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Genetically Determined Proteolytic Cleavage Modulates $\alpha7\beta1$ Integrin Function*

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The dystrophin-glycoprotein complex and the $\alpha7\beta1$ integrin are trans-sarcolemmal linkage systems that connect and transduce contractile forces between muscle fibers and the extracellular matrix. $\alpha7\beta1$ is the major laminin binding integrin in skeletal muscle. Different functional variants of this integrin are generated by alternative splicing and post-translational modifications such as glycosylation and ADP-ribosylation. Here we report a species-specific difference in $\alpha7$ chains that results from an intra-peptide proteolytic cleavage, by a serine protease, at the ^{603}RRQ^{605} site. Site-directed mutagenesis of RRQ to GRQ prevents this cleavage. This RRQ sequence in the $\alpha7$ integrin chain is highly conserved among vertebrates but it is absent in mice. Protein structure modeling indicates this cleavage site is located in an open region between the β-propeller and thigh domains of the $\alpha7$ chain. Compared with the non-cleavable $\alpha7$ chain, the cleaved form enhances cell adhesion and spreading on laminin. Cleavage of the $\alpha7$ chain is elevated upon myogenic differentiation, and this cleavage may be mediated by urokinase-type plasminogen activator. These results suggest proteolytic cleavage is a novel mechanism that regulates $\alpha7$ integrin functions in skeletal muscle, and that the generation of such cleavage sites is another evolutionary mechanism for expanding and modifying protein functions.

Integrins are α,β-heterodimeric membrane receptors for extracellular matrix proteins (for reviews, see Refs. 1–3). They are used by cells to sense and modify their environments and they are involved in a wide range of cellular processes including cell adhesion, migration, differentiation, proliferation, apoptosis, and cancer metastasis (for reviews, see Refs. 4–8). Integrins are present in all metazoans and they are highly conserved in structure and function. In general, both α and β subunits have a short cytoplasmic domain, a large N-terminal extracellular domain, and a single hydrophobic transmembrane segment (9). The N-terminal of the α subunit contains seven FG-GAP repeats forming a β-propeller domain that is important for ligand binding (10).

The $\alpha7$ chain is synthesized as a single 1135-amino acid polypeptide precursor, and like other integrin α chains, it is cleaved within the cell to form a heavy (≈100 kDa) and a light (≈30 kDa) chain connected by a disulfide bond (11). $\alpha7$ associates with $\beta1$ subunits and the $\alpha7\beta1$ integrin is expressed in skeletal and smooth muscle cells, neurons, Schwann cells, and cardiomyocytes where it functions as a receptor for laminin (12–14). Expression of $\alpha7$ integrin in skeletal muscle is developmentally regulated at the transcriptional level and by alternative splicing, resulting in at least two extracellular (X1 and X2) and two cytoplasmic isoforms (A and B) (12, 13, 15–17). The $\alpha7$A cytoplasmic isoform is only found in skeletal muscle, and it is enriched at myotendinous and neuromuscular junctions (12, 13, 15, 18). Unlike $\alpha7$A, the $\alpha7$B isoform is found throughout the sarcolemma and it is also expressed in other cell types (13, 19). Both $\alpha7$A and $\alpha7$B levels increase during myogenic differentiation, indicating that expression of the integrin is coordinately regulated with skeletal muscle maturation (11). The $\alpha7$X1 and $\alpha7$X2 isoforms differ in their extracellular regions and have different ligand preferences and binding affinities to laminin (16, 17). Whereas $\alpha7$X2 binds equally well to laminin-1 and laminin-2/4 (merosin), $\alpha7$X1 preferably binds to laminin-2/4, and may also bind laminin-8 and laminin-10/11 (17, 20–22). This suggests that diverse functions of the integrin can be achieved by varying the composition of its extracellular and cytoplasmic domains. The $\beta1$ cytoplasmic domain may also influence $\alpha7\beta1$ binding (23). Additional post-translational modifications, including glycosylation and ADP-ribosylation, may also regulate $\alpha7\beta1$ functions (24, 25). A novel clipped form of $\alpha6$ integrin has been reported in human prostate cancer (26–29) and various cleavage forms of the $\alpha4$ integrin subunit have also been reported (30–33), indicating proteolytic cleavage of integrin subunits is another post-translational mechanism that may regulate integrin function (34–36). Interestingly, both $\alpha7$ and $\alpha6$ integrins have recently been identified as tumor suppressors and inhibitors of metastasis in various malignances including prostate cancer (19). Thus, it is of interest to understand if proteolytic cleavage of the $\alpha7$ integrin chain occurs and how it may regulate $\alpha7$ integrin functions.

Myogenesis and regeneration of skeletal muscle involves myoblast activation, proliferation, migration, and subsequent fusion into myofibers (37–39). As in other examples of tissue remodeling, these processes involve localized proteolysis of extracellular matrix proteins and their receptors (40, 41). The activation of plasminogen is often used to generate such extracellular proteolytic activities (42–44). Major components of the plasminogen activation system include urokinase-type

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TABLE 1
Primers for amplifying and sequencing α7 integrin genomic DNA and cDNA regions spanning the cleavage site

	cDNA	Genomic DNA
Rat and L8E63		
Forward	5′-gtgcccagcagctacagc-3′	5′-ggggttgttccagtgagaaa-3′
Reverse	5′-gggtcagagggcaggttg-3′	5′-ctctcacaccaggagcactg-3′
Human and PC1		
Forward	5′-catgttccagctccaggaaaatgt-3′	5′-gggattgttccagtgaggaa-3′
Reverse	5′-gggtccgatggcaggttg-3′	5′-cagaacccatgctcacctct-3′
Mouse and C2C12		
Forward	5′-tgtgttccagctgcaggaaaacgt-3′	5′-tgggacttgggagttgtttc-3′
Reverse	5′-gggtcagagggcaggttg-3′	5′-gggtgctgtcctttcacatt-3′

plasminogen activator (uPA),[2] its cell surface receptor (uPAR), and plasminogen activator inhibitor-1 (PAI-1). These are all expressed in skeletal muscle and form a tripartite complex capable of converting plasminogen into active plasmin (45–48). Muscle injury induces synthesis of the components of the plasminogen activation system early during regeneration (49–53). Similarly, skeletal muscle disease and injuries also induce higher levels of α7 gene expression and its protein levels at the sarcolemma (54, 55). Increased α7 integrin can also alleviate muscular dystrophy in *mdx/utrn*$^{-/-}$ mice, an animal model of human Duchenne muscular dystrophy (56, 57). Therefore, it is of interest to determine whether the α7 chain is a target of the plasminogen activation system in skeletal muscle. In addition, exacerbation of the dystrophic phenotype of the *mdx* mice by defects in the plasminogen activation system (58) raises the question whether proteolytic cleavage of the α7 chain is also involved in muscle pathogenesis.

Here, we report the presence of a species-specific proteolytic cleavage of α7 integrin, its location at amino acid residues ^{603}RRQ^{605} in the α7 chain, and its function in regulating cell adhesion and spreading. Cleavage of the α7 chain is elevated upon myogenic differentiation, and may be mediated by urokinase-type plasminogen activator. These results suggest proteolytic cleavage as a novel mechanism that regulates α7 integrin functions in skeletal muscle, and that the generation of such cleavage sites is another evolutionary mechanism for expanding and modifying protein function.

EXPERIMENTAL PROCEDURES

Animals and Skeletal Muscle Tissues—Generation and genotyping of transgenic mice expressing rat α7BX2 integrin have been described (56, 57, 59). Five-week-old α7 transgenic mice and their wild type (SJ6/C57BL6) controls were used. Protocols for animal use were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign. Mice were euthanized by CO_2 asphyxiation. The gastrocnemius-soleus complexes were rapidly dissected and snap-frozen in liquid nitrogen.

Cell Culture and Transfection—C2C12 mouse myoblasts were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (Biomeda), 0.5% chick embryo extract (Biologos Inc.), and 2 mM glutamine (Invitrogen). Rat L8E63 myoblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum (Invitrogen). Human PC1 myoblasts were cultured in Ham's F-10 medium containing 15% fetal bovine serum, 0.5% chick embryo extract, 2 mM glutamine, and 1.2 mM $CaCl_2$. Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum. Upon confluence cells were switched to differentiation medium (Dulbecco's modified Eagle's medium containing 2% horse serum) to induce myotube formation. Transfections were carried out using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Stably transfected CHO cells expressing wild type and mutant α7 integrin were selected with Zeocin at a final concentration of 1 μg/ml in Ham's F-12 medium. Transfection efficiency was determined by immunofluorescence microscopy.

Plasmids and Site-directed Mutagenesis—Plasmids encoding the rat integrin α7 were generated by subcloning rat α7 integrin cDNA (11) into pcDNA4/TO-E (Invitrogen). Primers (5′-ggtctccaaacccctcgattagggaggcaagcgcctgacc-3′ and 5′-ggtcagcgcttgcctccctaatcgaggggtttggagacc-3′) were used to produce the desired point mutation in the rat α7 integrin with QuikChange II Site-directed Mutagenesis Kits (Stratagene) following the manufacturer's instructions. The sequence of the mutated α7 plasmid was confirmed by sequencing.

Reverse Transcription, PCR, and Sequencing—Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega). Total cellular RNA was isolated using TRIzol reagent (Invitrogen). 2 μg of total RNA was used to synthesize first strand cDNA with the Retroscript reverse transcription kit (Ambion). Primers used to amplify human, mouse, and rat α7 integrin from genomic DNA and cDNA are listed in Table 1. PCRs of genomic DNA or cDNA were carried out using high fidelity *Pfu* polymerase (Stratagene) in 50-μl reactions. PCR conditions were: 95 °C for 5 min, followed by 40 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen) and sequenced at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois, Urbana-Champaign.

Sequence Alignment and Protein Structure Modeling—Sequences of α7 integrin were obtained from the Ensemble data base and aligned using ClustalW (60). This program was also used to align human α3, α6, α7, and αV integrin sequences. Structural modeling of the α7 integrin chain was done in MODBASE (61) using the known integrin αV crystal structure (Protein Data Bank code 1JV2) in the protein data bank. The mod-

[2] The abbreviations used are: uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; PAI-1, plasminogen activator inhibitor-1; DPBS, Dulbecco's phosphate-buffered saline; CHO, Chinese hamster ovary; BSA, bovine serum albumin.

eled α7 integrin structure was viewed with Chimera Package (62) and superimposed on the αV integrin structure.

Antibody and Chemical Reagents—O26 monoclonal antibody was prepared as described (11). Preparation of rabbit polyclonal antibody (CDB347) against the α7B cytoplasmic domain has also been described (12). Polyclonal rabbit anti-rat integrin β1 antibody was kindly provided by Dr. Steffan Johansson (Uppsala University, Sweden). Active murine uPA was purchased from Chemicon. Amiloride hydrochloride hydrate was obtained from Sigma. Serine protease inhibitor mixture, α2-antiplamin, and uPA inhibitor PAI-1 were obtained from Calbiochem-Novabiochem (La Jolla, CA).

Western Blotting and Immunoprecipitation—Muscle tissue or cell pellets were extracted in lysis buffer composed of 2% Triton X-100, 20 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 7.5), 1/200 Protease Inhibitor Mixture III (Calbiochem), and 1 mM phenylmethylsulfonyl fluoride at 4 °C for 30 min. After centrifugation, supernatants were collected and protein concentrations were determined using the Bradford assay (Bio-Rad). Equal amounts of total protein were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes. Blocked membranes were incubated with the respective primary antibodies. Horseradish peroxidase-linked secondary antibodies (Jackson ImmunoResearch) were used to detect bound primary antibodies. Immunoreactive protein bands were detected by chemiluminescence using an ECL kit (Amersham Biosciences). The intensities of these bands were quantified using ImageQuant software.

For immunoprecipitations, polyclonal antibody against β1 integrin was covalently linked to AminoLink plus coupling gel using a mammalian co-immunoprecipitation kit (Pierce). 500 μl of total protein were added to the antibody-coupled gel and control non-antibody-linked gel. The mixtures were rotated at 4 °C for 4 h, and washed five times with cold Dulbecco's phosphate-buffered saline (DPBS). Bound protein complexes were eluted with ImmunoPure IgG Elution Buffer (pH 2.8) (Pierce) and loaded onto 8% SDS-PAGE gels and subjected to Western blotting.

Immunofluorescence—Expression of wild type and mutant rat integrin α7 was detected by immunofluorescence microscopy using O26 monoclonal antibody at 10 μg/ml, a concentration that detects only rat α7. Fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) diluted 1:150 was used to detect the primary antibody. Washes were done in serum-free media. Cells were then fixed in ice-cold methanol for 5 min and rehydrated in PBS for 30 min at 4 °C. Texas Red-labeled phalloidin (Molecular Probes) was used at a 1:200 dilution. Coverslips were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories) and sealed with Pro-Texx (Lerner Laboratories). Immunofluorescent images were acquired using a Leica DMRXA2 microscope, AxioCam digital camera (Zeiss), and Openlab software (Improvision) and immunofluorescence intensity was determined using ImageQuant software (GE Healthcare).

Flow Cytometry—Cells transfected to express wild type and mutant α7 integrin were detached and pelleted. After washing, cells were incubated with monoclonal antibody O26 in medium containing 1% BSA on ice for 1 h. After three washes the cells were incubated with fluorescein isothiocyanate-labeled secondary antibody (1:150 dilution) for 1 h on ice. Following three more washes the cells were fixed in 4% paraformaldehyde for 10 min, pelleted, and resuspended in DPBS containing 1% BSA. Vector-only transfected cells were used as a negative control. Flow cytometry analysis was performed using a BD Biosciences FACS 6 color cytometer (BD Biosciences). Data analysis was performed using FCS Express 3 (De Novo Software).

Cell Adhesion and Spreading Assays—The adhesion assay has been described (21). Briefly, mouse laminin (Invitrogen) in 0.1% BSA in DPBS was adsorbed onto 24-well polystyrene plates (Costar) overnight. The plates were washed three times with DPBS and blocked for 1 h at room temperature with DPBS containing 1% BSA. Ham's F-12 medium with 0.1% BSA was added to the wells and incubated for 1 h at 37 °C. Transfected CHO cells were washed once and suspended in F-12 medium containing 1% BSA. 5×10^4 cells were added to each well and incubated at 37 °C for 10, 20, and 30 min. Non-adherent cells were removed by gently washing with DPBS; the remaining cells were fixed in 95% ethanol for 5 min and stained with 0.1% crystal violet for 30 min. After destaining with distilled water, stained cells in 10 random fields were counted using a Nikon eclipse TS100 inverted microscope (×20 objectives) equipped with a Nikon Coolpix 950 digital camera.

For cell spreading assays, glass Lab-Tek chamber slides (Fisher Scientific) were coated overnight with 20 μg/ml laminin in DPBS containing 0.1% BSA. Chambers were washed three times with DPBS and 10^5 cells in 500 μl of Ham's F-12 containing 1% BSA were added. After 15, 30, 45, and 60 min, non-adherent cells were gently washed away with DPBS. Adherent cells were fixed with 4% paraformaldehyde in DPBS for 10 min and rendered permeable in DPBS containing 0.5% Triton X-100 for 10 min. Fixed cells were stained with Texas Red-labeled phalloidin. Cellular and nuclear areas were measured using Openlab software. The percent of spread cells in 20 randomly chosen fields per sample were counted. A cell was considered to have spread when its total area was at least 2-fold larger than the area of its nucleus.

Data Analysis—All averaged data are presented as the mean ± S.E. Multiple group comparisons were performed using one-way analysis of variance followed by Tukey's post hoc analyses. Comparisons of two groups were performed using unpaired two-tail Student's *t* test. Differences were considered significant at $p < 0.05$.

RESULTS

A Species-specific Proteolytic Cleavage Site in the α7 Integrin Chain—Two proteolytic cleavage sites were initially identified in the sequence inferred from the gene encoding the rat α7 integrin chain (Fig. 1*A*) (11). One cleavage takes place within the cell, at a RRRE site, and is common to many α integrin chains (1). This generates a heavy and a light chain that are disulfide bond linked (11). A second proteolytic cleavage site generates a 70-kDa fragment of the C-terminal portion of the unreduced full-length 120-kDa chain. The same 70-kDa α7 fragment is present in both human and rat skeletal muscle, but not in mouse (Fig. 1*B*). Likewise, the 70-kDa peptide was

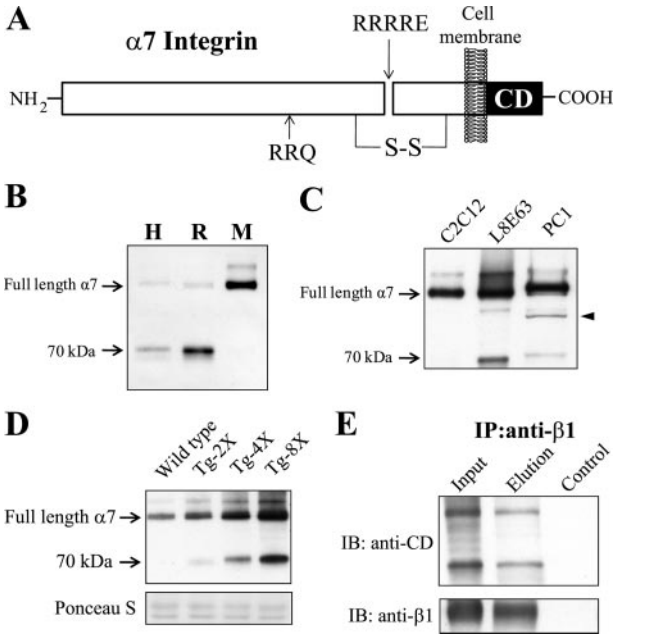

FIGURE 1. **Species-specific proteolytic cleavage of α7 integrin.** *A*, schematic of the rat α7 integrin chain. Proteolytic cleavage at the RRRE site generates a light and heavy chain linked by a disulfide bond. Another proteolytic cleavage site, RRQ, has been inferred from the rat integrin α7 cDNA. *CD* denotes the cytoplasmic domain. *B*, immunoblot analysis of non-reduced extracts, using an antiserum against the cytoplasmic domain detects a 70-kDa α7 peptide in human (*H*) and rat (*R*) skeletal muscle, but not in mouse (*M*). Full-length α7 integrin migrates at 120 kDa. *C*, immunoblot analysis of non-reduced extracts of myogenic cells detects the 70-kDa peptide in human (PC1) and rat (L8E63) myoblasts, but not in mouse (C2C12) myoblasts. *Arrowhead* indicates full-length, un-glycosylated α7 chain (100 kDa). *D*, immunoblot (*IB*) analysis of skeletal muscle from transgenic mice expressing 2-fold (*Tg-2X*), 4-fold (*Tg-4X*), or 8-fold (*Tg-8X*) rat α7 integrin. The levels of 70-kDa α7 peptide detected correlate with rat α7 expression. A section of Ponceau S-stained membrane demonstrates equal protein loading. $n = 5$. *E*, the 70-kDa α7 peptide is associated with the β1 subunit. Immunoprecipitates (*IP*) of L8E63 rat myoblast lysates with anti-β1 antibody were immunoblotted with anti-α7 integrin cytoplasmic domain antibody (*IB*). The 70-kDa α7 peptide is detected in the cell lysates (*Input*) and immunoprecipitates (*Elution*) but not in agarose beads only control elution (*Control*). The same blots were re-probed with the anti-β1 antibody to confirm the precipitation of β1 chain (*IB*: anti-β1). This experiment was repeated twice with identical results.

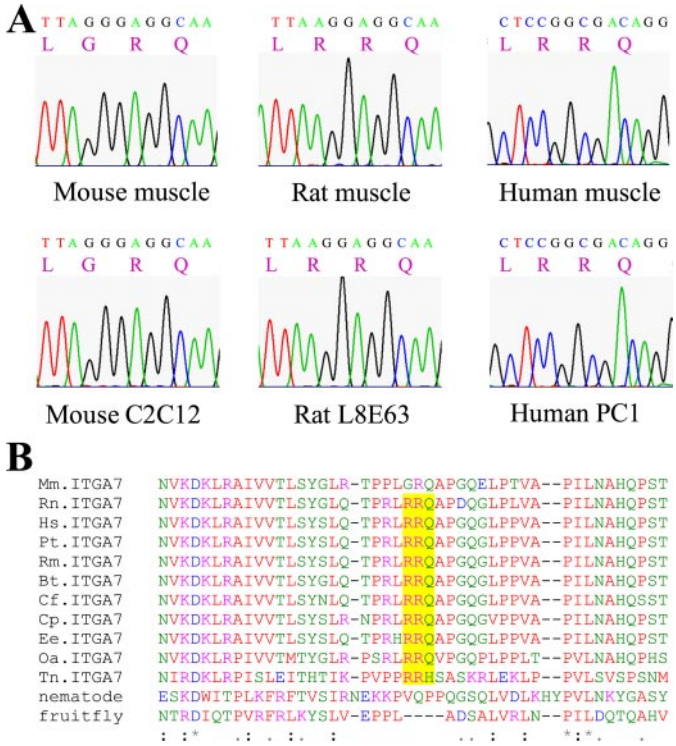

FIGURE 2. **DNA sequencing and α7 integrin protein sequence alignment confirm ^{603}RRQ^{605} as the potential proteolytic site.** *A*, sequencing chromatographs of amplified genomic DNA from normal human, C57BL/6 mouse, and Norway rat muscle, and human PC1, mouse C2C12, and rat L8E63 myogenic cells. The deduced amino acid sequence at the proteolytic cleavage site of mouse (GRQ) is different from that in humans and rat (RRQ). *B*, alignment of the α7 integrin protein sequences in the region containing the RRQ site deduced from the genomic DNA of mouse (*Mm*, Ensembl Gene ID ENSMUSG00000025348), rat (*Rn*, Ensembl Gene ID ENSRNOG00000007905), human (*Hs*, Ensembl Gene ID ENSG00000135424), chimpanzee (*Pt*, Ensembl Gene ID ENSPTRG00000005059), rhesus macaque (*Rm*, Ensembl Gene ID ENSMMUG00000013226), cow (*Bt*, Ensembl Gene ID ENSBTAG00000031123), dog (*Cf*, Ensembl Gene ID ENSCAFG00000000060), guinea pig (*Cp*, Ensembl Gene ID ENSCPOG00000007059), hedgehog (*Ee*, Ensembl Gene ID ENSEEUG00000002252), platypus (*Oa*, Ensembl Gene ID ENSOANG00000000604), puffer fish (*Tn*, Ensembl Gene ID GSTENG00027615001), and the sequences of the laminin binding integrin α subunits of *Caenorhabditis elegans* (nematode, Ensembl Gene ID F54G8.3) and *Drosophila* (fruit fly, Ensembl Gene ID CG1771) using ClustalW. The RRQ/H sites are highlight in *yellow*. This site is conserved among all vertebrates with the exception of mouse. Nematode and fruit fly laminin binding integrins do not contain this cleavage site.

detected in human PC-1 and rat L8E63 myogenic cells but not in the C2C12 line of mouse myogenic cells (Fig. 1*C*). The additional band of ~100 kDa detected in unreduced extracts from L8E63 and PC1 cells, using an antibody against the α7B cytoplasmic domain (Fig. 1*C*, *arrowhead*), likely represents the unglycosylated full-length α7 chain (12). Thus mouse, human, and rat α7 appear to differ in their post-translational processing in skeletal muscle. Transgenic mice expressing ~2-, 4-, and 8-fold more rat α7 chain have progressively more 70-kDa peptide indicating that mouse muscle has the required cleavage machinery (Fig. 1*D*). Cleavage of the α7 chain into the 70-kDa fragment was almost complete in human and rat muscle but it was less extensive in the transgenic mice and in human and rat myogenic cells in culture. Immunoprecipitation of extracts of L8E63 rat myotubes with polyclonal anti-β1 antibody and probing with antibody that recognizes the α7B cytoplasmic domain indicates the 70-kDa fragment is associated with the β1 subunit (Fig. 1*E*) and likely represents a functional species-specific variant of the α7β1 integrin. Because the α7 70-kDa peptide was detected in rat and human but not mouse, we believe this represents a species-specific difference in post-translational processing arising from differences in the mouse α7 protein sequence.

Identification of Amino Acid ^{603}RRQ^{605} as the Potential Cleavage Site by Alignment and Sequencing—Based on the apparent molecular mass of the 70-kDa fragment and its detection by antibody specific to the α7B cytoplasmic domain, ^{603}RRQ^{605} is the likely protease cleavage site in the rat and human α7 chains (Fig. 1*A*) (11). However, there are conflicting reports of the mouse α7 integrin sequence at this site in the NCBI data base (NCBI accession L23423 (^{603}RRQ^{605}) and NCBI accession L23423 (^{603}GRQ^{605})). To resolve this inconsistency and confirm the nucleotide sequence of this region in the α7 integrin gene and cDNA, PCR and reverse transcriptase-PCR were done on preparations from C57BL/6 and BALB/c mice, Norway rats, and humans, as well as C2C12, L8E63, and PC1 cells. Nucleotide sequencing of the amplicons (Fig. 2*A*)

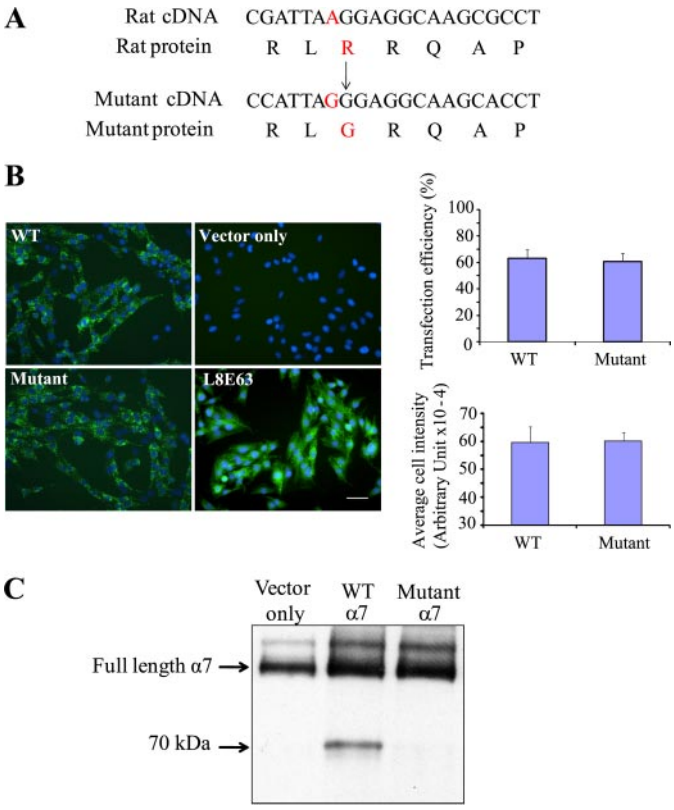

FIGURE 3. **A R603G mutation blocks cleavage of rat α7 integrin.** *A*, a point mutation in rat α7 integrin cDNA alters the proteolytic cleavage site. The mutated nucleotides and corresponding amino acids are in *red*. *B*, C2C12 myoblasts were transfected with wild type (*WT*) and mutant rat α7 integrin plasmids. Immunofluorescence staining of live cells, 36 h after transfection, using a monoclonal antibody specific for rat α7. Rat L8E63 myoblasts were used as a positive control. Immunofluorescence image intensities were quantified in six random selected fields using ImageQuant software. Transfection efficiency and average cell intensity were determined not to be significantly different from each other. *Bar* = 50 μm. *C*, the 70-kDa α7 peptide was only detected in C2C12 cells transfected with wild type rat α7 plasmid.

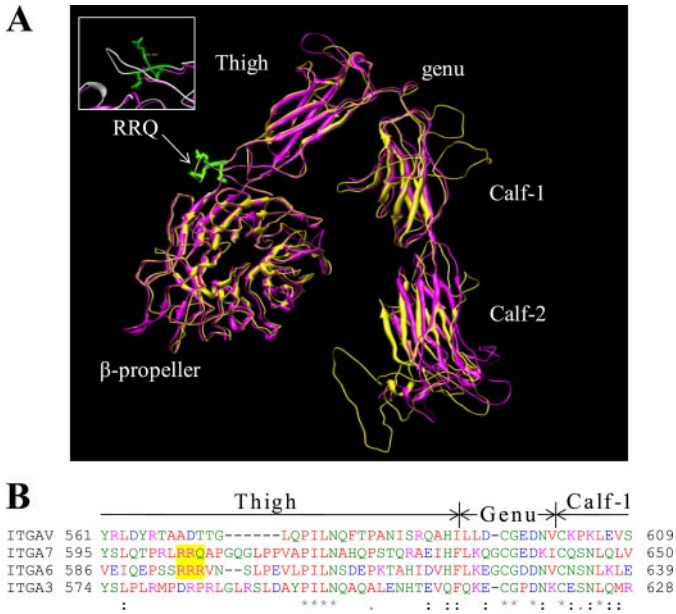

FIGURE 4. **A model of the extracellular portions of the human integrin α7 subunit (residue 34–1028) and human αV subunit based on the crystal structure of human αVβ3 integrin.** *A*, the spatial position of the α7 chain (*yellow*) and the αV chain (*magenta*) are indicated. The RRQ site in the α7 chain (*green*) resides in a relatively "opened" position between the β-propeller and thigh domains of the integrin. *Inset*, a magnified view comparing the RRQ site in human α7 integrin (*gray*) and the αV (*magenta*) structure. The first 33 amino acids of the α7 chain and the leader sequence of the αV chain are removed by post-translational processing and are not shown. *B*, sequence alignment of the human laminin binding integrin α subunits (α3, α6, and α7) and human αV using ClustalW. The RRQ sequences in α6 and α7 integrin are highlighted in *yellow*.

revealed the translated amino acid sequence at this site in mouse is GRQ, whereas it is RRQ in rat and human.

Alignment of the inferred amino acid sequences of α7 integrin from human, chimpanzee, mouse, rat, guinea pig, hedgehog, cow, pig, dog, duck-billed platypus, and puffer fish, and the laminin binding integrin from nematode and fruit fly, indicates the protease cleavage site RRQ (RRH in case of puffer fish) is highly conserved among vertebrates with the exception of the mouse, where it is GRQ (Fig. 2*B*). To exclude the possibility that the cleavage site in the C57BL/6 and BALB/c mice differ from other vertebrates due to a single nucleotide polymorphism, α7 integrin amino acid sequences from different mouse strains in the Jackson Laboratory mouse single nucleotide polymorphism data base (63) and NCBI dbSNP data base (64) were examined. No nucleotide polymorphism was detected at this site; all showed the same GRQ sequence. This confirms that the RRQ cleavage site that generates the 70-kDa polypeptide is present in all of the compared vertebrates but not in the mouse.

R603G Mutation Fully Blocks the Cleavage of Rat Integrin α7—To confirm the RRQ sequence in rat and human α7 integrin is the actual site of protease cleavage, site-directed mutagenesis was utilized to mutate RRQ in rat α7 cDNA to encode GRQ as in the mouse (Fig. 3*A*). C2C12 myoblasts transiently transfected with wild type rat and mutant rat α7 integrin constructs showed equal levels of rat α7 protein as seen by immunofluorescence using a rat-specific anti-α7 antibody (Fig. 3*B*). Quantification of fluorescence intensity revealed no significant differences in average cell intensities and transfection efficiencies. The 70-kDa protein was only detected in cells transfected with wild type rat α7 (Fig. 3*C*). This confirms that the RRQ sequence in the rat α7 integrin is the site of cleavage underlying production of the 70-kDa polypeptide although both rat and mouse cells are competent to cleave the α7 integrin at this site.

Structure Modeling Reveals Spatial Position of the α7 Integrin Cleavage Site—Integrins are a highly conserved family of proteins and different α subunits share high sequence and structural similarity (1). The crystal structure of the extracellular portion of human integrin αVβ3 identified four large domains (β-propeller, Thigh, Calf-1, and Calf-2) connected by smaller linker regions (10, 65). Sequence alignment of human laminin binding integrins (α3, α6, and α7) with integrin αV reveals the RRQ cleavage site in α6 and α7 integrin are near the knee-like bend (genu) region that links the thigh and calf-1 domains (Fig. 4*B*). To spatially locate the RRQ cleavage site, structural modeling of human α7BX2 was done using the structure of αVβ3 as a template (61). The comparison spanned amino acids 34 to 1028 of the human α7 chain that contains all four major regions of the extracellular domain. The modeling revealed high structural similarity between the α7 and αV chains, as shown in the composites of α7 (*yellow*) and αV (*magenta*) subunits (Fig. 4*A*). Although the alignment of primary amino acid sequences

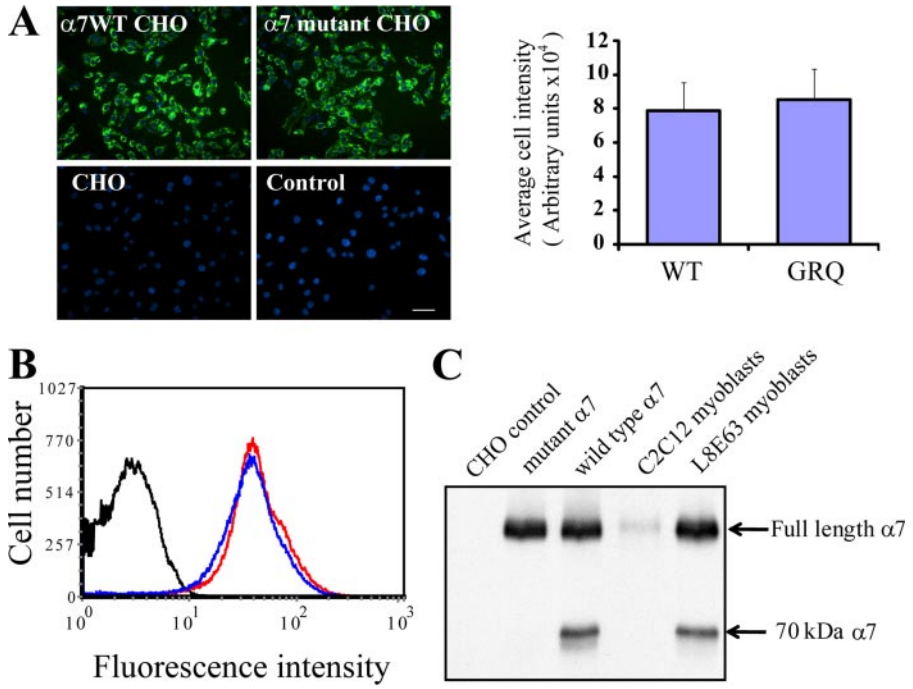

FIGURE 5. **Exogenously expressing wild type but not mutant α7 integrin is cleaved in CHO cells.** *A*, immunofluorescence localization of rat integrin α7 on the surface of stably transfected CHO cells reveals equivalent expression of wild type (*WT*) and mutant (*Mutant*) α7 proteins. Untransfected CHO cells and α7WT CHO cells stained only with secondary antibody were used as controls. *Bar* = 50 μm. *B*, flow cytometry confirms similar levels of expression of wild type and mutant α7 integrin on the surface of stably transfected CHO cells. *C*, immunoblotting with antibody against the B cytoplasmic domain of the α7 chain detects the 70-kDa α7 cleavage products in CHO cells transfected to express wild type but not mutant rat α7 integrin chains.

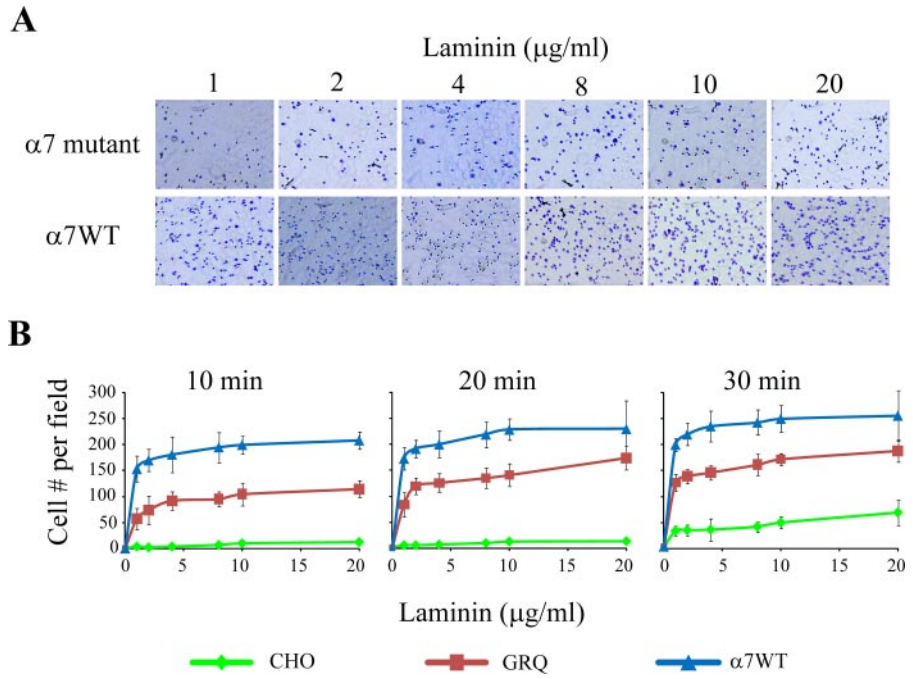

FIGURE 6. **Cleavage of α7 promotes cell adhesion.** Adhesion of stably transfected CHO cells expressing wild type and mutant α7 integrin on laminin. Cells were stained with crystal violet 10, 20, and 30 min after plating. *A*, representative images of adherent cells after 10 min are shown. *B*, quantification of adherent cells in 20 random selected fields after 10, 20, and 30 min. CHO cells expressing wild type (*WT*) α7 integrin adhere on laminin stronger compared with GRQ mutant α7 integrin expressing CHO cells.

located the RRQ cleavage site only 26 amino acids away from the genu region, structure modeling revealed this site to be in an exposed region between the β-propeller and thigh domain (Fig. 4*A*, *arrow*). Compared with integrin αV at this site, the α7 RRQ sequence (shown in *green*) extends further into the gap between the thigh and β-propeller domains (Fig. 4*A*, *inset*). In the cleaved α7 integrin, the β-propeller and the majority of the thigh region are disconnected from the genu and calf domains. The resulting 70-kDa α7 peptide includes the Calf-1, Calf-2, and genu domains and about 30 amino acids of the thigh domain.

Cleavage of α7 Promotes Cell Adhesion and Spreading—α7β1 integrin mediates the adhesion and migration of muscle precursor cells on laminin (21). To determine whether cleavage of α7 affects its ability to mediate cell adhesion and spreading, CHO cells with no endogenous integrin α7 were transfected with wild type and mutant α7 constructs and stably transfected cells were selected with Zeocin. Normally, CHO cells have minimal laminin binding ability (66). Immunofluorescence staining of live cells revealed comparable intensities of α7 at the cell membrane of cells stably transfected with either mutant or wild type constructs (Fig. 5*A*). Untransfected CHO cells did not show staining and confirmed the specificity of the antibody. Fluorescence-activated cell sorter analysis also showed equal amounts of wild type and mutant α7 on the cell surface (Fig. 5*B*). Western blots detected the α7 70-kDa fragment only in CHO cells transfected with the wild type α7 construct (Fig. 5*C*). The absence of the 70-kDa polypeptide in CHO cells transfected with the mutant α7 construct confirmed that the RRQ site is essential and sufficient for the α7 chain cleavage.

To determine whether cleavage of the α7 chain affects cell adhesion, CHO cells stably transfected with wild type and mutant α7 constructs were plated onto different concentrations of laminin for 10, 20, and 30 min. After non-adherent cells were removed, adherent cells were fixed and stained with crystal violet. Representative images are shown in Fig. 6*A*. Cell numbers were quantified in 10 random fields for each condition. The results demonstrate that both

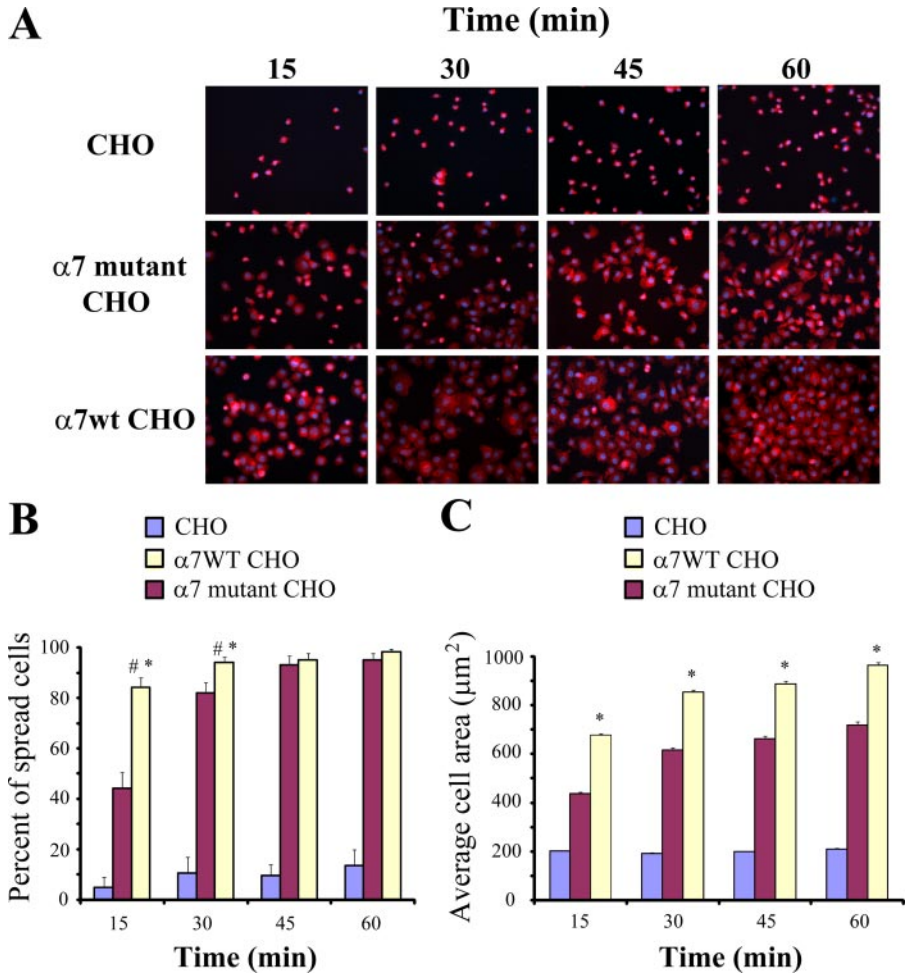

FIGURE 7. **Cleavage of α7 integrin promotes cell spreading.** *A*, spreading of stably transfected CHO cells expressing wild type (*WT*) and mutant rat α7 integrin on laminin (20 μg/ml). Adherent cells were fixed and visualized by staining with Texas Red-labeled phalloidin. *B*, quantification of cell spreading on laminin. Cell and nuclei areas, in 20 random fields for each condition, were measured using Openlab software and the percentages of spread cells are given. Differences in spreading promoted by wild type and mutant α7 are statistically significant (#, $p < 0.001$) at 15 and 30 min and are also significantly different from the untransfected control (*, $p < 0.001$). *C*, cell areas were determined for at least 1000 cells for each condition at each time point. Cells expressing wild type α7 integrin have larger average cell areas at all time points measured and the differences are statistically significant (#, $p < 0.001$). *Error bars* indicate S.E.

wild type and mutant α7 chain can promote adhesion of CHO cells on laminin in a concentration-dependent manner. However, more CHO cells expressing cleavable wild type α7 attached than the cells that express the non-cleaved mutant form at all three times measured (Fig. 6*B*). Whereas cell attachment results from meeting a threshold of adhesion, the cleaved integrin is more effective than the uncleaved form in meeting this criteria. Although it is not practical to measure the K_a of cell attachment, it is reasonable to conclude that cleavage promotes stronger integrin binding to laminin. In addition, we did not detect differences in the rates of attachment within the concentration range of laminin used.

In wells coated with high concentrations of laminin, more cells transfected with wild type cleavable α7 were spread compared with cells transfected with the mutant α7 integrin. In the same interval of time almost no untransfected cells were spread. Representative images of cells fixed after 15, 30, 45, and 60 min of incubation are shown in Fig. 7*A*. The proportions of spread cells in 20 random fields were determined for each condition (Fig. 7*B*). CHO cells expressing wild type, cleavable α7 spread faster than the cells with the mutant α7. The differences observed between cells expressing the wild type and mutant α7 integrin at 15 and 30 min were significant and both were higher than the vector controls. The areas of cells expressing α7 were consistently greater than the area of cells containing the uncleaved mutant form (Fig. 7*C*). Thus cleavage of α7 facilitates ligand binding and results in faster and more extensive cell spreading.

Cleavage of the α7 Integrin Chain Is Elevated during Myogenic Differentiation—Increased levels of total α7 integrin during myogenic differentiation and skeletal muscle regeneration have been reported (12, 54, 55, 67, 68) and suggest muscle fibers require more α7β1 integrin for adhesion. We therefore determined if levels of the 70-kDa polypeptide are altered during myoblast differentiation. During the first 2 days of differentiation of rat L8E63 myogenic cells, the amount of the 70-kDa polypeptide increased sharply and was maintained at high levels thereafter (Fig. 8*A*). Quantification of band intensities showed that the relative amount of the 70-kDa peptide compared with total α7B integrin increased from 14 to 44% during the first 2 days of differentiation (Fig. 8*B*). The increase in cleaved α7 at the beginning of differentiation may reflect the requirement of myotubes to adhere, and/or increased protease activity at this time. However, the cleavage of α7 integrin is not essential for myogenesis as mouse myoblasts differentiate *in vivo* and *in vitro* without α7 cleavage.

Inhibitors of Urokinase-type Plasmingen Activator Reduce Cleavage of α7 Integrin—αVβ3, α3β1, and α6β1 integrins interact with uPAR and regulate extracellular matrix remodeling and cell migration (69, 70). α6 can also be cleaved by uPA to produce a functionally active variant with similar molecular mass as the α7 70-kDa fragment (28, 34). In addition, levels of uPA and uPAR increase during myogenic differentiation *in vitro* (46, 47, 53, 71) and correlate with the increase in α7 70-kDa levels. To test if uPA can cleave α7 and produce the 70-kDa fragment, purified active uPA, serine protease inhibitors, and uPA inhibitors (Amiloride) were added to L8E63 cells, and the levels of the 70-kDa peptide were determined. Brief treatment of these cells with active uPA resulted in higher levels of integrin cleavage as shown by Western blots (Fig. 9*A*), indi-

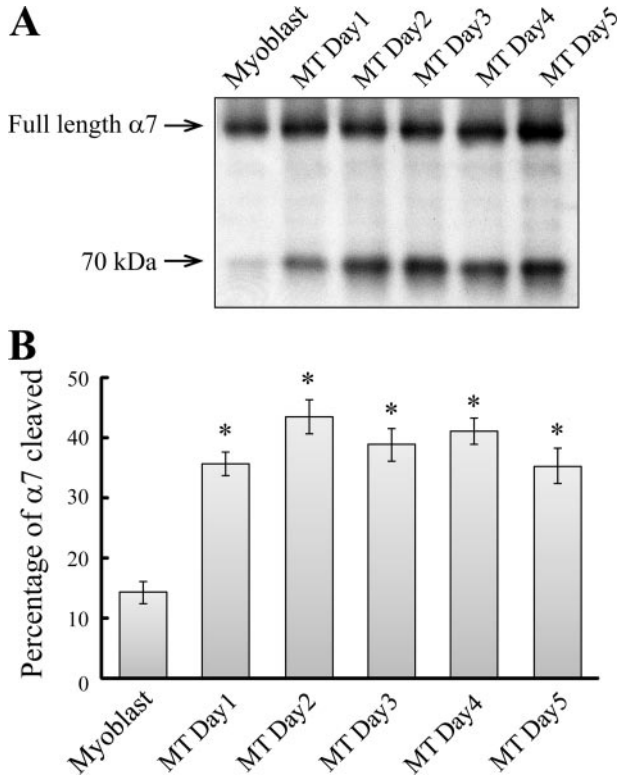

FIGURE 8. **Cleavage of α7 integrin chain increases during myogenic differentiation.** *A*, immunoblot detection of full-length and 70-kDa α7 peptide during rat L8E63 myoblast differentiation using antibody against the α7 chain cytoplasmic domain. Myoblasts were grown, switched to differentiation medium, and harvested 1–5 days after initiation of myotube (*MT*) formation. *B*, the percent of total α7 that is cleaved increases during differentiation from about 14% in myoblasts to 44% within the first 2 days of differentiation. Experiments were done in duplicate and repeated three times. *, $p < 0.05$ compared with other days.

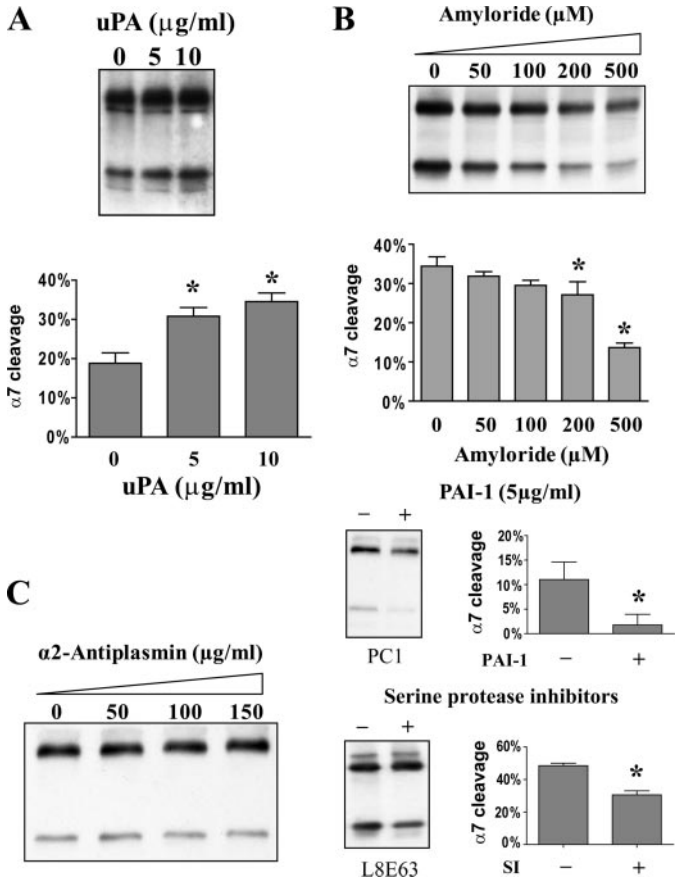

FIGURE 9. **uPA promotes cleavage of α7 integrin chain.** *A*, immunoblot analysis of α7 cleavage upon treatment of L8E63 cells with uPA for 1 h. Quantification of band intensities shows a significant increase of cleaved α7 in cells treated with uPA. *B*, amiloride hydrochloride hydrate (*Amyloride*) and human plasminogen activator inhibitor-1 (*PAI-1*), inhibitors of uPA, reduced α7 cleavage in L8E63 myoblasts and human PC1 cells, respectively. Treatment of L8E63 cells with a mixture of serine protease inhibitors (*SI*) also inhibits cleavage of α7 chain. Quantification of the band intensities shows amiloride treatment significantly reduces α7 cleavage and the decrease is significant at 500 mM. *C*, α2-antiplasmin does not affect α7 cleavage in L8E63 cells indicating integrin cleavage is independent of plasmin activity. *, $p < 0.05$.

cating that α7 is a substrate of uPA-mediated extracellular proteolyic activity. Blocking uPA activity with uPA inhibitors or serine protease inhibitors reduced the amount the 70-kDa peptide (Fig. 9*B*). In addition, a more apparent reduction of the 70-kDa peptide was observed (Fig. 9*B*) when human PC1 myoblast were treated with a mutant human PAI-1 recombinant protein that is constitutively active and stable at 37 °C.

As many effects of uPA are mediated by the serine protease plasmin (42), we determined if plasmin is essential for α7 chain cleavage. Addition of α2-antiplasmin to L8E63 myoblast cultures for 3 days did not affect the extent of α7 chain cleavage (Fig. 9*C*) indicating that, like α6 integrin, α7 chain cleavage mediated by uPA is independent of plasmin. These results suggested that uPA but not plasmin is at least partially responsible for α7 integrin cleavage at RRQ sites.

DISCUSSION

We have previously shown that α7β1 is the major laminin binding integrin in skeletal muscle and it is involved in regulating myogenic cell proliferation, migration, differentiation, and muscle fiber attachment to the extracellular matrix (11, 13, 21, 72). Increased levels of α7β1 were detected in muscular dystrophy characterized by defects in dystrophin (54) and this increase was proposed to compensate for the lack of dystrophin and the compromised cytoskeleton-extracellular matrix linkages (54). This hypothesis was confirmed by showing that transgenic overexpression of rat α7 integrin in *mdx*/*utrn*$^{-/-}$ mice alleviates muscle pathology and extends longevity (56, 57).

In the present study, we report a species-specific proteolytic cleavage that modulates α7β1 function. Site-directed mutagenesis confirmed the ^{603}RRQ^{605} amino acid residues in the rat α7 chain as the proteolytic cleavage site that results in production of a 70-kDa polypeptide. This RRQ sequence is a canonical di-basic site recognized by serine proteases (11, 32). The corresponding sequence in mouse α7 integrin is GRQ and the 70-kDa α7 peptide is not detected in mouse myogenic cells and muscle tissue. A 70-kDa fragment of radioactive-labeled mouse α7 has been reported (73), however, trypsin used in that experiment may have cleaved at the arginine of the GRQ site. Our results (Fig. 1) indicate it is not representative of the *in vivo* status of the mouse α7 integrin chain. From the amino acid sequences, the predicted molecular mass of this fragment is 58 kDa, yet in Western blots it has an apparent molecular mass of 70 kDa. This difference is likely due to glycosylation at the four *N*-glycosylation sites present in the 70-kDa polypeptide and

full-length α7 chain (12). The cleaved α7 70-kDa fragment remains associated with the β1 integrin subunit as does the NH_2-terminal half of the molecule that contains the ligand binding site. As the conformations of integrin cytoplasmic tails are dynamic and dependent on ligand binding (12, 74), the cleaved α7β1 complex may differ in its intracellular signaling capacity and perhaps its association with molecules that interact with α7β1 cytoplasmic domains (75, 76). Comparison of α7 integrin amino acid sequences from a variety of vertebrates demonstrates that the RRQ sequence responsible for cleavage is highly conserved among vertebrates with the exception of the mouse. Although RRQ at this site in mouse α7 was reported (16), our sequencing results for DNA and RNA from C2C12 cells and muscle from C57Bl6 and BALB/c mice demonstrated GRQ as the actual sequence encoded. Additional sequence data from other strains of mice confirm this and rule out nucleotide polymorphism at this site. Comparative sequence analysis in vertebrates and the absence of cleavage in this region of the laminin binding integrin of invertebrates, suggest that the RRQ site has evolved after vertebrates separated from invertebrates. The presence of GRQ at this site in the mouse instead of RRQ indicates mouse α7 integrin might have diverged further after mice separated from other rodents during evolution. Furthermore, the absence of single nucleotide polymorphisms among different mouse strains at this cleavage site suggests that this arginine to glycine conversion happened in a common mouse ancestor before mice diverged into strains. The selective advantage this change presents to mice is unclear but it likely relates to the regulation of α7β1 ligand binding.

A homology model of the α7 chain, based on the αV integrin crystal structure, was developed to study the spatial location of the RRQ site. Although the primary sequence of the RRQ site indicates it is near the flexible genu region, structural modeling reveals it is located at a unique site between the β-propeller and thigh domains. This site is readily accessible to interact with other proteins, including proteases. It has been shown that several other integrin α subunits (α3, α4, α5, αV, α6, and α9) interact with either uPAR or uPA at their β-propeller domain (69, 70, 77). Ease of accessibility of the RRQ site in α7 to potential proteases such as uPA and plasmin suggests α7 may also transiently interact with these proteases and function as a target. Although the sites of cleavage of α4, α6, and α7 are in the same physical proximity, proteolysis of α4 takes place at a KRS site and may be mediated by a distinct enzyme (30, 32).

Studies of the αV structure predict a 100° rotation between the β-propeller and thigh domains upon ligand binding (65, 74, 78). Proteolytic cleavage at the RRQ site of α7 may diminish the tension produced by rotation between the two domains and as a consequence, cleaved α7 integrin may bind its ligand faster and/or stronger. This prediction is supported by our results demonstrating better adhesion and faster cell spreading when the α7 chain is cleaved. In addition, we and others have reported that engagement of the α7β1 integrin by ligand or antibody results in conformational changes in the cytoplasmic domain of the integrin and its association with the cytoskeleton (12, 79, 80). Using affinity chromatography, cleaved α7 chain appears to preferentially bind to laminin (21). It has also been reported that interaction between laminin and the α7β1 integrin during development initiates allosteric changes that result in an activated integrin capable of transducing signals (80). Based on these findings and the results herein, we propose that proteolytic cleavage of α7 integrin facilitates or enhances conformational changes in the cytoplasmic domain, ligand binding, and integrin-mediated signaling.

The increased expression of α7 integrin when myogenic cells differentiate is accompanied by increased myofiber attachment to the extracellular matrix (12, 15, 68). Cleavage of the α7 chain is also increased upon muscle differentiation and remains relatively high thereafter. Components of the fibrinogen system (uPA, uPAR, and PAI-1) are also increased at this time and are believed to mediate cell adhesion, migration, and fusion (46, 47, 53, 71). The concurrence of elevated α7 cleavage and components of the fibrinogen system during myogenic differentiation prompted us to examine if inhibiting serine protease activity, especially uPA and plasmin, can block the formation of the 70-kDa polypeptide. Using serine protease inhibitor mixtures, uPA- and plasmin-specific inhibitors, we demonstrated that uPA but not plasmin could cleave the α7 chain. A decrease in α7 cleavage was only detected 3–5 days after treatment with uPA inhibitors and suggests that either the 70-kDa polypeptide is relatively stable, or uPA might only partially account for cleavage of the integrin. Consistent with this, wild type rat α7 expressed in CHO cells is also cleaved, and CHO cells have been shown to also produce high levels of uPAR and uPA (77, 81, 82).

Muscular dystrophies are characterized by progressive degeneration and regeneration. Proteomic studies have identified elevated levels of extracellular protease inhibitors in dystrophic muscles possibly in response to the increased proteolytic activity (83, 84). In addition, satellite cells derived from Duchenne muscular dystrophy patients express more uPAR and PAI-1 but less uPA (85, 86). Although α7 levels are elevated in Duchenne patients (54), lack of a corresponding increase in cleavage might impact its capacity to ameliorate the development of pathology. Therefore, it will be interesting to determine the status of α7 chain cleavage in these patients.

Skeletal muscle regeneration closely resembles muscle development and requires myogenic cell activation, proliferation, adhesion, and migration (38, 39). The α7β1 integrin plays important roles during these processes (13). Interestingly, the uPA system is also important to skeletal muscle regeneration (41, 49, 52, 87, 88). Mice deficient in uPA and uPAR have impaired muscle regeneration after chemically induced damage, whereas mice lacking the inhibitor PAI-1 have improved regeneration (52, 53, 87, 89). Our evidence that cleaved α7 mediates better cell adhesion and spreading suggests that cleavage of α7 in regenerating muscle by uPA may promote myoblast adhesion, migration, and subsequent muscle repair. Muscle fibers also express high levels of α7β1 integrin, particularly at myotendinous and neuromuscular junctions, where increased integrin-mediated adhesion provides junctional stability. In the absence of the integrin at these sites (in α7 null mice), or in the absence of the dystrophin glycoprotein complex that also provides junctional integrity, muscle function is compromised. As the α7β1 integrin can also protect muscle from exercise-induced damage (59), it will be of interest to determine the extent of α7 cleavage in skeletal muscle after exercise.

Last, it was recently reported that the α6 and α7 integrins also function as tumor suppressors (19, 90). The uPA system, responsible for the cleavage of the laminin binding integrins (α6 and α7), is also involved in oncogenesis and metastasis (for review, see Refs. 91 and 92). Therefore, the cleavage status of these integrins in tumor tissues and how proteolysis of integrin α chains may relate to tumorigenesis and metastasis will be interesting to explore. An understanding of how the uPA system can regulate integrin cleavage and functions in cancer cells may help in developing effective therapies (93, 94).

In summary, we have shown that the rat α7 integrin chain can be cleaved at a species-specific proteolytic cleavage site ^{603}RRQ^{605}. This same site is present in human α7. The serine protease uPA can mediate this cleavage in cell culture and it is a prime candidate enzyme for mediating integrin cleavage *in vivo* and in cancer tissues. Cells expressing cleaved α7 demonstrated better adhesion and faster cell spreading on laminin. Our results also suggest proteolytic cleavage as an evolutionary mechanism to regulate integrin structure and function.

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