers. Whether site-specific alternative splicing underlies the localization of the $\alpha 7 \beta 1$ isoforms remains an interesting question to resolve.

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