A Novel Periodic Localization for al Integrin in Skeletal Muscle

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Abstract

Integrins influence myogenic mechanisms. A number of integrin subunits have already been demonstrated to have specific roles in various aspects of the developmental process. Data is presented that demonstrates a novel localization for al integrin in avian skeletal myofibers. Cultured chick pectoralis muscle was immunofluorescently labeled with antibodies to al integrin and several muscle specific proteins, al integrin was detected in postmitotic, mononucleated, elongated myocytes with a punctate distribution along the myofiber. As early as 3 days in culture, al integrin reorganized from a punctate pattern to one with a sarcomeric periodicity. The periodic al integrin band, which appeared as a single band in contracted muscle, was observed as a doublet band when the muscle was relaxed with one half of each doublet band localized on either side of the Z line. The identical periodic localization for ai integrin was found on intact myofibers from chick embryonic day 17 hind limb and cardiac muscle as well as on murine C2C12 myotubes. The temporal regulation of the punctate to periodic distribution indicates a possible role for this integrin subunit in the assembly of the myofiber early in myogenesis. Key words: al integrin, myogenesis, skeletal muscle, development, avian.

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Integrins are a large family of transmembrane proteins that have been identified to play a role in multiple cellular events including cell adhesion [17, 25, 26, 32, 60], proliferation [14, 34, 57, 73], differentiation [8, 10, 52, 57, 73], migration [17, 71, 92] and signaling [2, 3, 7, 44, 72, 81, 88]. Functionally integrins exist as heterodimers, consisting of one α and one β subunit (For reviews [1, 15, 44, 88]). A large percentage of the known a and p integrin subunits have been reported to be expressed by skeletal muscle at various times throughout development and to affect the course of myogenesis. Studies have shown that while αl, α3, α4, α5, α6, α7. α9, αυ, α_{mt}, β1 and β3 have all been identified, elucidation of the regulation and function of many of these subunits has yet to be fully appreciated. Changes in integrin subunit levels have been documented during embryonic, fetal and adult stages in myocytes, primary and secondary myotubes and satellite cells as well as in muscle cell lines. Some changes in integrin subunit levels can be associated with particular myogenic events. Specific integrins can influence the differentiation pathway of skeletal muscle precursors. Interference with pi integrin function was found to block skeletal muscle differentiation [57]. Initially, α5 βl was suggested to be important in the differentiation of muscle [25, 10]. Subsequently, muscle differentiation was shown to be influenced by both the α5 and α6 integrin subunits [72, 73]. While α5 appeared to preferentially maintain muscle precursors in the proliferative phase, α6 permitted them to withdraw from the cell cycle and differentiate. More recently, a decrease in the expression level of the β3 subunit of αυβ3 integrin has been reported to be essential for the differentiation of muscle satellite cells [8]. Integrins can be affected by differentiation as well as effect a developmental change. α_{ml} integrin levels have been shown to be up-regulated upon myogenic differentiation in human fetal myoblasts from the cell line G6 [36].

Developmental cues can also be modulated by specific α integrins and their interaction with extracellular matrix molecules. The regulation of α5 integrin as well as α6 and α7 integrin in muscle cells has been shown to correlate with their respective extracellular matrix ligands. Protein levels of the fibronectin receptor, α5

integrin, were found to be up-regulated in chick muscle from embryonic days 4 through 17, when muscle associated fibronectin levels were high [59]. However decreased levels of α5 integrin were detected in the adult along with decreased levels of fibronectin. Up-regulation of the laminin receptor, α6 integrin, in chick muscle was seen throughout stages 8 to 35 [12]. Cells positive for α6 integrin were detected in regions destined to give rise to muscle including the somite and the limb. In these regions, the cells which terminally differentiated into skeletal muscle were also identified as positive for 0.6 integrin. The region surrounding these skeletal cells was positive for laminin. Like α5 integrin, α6 integrin was reported to be down-regulated in adult chick muscle. However, in this case, even though 0.6 integrin decreased, laminin was still detectable. Finally in mouse, αv integrin, a receptor for vitronectin and fibronectin, was reported to be up-regulated in cells of the myotome and in premuscle masses in the body wall and the limbs [40]. The αv integrin staining appeared to be concentrated at the ends of the cells indicating association with myotendinous junctions, αv integrin was seen to be down-regulated in adult muscle. In cultured mouse myotubes, αv integrin was found to be enriched at myotube ends [40]. However, in chick muscle plated under different culture conditions, αv integrin was found to be up-regulated with a localization at the Z-line [56]. In both cases αv integrin seems to be involved in an anchoring mechanism, αv is known to form heterodimers with a number of β integrin isoforms. Since it has been shown that the αvβ 3 integrin heterodimer is down-regulated during muscle development, the β integrin partner for av integrin at the Z line is unlikely to be β 3 but one of the other β integrin subunits. While αv integrin localizes to the myotendinous junction early in development, α7 integrin, a laminin receptor, along with its appearance in the neuromuscular junction [54] appears to be associated with the myotendinous junction in later developmental stages and in the adult [4]. α7 integrin has been identified on both primary and secondary myocytes [34]. In primary myocytes, α7 integrin was only observed after the muscle specific intermediate filament, desmin, was already being synthesized. In secondary myocytes, α7 integrin was detected prior to the appearance of desmin. It was proposed that the appearance of α7 integrin influenced the proliferation of secondary myocytes and the alignment of secondary myocytes around primary myotubes. It has also been suggested that the interaction of laminin with α7 integrin can regulate the proliferative state of muscle fibers [34].

Integrins can influence anchoring not only to the substrate but also between cells and provide signals for fusion. It has been shown that interaction of α4 integrin, the a subunit of very late activation antigen 4 (VLA-4) and its receptor the vascular cell adhesion molecule 1 (VCAM-1), has an affect on myogenesis [70]. Up-regulation of a4 integrin protein levels has been shown to occur after the fusion of primary myoblasts into primary myotubes [76, 19]. In C2C12 cells, blocking of the VCAM-1 and VLA-4 receptors with the appropriate antibodies resulted in an approximate 60% decrease in myotube formation. Secondary myocytes then exhibit VCAM-1 on their surface as they align along primary myotubes. The expression of a4 on primary myotubes and that of VCAM-1 on secondary myotubes has been proposed to correlate with the regulation of secondary myotube formation. Two other a integrin subunits, a 1 and α9 integrin, which as yet have no defined myogenic event associated with them, have also been identified in skeletal muscle. Investigations of the α9 integrin subunit have predominantly been limited to distribution studies [64, 85]. In mouse, α9 has been identified in skeletal muscle of the diaphragm and tongue as early as embryonic day 12.5 and in skeletal muscle of the trunk and limb by embryonic day 14.5. The presence of the αl integrin subunit in skeletal muscle has also been demonstrated. In embryos, αl integrin was detected in developing myotomes and in cells migrating from them but was not observed in adult skeletal muscle [24, 32] except at the neuromuscular junction in postnatal rats [54]. In skeletal muscle cultures al integrin was detected in older cultures, i.e. 25 days [56].

The complexity of the integrin story in myogenesis has been increased by the mounting number of muscle associated integrin isoforms. Variant, alternatively spliced forms of α3, α6, α7 and β1 are differentially expressed during development. Using RT-PCR, α3A mRNA was detected in all mouse tissues examined, while α3B mRNA was detected only in skeletal, heart and brain tissue [21]. By immunohistochemistry, however, α3B protein levels were detected on only endothelial cells in heart and brain. The α3 integrin expression detected on skeletal muscle during development [56] is likely to be the α3A variant. a6 integrin and α7 integrin have variants in both the cytoplasmic [41, 78, 79, 94] and the extracellular domains [20, 94]. It has been hypothesized that the appearance of the different α6β 1 and α7β 1 isoforms, which bind exclusively to laminin, may be related to their interaction with various laminin isoforms and as such influence development. Unlike for the isoforms of α7 integrin, although α6 integrin has been shown to be present on skeletal muscle [12], a detailed analysis of the appearance of the α6 integrin isoforms during development is not yet available. α7 integrin has been shown to be a component of both the myotendinous junction [4] and the neuromuscular junction [54]. The α7A, α7B and α7C integrin isoforms are differentially

expressed in skeletal muscle development both *in vivo* [34, 48, 83] and in cell lines [16, 78, 92, 94], In myotomal muscle, α*l* integrin is found diffusely distributed along the sarcolemma but especially concentrated at the myotendinous junctions [4, 48, 83]. This distribution continues postnatally at which time α7A and α7B integrin are reported to be isolated to synaptic regions while a7C can be detected at both synaptic and extrasynaptic regions [54]. The α7B isoform is detected in myoblasts prior to terminal differentiation. After differentiation, although present, the α7B transcript is down-regulated and the α7A and α7C transcripts are up-regulated [78]. The α7A isoform is detected exclusively in skeletal muscle tissue [16]. There is also a differential distribution of the a7 integrin extracellular isoforms, XI and X2 [94, 17]. Both the XI and the X2 isoforms were found in myoblasts while in adult skeletal muscle the X2 isoform was found exclusively [94]. Four isoforms have also been identified for the (β1 integrin subunit, β1A, β1B, β1C and β1D. Of these, only β1A and β1D are found in skeletal muscle [2, 7, 82, 93]. In cells lines [82] and *in vivo* [93], β1D mRNA levels increase in maturing myotubes while βlA mRNA levels decrease. The βlD isoform was localized in adhesion junctions as well as with a sarcomeric periodicity in the skeletal muscle sarcolemma [7].

The loss of β integrin has resulted in functional defects in skeletal muscle. Competitive inhibition of the ligand binding site using anti-β1 integrin antibodies in primary skeletal cultures has been demonstrated to cause the detachment of postmitotic myocytes from the substratum [60, 57], while the inhibition of β1 integrin expression via antisense oligonucleotides in cultured satellite cells resulted in the detachment of myoblasts from the substratum [14]. Even more dramatic than the loss of skeletal muscle adhesion, are the effects of integrin loss on myogenesis due to gene deletions. Several reports detail abnormal muscle function as a result of the deletion of an integrin gene. Newman and Wright [61] have described a *Drosophila* embryonic lethal mutant, myospheroid, that lacks the PS3 antigen (homologue to vertebrate P integrin). The loss of integrin in the skeletal muscle of this mutant phenotype has been shown to result in defective muscle [61]. In cultured cells grown from these myospheroid mutant embryos, Z bands do not form [84]. *In vivo,* the mutant embryos appear to develop normally until the first muscle contraction [84, 95]. Subsequent contractions are weak or absent and embryos die at or near hatching [61, 71]. The myocytes appear to fuse normally into myotubes but fail to establish normal sarcomeres. A similar scenario has been reported for the *C. elegans* lethal mutant, Pat3, which also lacks p integrin [35]. In the muscle of Pat3 mutants, neither actin nor myosin is properly organized into sarcomeres as compared to wild-type embryos [87]. The mutants are paralysed at later stages and elongation is arrested at the two fold stage. The loss of integrin in these mutants and the resultant sarcomeric disorder suggested that the integrins played a role in myofibril assembly [43, 87]. An alternative proposal was that the sarcomeres assembled normally, but due to a disruption in cytoskeletal attachment to the membrane, became disorganized after the first few muscular contractions which subsequently led to muscle dysfunction. Based upon this scenario, it was also suggested that integrins might play a role in anchoring the cytoskeleton to the membrane [43, 87]. In vertebrates, there is one report describing the loss of β1 integrin. Mice, in which more than 50% of embryonic stem cells were lacking β1 integrin were examined [28]. Adhesive and migratory cell properties were reduced in homozygous mutants but unaffected in heterozygous mutants.

The examination of genetic mutations of a integrin subunits has not been as instructive as the mutations for β integrin. Generation of mice with null alleles for αl [32], α4 [90], α5 [86, 89], α6 [33] and α7 [55] integrin subunits have, in general, showed no obvious effects on muscle development. Mice with null alleles for α4 and α5 integrin resulted in embryonic lethality before skeletal muscle development had progressed very far. Although, in the case of α4 integrin it has been shown that α4-null embryonic stem cells were able to contribute to skeletal muscle cultures in which the myofibers appeared morphologically normal [91]. Mice with the null allele for 0.6 died at birth with blistering of the skin and other epithelia but with no obvious affects on their skeletal muscle. Mice with null alleles for al [32] and α7 [55], unlike mice with null alleles for α4, α5, and α6, remained viable. The skeletal muscle of the cd-null allele mice appeared normal [32]. The skeletal muscle of the α7-null allele mice appeared to develop normally but after birth began to show signs of myopathy characteristic of muscular dystrophies [55]. The effects of the absence of α7 integrin appears to be not so much on skeletal muscle development but on the maintenance of the integrity of the myofiber as was seen in *Drosophila* and *C. elegans* embryos lacking pi integrin.

Certain integrins have been shown to exhibit a sarcomeric periodicity along the sarcolemma in muscle cells. This periodic localization suggests that these integrins are involved in linking the cytoskeleton to the membrane. In the skeletal muscle of *Drosophila* embryos, antibody to the PS3 antigen (pi integrin homologue) localizes with a sarcomeric staining pattern that aligns with the Z band [84]. In C. *elegans* larvae, p integrin has been localized to dense bodies (Z line analogs) as well as M-lines [87]. In cultured avian

skeletal muscle, (31 integrin [7, 11, 93] and αv integrin [56] were detected with an obvious sarcomeric periodicity that was also reported to align at the Z line. In addition, weak periodic al integrin staining was reported in older chick skeletal muscle cultures, i.e. 25 days [11]. Although the presence of al integrin in skeletal muscle is known, its precise localization along the sarcolemma has not been fully documented. Data is presented that demonstrates a novel periodic localization for αl integrin along avian skeletal myofibers as well as a temporal appearance that is earlier than has previously been reported. Avian skeletal myofibers, both *in vitro* and *in vivo,* and C2C12 cells were immunofluorescently labeled with anti- αl integrin as well as with antibodies to muscle specific proteins. In nascent, postmitotic, myocytes αl integrin was localized with a punctate distribution along the sarcomere. However, in more mature muscle the αl integrin reorganized to reveal a sarcomeric periodicity that was isolated to the Al region of the sarcomere and as such with the elastic region of the membrane, αl integrin could be detected with this sarcomeric periodicity as early as 3 days in primary skeletal cultures, earlier than has previously been reported.

The temporal reorganization of al integrin in skeletal muscle suggests a role for this protein in the assembly of the myofiber early in myogenesis.

Material and Methods

All studies were carried out using white leghorn chicken embryos staged according to Hamburger & Hamilton [37].

Purification of αl integrin antibody

The αl integrin antibody is a polyclonal antibody made against an 11-mer peptide (CKRPLKKKMEK) to the cytoplasmic portion of the protein [58]. The al integrin antibody was purified using the Pierce Ultralink immobilized carboxy system (Pierce, Rockford, IL) according to the manufacturers instructions. Briefly, the antibody serum was passed over a column to which the original peptide immunogen was bound. Serum that did not bind to the column was saved as the column run-off. Antibody that bound to the column was eluted with 0.1 M glycine - pH 2.8 and pooled. The pooled eluate was concentrated by centrifugation at 2000 x g (4°C) in an Amicon centriprep ultrafilter with a 10,000 nominal molecular weight cut (Amicon, Beverly, MA). Identical results were obtained using either the purified or unpurified anti- αl integrin. The purified αl integrin antibody was used for the immunohistochemistry studies.

Preparation of isolated intact myofibers

Hind limb or cardiac muscle was isolated from day 17 chick embryos. Embryos were removed from the shell and placed in Hanks balanced salt solution (BSS - Life Technologies, Gaithersburg, MD) in a 100 mm Petri dish (Falcon brand - Fisher Scientific, Pittsburgh, PA). For skeletal muscle, the skin over the leg was removed and hind limb muscle was clipped at the tendon away from the bone. For cardiac muscle, the skin over the sternum was removed, the sternum split in half to expose the heart and the heart was removed. The remainder of the protocol was identical for both tissues. Muscle was placed into fresh BSS in 35 mm Petri dishes. The tissue was then stretched by pinning the muscle ends into either silicone (dissecting dishes with silicone: Electron Microscopy Sciences, Ft. Washington, PA) or wax disks with 0.2 mm stainless steel minutien pins (Fine Science Tools, Foster City, CA). (To make the wax disks, paraffin was heated until it was liquid, poured into 100 mm glass Petri dishes to a thickness of approximately 1/4" and then allowed to solidify.) Each piece of pinned tissue was covered with a small amount of BSS to prevent drying while the remaining pieces were pinned to the wax. Residual BSS was removed and the tissue was fixed with 2% formaldehyde in potassium acetate buffer (PAB: 100 mM $C_2H_3O_2K$, 15 mM KH_2PO_4 with 5 mM EOT A, 20 mM iodoacetic acid; pH 6.3) for 30 minutes. lodoacetic acid and EGTA were added to the buffer to reduce residual muscle contractions. The tissue was extracted using 0.5% Triton-X 100 in phosphate buffered saline (PBS: 0.015 M NaCl, 0.83 mM Na_2HPO_4, 0.16 mM $NaH_2PO4'H_2O$) overnight. Using a stereomicroscope, muscle fibers were teased from the main muscle mass and immunostained in 96 well plates (see below).

Preparation of primary skeletal muscle cultures

Skeletal muscle cultures were prepared from embryonic day 10 pectoralis muscle as previously described [23, 39]. Briefly, pectoralis tissue was isolated and as much contaminating tissue as possible (i.e. connective tissue and blood vessels) was removed. The cleaned, isolated tissue pieces were minced then trypsinized in Ca^{2+}-Mg^{2+} free balanced salt solution (CMF-Life Technologies) with trypsin (final concentration: 0.05% trypsin (volume to volume) - Life Technologies) at 37°C for 15 minutes. Cells were spun at 200 x g for 10 minutes and resuspended in Minimal Essential Medium (MEM) with Earle's salts and L-glutamine to which was added 10% horse serum, 5000 U/ml penicillin, 5000 µg/ml streptomycin, 1% fungizone (250 jig/ml amphotericin B and 205 µg/ml sodium deoxycholate), 200 mM glutamine (all from Life Technologies) and 10% chicken embryo extract (prepared from 11 day chick embryos [23]. Cells were plated on collagen coated Aclar (Allied Signal, Inc., Morristown, N.J.) at a density of 4.0 x 10^5 cells per 35 mm tissue culture dish (Falcon brand - Fisher scientific). Cultures were fed every other day with

MEM containing the same components as the MEM described for plating the cells. To ensure maximum relaxation of the muscle fibers during fixation, cultures to be fixed were first incubated in Ca^{2+} free medium containing iodoacetic acid and EGTA for 1 hour. The Ca^{2+} free medium was removed and the cultures were immediately fixed with 2% formaldehyde in potassium acetate buffer (PAB) containing iodoacetic acid and EGTA for 30 minutes. Cells were extracted for 30 minutes using 0.5% Triton-X 100 in PBS and immunostained as described below.

Preparation ofC2C12 cultures

The C2C12 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in the recommended media (DMEM with 10% fetal calf serum (Life Technologies)) and routinely split before they reached confluency in order to retain their myogenic potential. To obtain differentiated muscle, cells were plated into 35 mm tissue culture dishes (Falcon - Fisher Scientific) containing collagen coated aclar and switched to DMEM with 10% horse serum (Life Technologies). Cultures were fed twice a week with the appropriate media. To ensure maximum relaxation of the muscle fibers during fixation, cultures were incubated in Ca^{2+} free medium containing iodoacetic acid and EGTA before they were fixed with 2% formaldehyde as described for primary skeletal muscle cultures. Cells were immunostained as described below.

Immunofluorescent staining

Staining protocols have previously been described [53]. Briefly, tissue or cultures were fixed, extracted, incubated in primary antibody for 1 hr at 37°C in a humid chamber, washed 3x's with PBS-0.5% TritonX-100, incubated in secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 1 hr at room temperature, washed again 3x's with PBS-0.5% TritonX-100, incubated with Hoechst bis-benzamide for nuclear identification (#33258, 1:1000 dilution of 1 µg/ml stock, Sigma Chemical Co., St. Louis, MO), washed with PBS for 10 minutes and mounted. For cultures, which were grown on plastic aclar squares, the aclar square with the adherent cells was removed from the dish. The staining protocol was executed with the cells attached to the aclar. For primary antibody incubations, the aclar was placed cell side up in a Petri dish and antibody was added directly to the cells. For secondary antibody incubations, the aclar was placed cell side up in a 35 mm Petri dish with 1 ml of secondary antibody solution. For isolated myofibers, the myofibers were placed in individual wells of a 96 well plate. Fibers were moved to a new well for each incubation and washing step.

For double labeling, cultures were incubated sequentially: 1) primary antibody; 2) first secondary antibody; 3) second primary antibody; 4) final secondary antibody; 5) nuclear label. Cultures were washed between each step as described for single antibody staining. Both cultures and intact myofibers were mounted using 100% glycerol containing anti-bleaching agents [77]. Intact myofibers were mounted on cover glasses (22 mm X 60 mm - A.H. Thomas Co. Swedesboro, NJ) and covered with glass cover slips (22 mm X 22 mm - A.H. Thomas Co.). Narrow strips of aclar (< 0.25 mm) were glued to the bottom cover glass to act as supports for the top cover slip. These supports prevented tissue compression. Intact myofibers were examined either by conventional fluorescence microscopy or with a Biorad 600 laser scanning confocal microscope. For laser scanning confocal microscopy, optical serial sections were taken and projected to obtain the final micrographs. Prints were made on a Sony color video printer. Cultures were examined using a Nikon conventional fluorescence microscope. Photographs on the Nikon microscope were taken with T-max-400 film (Kodak, Rochester, NY) or Hypertech film (Penguin Studios, Huntington Station, NY).

Antibodies

Polyclonal antibodies directed against peptide sequences from the cytoplasmic domains of al [58] and PIA [25] have previously been characterized. A second polyclonal antibody against the cytoplasmic domain of PIB was a generous gift from Dr. G. Tarone, University of Torino, Torino, Italy [2]. Monoclonal antibodies against titin (clone#Tll) and against sarcomeric-a-actinin (clone#EA-53) were from Sigma Chemical Co. (St. Louis, MO). The monoclonal antibody, CSAT, against β1 integrin was a generous gift from Dr. A.F. Horwitz [60]. CSAT was also obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, along with the monoclonal antibody against Ca^{2+}ATPase developed by Dr. D.M. Fambrough [47] and a second monoclonal antibody against β1 integrin (V2E9) developed by Dr. A.F. Horwitz [38].

Results

Periodic localization ofal in skeletal muscle

Integrins have clearly been demonstrated to play a role in skeletal muscle development. The identification of al integrin in skeletal muscle is suggestive that it too has a functional role in muscle. The exact localization of the protein within the muscle fiber is critical in assessing a potential role for this protein. Embryonic day 10 pectoralis cultures were

immunofluorescently labeled with antibody to al integrin to determine its distribution along the myofiber. The αl integrin antibody has previously been characterized by Menko & Philp [58]. αl integrin staining was detected with an obvious periodicity in elongated, mononucleated myocytes or multinucleated myotubes as early as 3 days in culture (Fig. 1A). The exact time of the reorganization varied with the culture preparations, al integrin was detected in postmitotic, mononucleated, myocytes which were bipolar, however, the staining pattern was observed as diffusely punctate rather than periodic (Fig. IB). Skeletal muscle cultures at 10 days, immunofluorescently labeled with the flowthrough portion of serum from an antibody purification that did not bind to the column (Fig. 1C) or with fluorescently conjugated secondary antibody alone (data not shown), did not exhibit periodic staining along the myofibrils. A doublet pattern of al integrin staining was apparent only when the muscle was sufficiently relaxed before it was fixed. To prevent contraction, the cells were exposed to iodoacetic acid and EGTA before and during fixation in order to deplete, respectively, ATP and residual Ca^{2+} which can cause the myofibrils to contract. If the muscle was in a state of contraction during fixation, αl integrin was detected as a series of single bands. This situation has previously been described for anti-vinculin staining of chick skeletal muscle by Pardo et. al. [65].

To confirm that the contraction state of the muscle dictated the degree of separation of the al integrin doublet bands, 10 day cultures were immunofluorescently doubly labeled with antibodies against α1 integrin (Fig. 2 A, C & E) and α-actinin (Fig. 2B, D & F) in both the contracted (Fig. 2A & B) and the relaxed state (Fig.2C, D, E & F). Sarcomere length was determined by measuring the distance between two Z lines which were demarcated by α-actinin, a Z line protein. On the same myofiber, doubly stained with anti- αl integrin, the distance between the two bands of the αl integrin doublet was observed. Measurements of a series of individual sarcomeres for each state of muscle contraction are detailed in Table 1. The typical sarcomere length is between 2.0 μ. and 2.5 μ. When the muscle was contracted (Fig. 2 A & B), sarcomere length measured on average 1.99μ. The αl integrin staining on this myofiber revealed that only single bands could be detected that appeared to colocalize in the region of the Z line. When the muscle was relaxed the distance between the sarcomeres was increased and sarcomere length measured on average between 2.14 μ, and 2.36 μ.. Relaxation of the muscle was accomplished by bathing the muscle in media that would reduce the potential for contractions (see materials and methods). On the relaxed myofibers (Fig. 2C, D, E and F), al integrin staining (Fig. 2C and E) revealed periodic doublet bands that appeared to localize on either side of the Z line. The spacing between the doublet bands could now be resolved. Increasing sarcomere length correlated with the ability to detect α1 integrin doublet bands, thus in order to detect α1 integrin doublet bands muscle had to be in the relaxed state. As the state of muscle contraction increased, resolution of the doublet into two distinct bands became increasingly more difficult to detect.

Table 1. al integrin distribution in relaxed versus contracted sarcomeres.

Contracted muscle (Fig. 2 A and B)		Relaxed muscle (Fig. 2 C and D)		Relaxed muscle (Fig. 2 E and F)	
2.05μ	2.0μ	2.25μ	1.94μ	23μ	2.38μ
2.02μ	2.0 μ	1.88μ	2.1lμ	2.33μ	2.38μ
1.95μ	1.95μ	2.05μ	2.16μ	2.38μ	2.5μ
1.88μ	2.05μ	2.16μ	2.11μ	2.44μ	2.33μ
2.05μ	1.88μ	2.05μ	2.33μ	22μ	2.33μ
1.94μ	1.94μ	2.11μ	2.2μ	2.25μ	2.44μ
2.11μ	2.0μ	2.2μ	2.05μ	2.3μ	2.44μ
2.0μ	2.11μ	2.25μ	2.25μ	2.3μ	2.33μ
1.95μ	2.0μ	2.0μ	2.3μ	2.33μ	2.5μ
2.0μ		2.2μ		2.33μ	
Avg. sarcomere length: 1.99μ SD:0.06μ,n= 19		Avg. sarcomere length: 2.14μ SD: 0.12μ, n = 19		Avg. sarcomere length: 2.36μ SD: 0.08μ, n = 19	

The length of sarcomeres from intact myofibers in both contracted and relaxed states was measured and compared. Intact myofibers were immunofluorescently labeled with anti α-actinin and anti- αl integrin. The distance from Z line to Z line (α-actinin staining) was determined. Representative sarcomere lengths are given in microns for contracted and relaxed muscle.

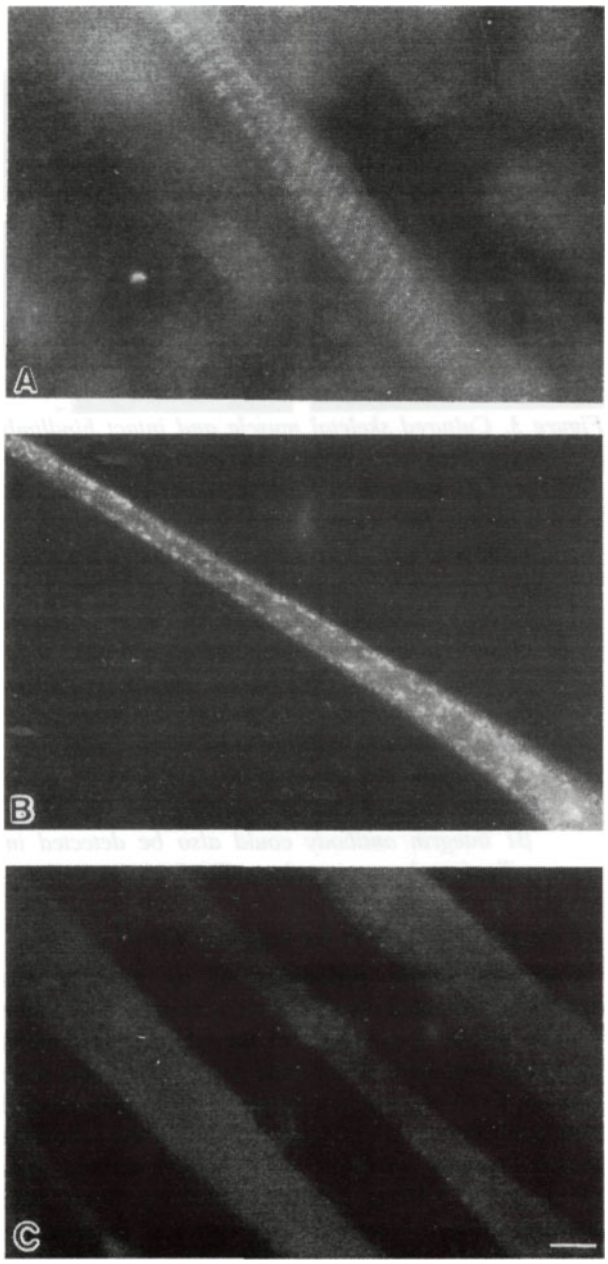

Figure 1. Cultured pectoralis muscle immunofluorescently labeled with anti- αl integrin (A & B), or anti-al integrin flowthrough (C). As early as 3 davs in culture, α1 *integrin was detected on skeletal myofihers with a sarcomeric periodicity (A) while in postmitotic. mononucleated myocytes, al integrin displayed a punctate distribution (B). No positive staining was detected in 10 day skeletal muscle cultures that were labeled with the column flowthrough from the* α1 *integrin antibody purification (C). Scale bar = 5μ.*

Periodic localization of β*1 integrin*

The detection of αl integrin with a sarcomeric periodicity along the myofiber, led to the expectation

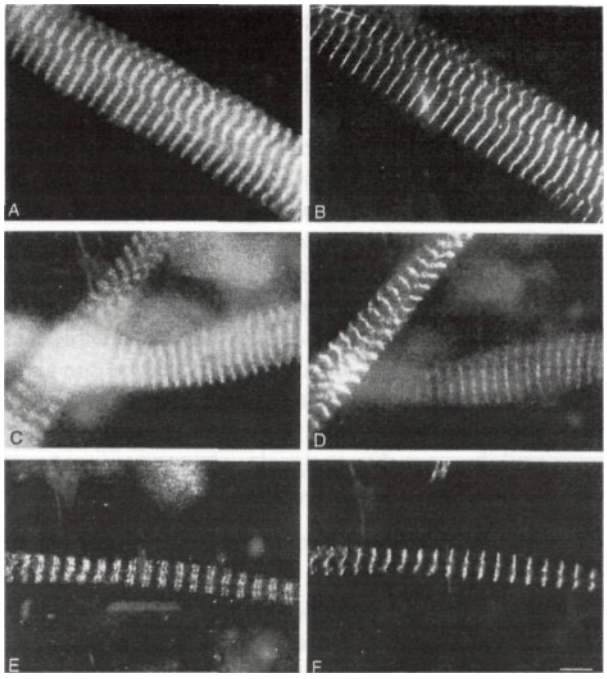

Figure 2. 10 day cultured skeletal muscle in different states of contraction which was immunofluorescently doubly labeled with antibodies to al integrin (A, C, E) and a-actinin (B, D, F). Sarcomere length was determined hv measuring the distance between Z bands that were labeled with anti- α*-actinin. Sarcomere measurements for each state of muscle contraction are provided in Table I. Sarcomere length measured on average 1.99 \JL in the most contracted muscle (B) and* αl *integrin was detected with a sarcomeric periodicity that appeared as single bands (A). Sarcomere length in muscle in two different states of relaxation measured on average 2.14 H (D) and 2.36 μ (F). In the relaxed muscle, periodic* αl *integrin was detected as doublet bands (C & E). The more relaxed the muscle the greater the degree of separation between the two components of the doublet band. The ability to resolve the* αl *integrin doublet bands was directly related to the relaxation state of the muscle. Scale bar = 5μ.*

that β1 integrin, its potential heterodimeric partner, would be similarly localized. Skeletal muscle cultures at 10 days were immunofluorescently labeled with antibodies to β1 integrin and analyzed. Both the β1 integrin antibody known as CSAT [60] and the one designated V2E9 [38] exhibited positive staining that demonstrated sarcomeric periodicity both in culture (Fig. 3A) and *in vivo* (Fig. 3C). Like anti-al integrin, anti-β1 integrin was detected in postmitotic. mononucleated, bipolar myocytes in a punctate configuration (Fig. 3B). Staining with a sarcomeric

periodicity was observed in either elongated, mononucleated myocytes or multinucleatcd myotubes as early as day 3 in culture. The V2E9 β1 integrin antibody was also found to localize in adhesion plaques (Fig. 3D). The integrin antibody against the cytoplasmic β1B isoform, as previously reported [2], exhibited no periodic staining. No staining was detected with antibody against the cytoplasmic domain βl A [25] integrin isoform. Although βl A integrin has been shown to persist in maturing skeletal muscle, it has been demonstrated that the level of βl A decreases while that of β1 D increases [7]. Lack of staining may be due to an insufficient amount of protein available for detection but alternatively it may be the consequence of inaccessibility to the antigenic site as a result of occupation of the integrin receptor.

Periodic localization of αl integrin in mvoftbers in vivo and C2C12 cells

To ensure that the periodic doublet localization of al integrin was a general property of skeletal muscle and not the result of particular culture conditions, the periodicity of al integrin staining was confirmed in independent muscle systems. Since the localization of the protein *in vivo* was as critical a factor to be determined as the presence of the protein, it was essential to examine embryonic muscle in such a way as to be able to visualize contiguous sarcomeres. Examination of isolated, intact, embryonic myofibers ensured that any al integrin sarcomeric periodicity would be readily apparent. Isolated myofibers from the hind limb and heart of day 17 chick embryos were immunofluorescently labeled with anti- αl integrin and examined with a confocal laser scanning microscope. Serial optical sections were scanned and the images were projected together to create the final micrographs. In intact hind limb myofibers, the distribution of al integrin was identical to that which was seen in cultured skeletal myofibers. al integrin was detected as doublet bands that were localized with a sarcomeric periodicity in the region of the Z band (Fig. 4A). The localization of al integrin in isolated cardiac myofibers was akin to that seen in isolated skeletal myofibers. al integrin exhibited doublet bands that were localized with a sarcomeric periodicity (Fig. 4B). To extend these results, C2C12 cells were plated, switched into media that promoted myogenesis, fixed and immuno-fluorescently labeled with antibodies to αl integrin and α-actinin. In mature cultures, multinucleated myotubes, in which well striated myofibrils were demonstrated with α-actinin, were also shown to exhibit periodic staining with αl integrin that could be found localized in the region of the Z line (Fig. 4C).

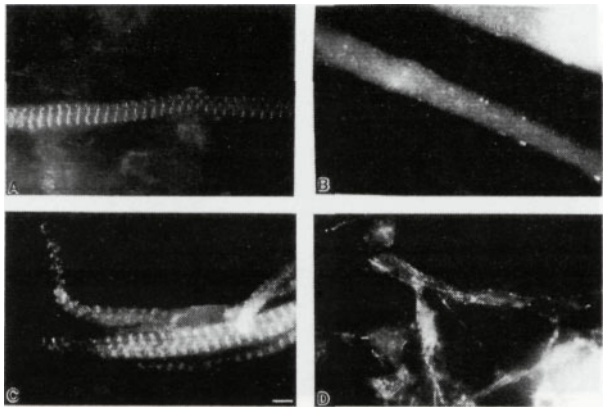

Figure 3. Cultured skeletal muscle and intact him/limb mvofibers were immunofluorescently labeled with anti β1 integrin (V2E9). As early as 3 davs in culture, β1 integrin was detected on skeletal myofibers with a sarcomeric periodicity (A) while in postmitotic, mononucleated myocytes from younger cultures, β1 integrin was observed diffusely distributed along the sarcolemma in a punctate pattern (B). Isolated intact myofibers from embryonic dav 17 hindlimb demonstrated a periodic staining pattern when labeled with anti β1 integrin (C) identical to that seen in more mature cultured skeletal myotithcs (A). The V2E9 β1 integrin antibody could also be detected in adhesion plaques in culture (D). Scale bar = 5μ.

αl integrin localizes in the region of the AI junction

Once sarcomeric periodicity of αl integrin was established, isolated myofibers were examined to define a more precise localization of the αl integrin doublet along the sarcomere. Embryonic day 17 myofibers were relaxed, fixed and immunofluorescently doubly labeled with antibodies to αl integrin and one of the following muscle specific proteins; sarcomeric- α-actinin, titin (Til), or Ca^{2+}ATPase. Doubly labeled myofibers were scanned using a laser scanning confocal microscope. Serial optical sections were scanned in the individual fluorescent channels and projected separately to obtain the final images for each antibody.

A comparison of αl integrin to sarcomeric- α-actinin indicated that one half of each αl integrin doublet (Fig. 5A) was localized on either side of the sarcomeric-a-actinin band (Fig. 5B). It was clear that the α-actinin band (arrows) fell between the two halves of the al integrin doublet (arrows). It can not be overemphasized that if the muscle was contracted during fixation, the integrin periodicity appeared to be Z line related. However, when the muscle was sufficiently stretched prior to fixation, the two bands of the α1 integrin doublet were seen on either side of the Z line.

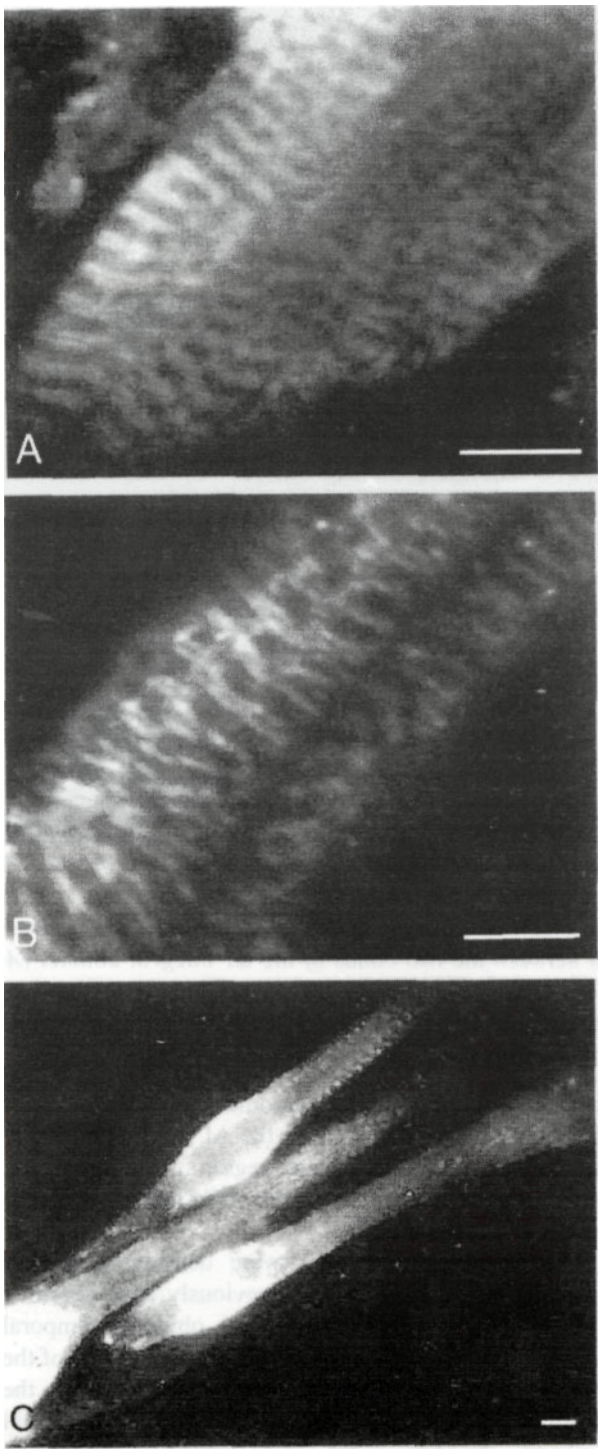

Figure 4. Intact myofibers from embryonic day 17 cluck skeletal hind limb (A) and embryonic day 17 chick cardiac muscle (B) as well as 13 day cultured C2C12 cells (C) were immunofluorescently labeled with anti-al integrin. The intact myojihers were examined by laser scanning confocal microscopy while the C2C12 cells were examined by conventional microscopy, al integrin wax obsen'ed with a sarcomeric periodicity in muscle fibers from all three srstems. Scale bar=10μ.

Based on the approximate distance between the Z line and the αl integrin doublet, it appeared that αl integrin was positioned within the I band. To verify this, isolated myofibers were doubly labeled with anti-cd integrin (Fig. 5C) and anti-Ca^{2+}ATPase (Fig. *5D)* which has previously been shown to localize in the sarcoplasm reticulum within the I band [47]. Each half of the al integrin doublet (arrows) was detected at the border of, but within, the region positively labeled for Ca^{2+}ATPase (arrowheads). The degree of muscle contraction was estimated by the width of the I band. The greater the degree of muscle contraction, the smaller the 1 band appeared and the closer the two halves of the integrin bands were drawn together. As previously described, eventually the two doublet bands came close enough together to appear essentially as a single band.

To refine the localization of the αl integrin doublet along the I band, skeletal muscle was doubly labeled with antibodies to αl integrin (Fig. 5E) and titin (Fig. 5F), a structural protein that spans almost the full length of the sarcomere. Furst et al. [31] have characterized antibodies to specific epitopes along the length of the titin molecule. The titin antibody used in this study, Tl I, was to an epitope located within the I band, specifically 0.05 u from the Al junction on either side of the Z line. This epitope has been reported to be in the elastic region of the sarcomere and to be influenced by the contraction state of the muscle [31]. The titin antibody presented a periodic doublet pattern of staining almost identical to that of the αl integrin doublet. The colocalization of the αl integrin doublets (arrowheads) and the titin doublets (arrowheads) is obvious.

Discussion

Integrins are involved in multiple aspects of myogenesis including migration, proliferation, differentiation, myofibril assembly and contraction via adhesion, mechanotransduction and signaling events. In order to define mechanisms of muscle dysfunction related to integrin abnormalities, interactions in normal muscle between the cytoskeleton and its membranes with relation to these molecules must be defined. The integrin subunits identified in skeletal muscle have been reported to be either diffusely distributed across the sarcolemma or associated with specific adhesion structures such as adhesion plaques [4, 26, 65], myotendinous junctions or costameres. Specific integrins, including αv and β1, have been demonstrated to localize with a sarcomeric periodicity [11, 56. 84, 87]. The absence of β1 integrin, which has such a periodic localization, has been shown to result in the loss of normal muscle function as a result of abnormal muscle development [84, 87]. It has not, however, been definitively determined whether the disruption of

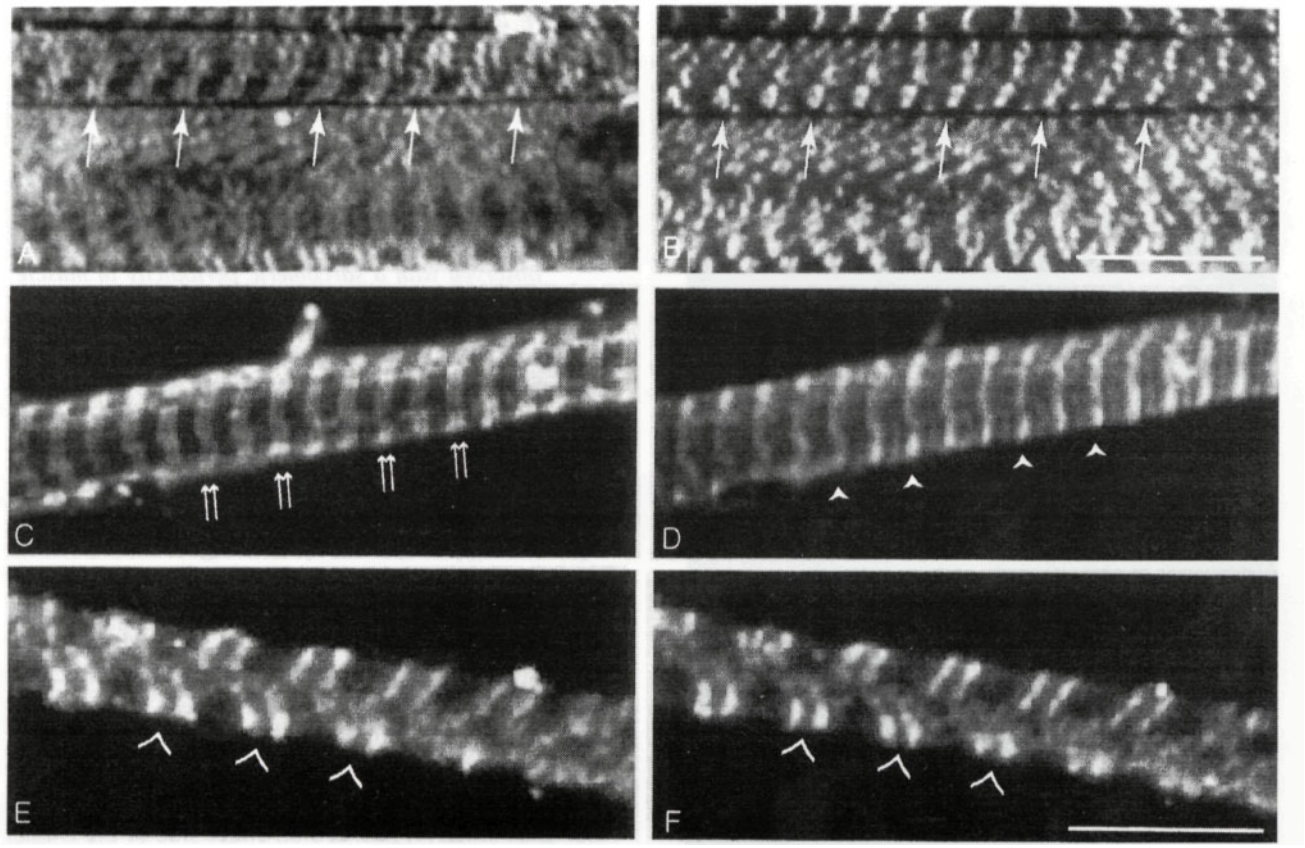

Figure 5. Intact myofibers from stage 17 chick hind limb immunofluorescently doubly labeled with anti- αl integrin (A, C. E) and anti- α-actinin (B), anti-Ca^{2+}ATPase (D) or anti-titin (F). The myofibers were examined with a laser scanning con focal microscope. Arrowheads and arrows mark the distribution of one protein relative to the other, α-actinin that was localized at the Z line (B-arrows) fell between the two bands of the α1 *integrin doublet (A-arrows). A comparison of* α1 *integrin (C-arrows) to Ca^{2+}ATPase (D-arrowheads) demonstrated that the* αl *doublet band was localized within the I band. More precisely,* αl *integrin (E-arrowheads) colocalized with an epitope of titin (F-arrowheads) that is within the 1 band but borders the Al junction. Scale bar = 5μ.*

sarcomeres was due to the loss of myofibril assembly competence or was a result of insufficient maintenance of myofibril/membrane interactions.

The data collected in this study revealed that al integrin could be detected diffusely distributed along the sarcolemma in nascent, postmitotic myofibers. In more mature cultured myofibers, al integrin was determined to localize in periodic doublet bands associated with the Al junction of the sarcomere. The periodic αl integrin staining was found to be a series of doublet bands rather than a series of singlet bands which is what has been previously reported for other integrins that exhibit a sarcomeric periodicity in skeletal muscle [11, 84, 87]. Doublet bands were most obvious when the muscle was in a state of relaxation. If contracted muscle was examined, the distance between the individual bands of the doublet decreased and al integrin was detected as apparent singlet bands instead of doublet bands due to their close apposition and a lack of sufficient resolution, al integrin was found to colocalize with the Tl 1 epitope of titin [31], which has been pinpointed along the sarcomere to be within the 1 band specifically 0.05μ from the Al junction on either side of the Z line in an elastic region of the sarcomere. This suggests that al integrin also falls within the elastic region of the myofiber.

The αl integrin doublets were detected earlier in cultured myocytes than had previously been reported [11]. There was, however, no obvious temporal correlation between the age of the culture, the age of the myotubes or the girth of the myotubes and the reorganization of the αl integrin into doublets, αl integrin doublets were also identified in intact myofibers *in vivo* and in C2C12 cells in culture with a localization identical to that observed in cultured skeletal myofibers. Antibodies to the β1 extracellular domain exhibited a sarcomeric periodicity along the same temporal sequence as was found for al integrin. The cytoplasmic domain β1B integrin antibody, as previously reported [2], was not observed to stain in this periodic pattern, however, neither did the β1A cytoplasmic domain integrin antibody. The β1A

integrin isoform has been reported to decrease in maturing skeletal muscle. The lack of detectable staining may be a result of insufficient antigen or a lack of accessibility due to occupation of the integrin receptor. To date, only one β1 integrin cytoplasmic domain antibody, βlD, has been reported to stain skeletal muscle with a sarcomeric periodicity [7, 93]. βlD has also been identified in C2C12 cells that have undergone muscle differentiation [82]. Although αl integrin has been shown to be present during myogenesis [11, 24], the absence of αl integrin in the form of null alleles has not elucidated any specific functional role for this protein [32].

The reorganization of al integrin from a periodic distribution in nascent myocytes to one with a novel sarcomeric periodicity in more mature myofibers suggests that this protein plays a discrete role in the process of myogenesis. The temporal regulation of the protein in culture occurs at a time that is coincident with myofibril assembly and as such suggests a role for al integrin in either signaling a part of this process or as a transient link for some particular sarcomeric protein. As a1 integrin has not been identified in adult muscle, any early linkage would presumably be replaced as the myofibers mature. Localization of periodic al integrin, to the same region of the sarcomere in which the sarcoplasmic reticulum (SR) is established, is indicated by its colocalization with Ca^{2+}ATPase, an SR protein. The presence of al integrin within the I band may be due to a compartmentalized translation or storage of the protein in the SR for subsequent transport to other cellular locations. However, proteins such as Ca^{2+}ATPase [45] and calsequestrin [46] that are localized to specific areas of the sarcomere have been shown to be stored in regions that are germane to their functionality, al integrin is most likely to be associated with those regions of the myofibril with which it has a functional role.

While specific functional data has yet to be uncovered, the localization of αl integrin permits some interesting speculations regarding possible roles for this protein in muscle development. In muscle disorders in which the integrity of the muscle myofiber is compromised, it has been proposed to be the result of an inability of the cytoskeleton to remain adequately associated with its membranes [51, 67]. One currently postulated element of interaction between the myofibrillar cytoskeleton and its delimiting membrane has been designated "the costamere". Costameres were originally characterized by localization of the protein vinculin [65] at the Z line. It has been proposed that the costamere in skeletal muscle is involved in the interaction of the myofibrillar cytoskeleton with the sarcolemma at the Z band [65] and possibly in the generation of force transmission [75]. Costameres have also been implicated as mechanical connections in the generation offeree production in cardiac muscle [18]. Like integrin, vinculin was detected as singlet bands when muscle was contracted and as doublet bands when the muscle was sufficiently relaxed [65]. The similarity in localization of vinculin and al integrin suggests a potential transient role for al integrin in relation to the costamere early in muscle development. Functional similarities also exist between vinculin and integrin. Skeletal muscle in *C. Elegans* mutants that lacked vinculin exhibited abnormalities [6] as did *C. elegans* mutants that were β integrin deficient [87]. In addition to vinculin, a number of other molecules including talin [13, 42], a-actinin [63, 66], tensin [9] and pp^{125FAK} [3, 81], that are known to interact with integrins, are found in the region of the IZI complex. Like vinculin, the loss of a-actinin has been shown to result in an alteration of muscle structure. Schultheiss et al. have demonstrated that introduction of a truncated α-actinin into cultured chick skeletal muscle initially involved the incorporation of the truncated molecule into nascent Z bands but resulted in the eventual fragmentation of the myofibril [74]. The accumulated data indicate that there are likely to be a host of other significant molecules that play a role in linking the myofibrillar cytoskeleton to the extracellular matrix through the membrane. In fact, given the increasing number of molecules being found to interfere with normal muscle development and function, it can be postulated that there are multiple mechanisms that play important roles in establishing and/or maintaining the structural integrity of the skeletal myofiber.

An alternative to the action of α1 integrin as a linkage component through the membrane of the sarcolemma, would be al integrin associated with the additional internal skeletal muscle membranes. In the highly specialized environment of a muscle cell, a transmembrane protein might be an essential component of the more specialized internal muscle membranes that are found in striated muscle associated with each individual myofibril; 1.) the SR and 2) the transverse tubules (T-tubules) [30]. Briefly, the SR, a specialization of the cells' endoplasmic reticulum, consists of channels which store Ca^{2+} that is used to initiate muscle contraction. T-tubules, a system of tubes connected to the SR at specialized junctions [29], rapidly disseminate action potentials from the surface of the myofiber to the interior. In chick, SR is established in the region of the IZI complexes and subsequently, T-tubules appear in association with the SR [30]. In the papillary muscle of sheep [49], a dystrophin-glycoprotein complex has been demonstrated to form a network in the internal aspect of those myofibers that were associated with T-tubules. The complex has been proposed to link the actin portion of the cytoskeleton with the extracellular matrix [27]. In skeletal muscle,

however, the complex has only been localized to the sarcolemma [22, 62]. The differences observed in the localization of an anchoring complex in different types of striated muscle further substantiate the likelihood that there are alternate or perhaps parallel mechanisms that link the myofibrillar cytoskeleton to the various muscle membranes. Recent evidence in mice which lack the α7 integrin subunit and eventually develop muscular dystrophic like symptoms, demonstrates that integrins can be components of such alternative mechanisms [55].

Specific connections between the SR and the myofibril have also been elusive. Both intermediate filaments and microtubules have been invoked as potential candidates, although, neither has been positively demonstrated to function in this capacity. The colocalization of al with Ca^{2+} ATPase (an SR component - [47]) at the outer edges of the SR terminal cisternae is suggestive of an intimate connection between the SR and al integrin at those times when al integrin is so localized in the membrane. A working hypothesis asserts that integrin receptors could be established at an early stage of development relative to the appearance of the internal muscle membranes. The presence of the integrin(s) might function as part of a linking mechanism for the SR or T-tubules, or alternatively, as a molecule that would signal the docking of one or more membrane components. Integrin would localize in the membrane prior to the establishment of a specialized membrane. The integrin would then need to be functionally engaged by some certain time or event. Otherwise, failure to be engaged would lead to removal from the membrane and degradation. This raises the question of the binding target for the extracellular domain of a 1(31. The cytoplasmic domain of integrin is proposed to bind to the myofibril but it is unclear to what in the SR or T-tubules the extracellular domain of an integrin could be tethered. The Z line region in vertebrate muscle contains the actin cytoskeleton [5] as well as the dihydropyridinc receptor and the ryanodine receptor, both components in the skeletal excitation/contraction (E-C) coupling mechanism [29] associated with the SR. In dysgenic muscle, dysfunction is a result of a disruption in this E-C coupling. A recent report has indicated the likelihood of binding proteins in the membrane influencing the alignment of these two receptors which subsequently interact [68]. An integrin in the appropriate membrane location would be a likely candidate for such a signaling molecule.

In general, for any developmental stage at which al integrin has been hypothesized to act as a structural linking molecule, speculation can also be made as to a role for al integrin acting as a developmental cue to signal an event in myogenesis. It has been shown that integrins can mediate signals into the cell via the tyrosine phosphorylation of specific proteins [50, 69]. Paxillin [81] and ERK [52] are proteins that have been associated with tyrosine phosphorylation in skeletal muscle and appear to be highly regulated during development. It is more than likely that there is an assortment of other proteins that can transmit signals into cells. Alone or as part of a cascade, al integrin, in conjunction with such a protein, might be involved in signaling a step in the regulation of myofibril assembly, early cytoskeletal anchorage or the establishment of the SR or T-tubule membrane systems. Studies are currently underway to evaluate whether, for an individual myofibril, sarcomeres are assembled prior to or subsequent to the appearance of the al integrin doublet. In addition to assessing a potential role for a 1 integrin in myofibril assembly by examining the temporal regulation of α1 integrin in relation to that of the muscle specific proteins that comprise the sarcomere, the temporal regulation of αl integrin in relation to that of the assembly of the specialized muscle membranes is also being investigated.

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