

The Dystrophin Complex Forms a Mechanically Strong Link Between the Sarcolemma and Costameric Actin

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Abstract. The absence of dystrophin complex leads to disorganization of the force-transmitting costameric cytoskeleton and disruption of sarcolemmal membrane integrity in skeletal muscle. However, it has not been determined whether the dystrophin complex can form a mechanically strong bond with any costameric protein. We performed confocal immunofluorescence analysis of isolated sarcolemma that were mechanically peeled from skeletal fibers of mouse hindlimb muscle. A population of γ -actin filaments was stably associated with sarcolemma isolated from normal muscle and displayed a costameric pattern that precisely overlapped with dystrophin. However, costameric actin was absent from all sarcolemma isolated from dystrophin-deficient *mdx* mouse muscle even though it was localized to costameres in situ. Vinculin, α -actinin, β -dystroglycan and

utrophin were all retained on *mdx* sarcolemma, indicating that the loss of costameric actin was not due to generalized membrane instability. Our data demonstrate that the dystrophin complex forms a mechanically strong link between the sarcolemma and the costameric cytoskeleton through interaction with γ -actin filaments. Destabilization of costameric actin filaments may also be an important precursor to the costamere disarray observed in dystrophin-deficient muscle. Finally, these methods will be broadly useful in assessing the mechanical integrity of the membrane cytoskeleton in dystrophic animal models lacking other costameric proteins.

Key words: dystrophin • actin • muscular dystrophy • membrane skeleton • costameres

Introduction

In skeletal muscle, dystrophin is associated with a large, oligomeric complex of proteins that includes dystroglycans, sarcoglycans, dystrobrevins, syntrophins and sarcospan (Straub and Campbell, 1997). The dystrophin complex is generally thought to link the actin-based cortical cytoskeleton and laminin-2 in the extracellular matrix (Ervasti and Campbell, 1993). Genetic ablation of dystrophin or other components in the complex results in a variety of muscular dystrophies, which are often associated with defects in sarcolemmal membrane integrity (Petrof et al., 1993; Straub et al., 1997; Duclos et al., 1998; Cote et al., 1999). Thus, it is hypothesized that one important function of the dystrophin complex is to mechanically stabilize the sarcolemmal membrane from shear stresses imposed during eccentric

muscle contraction (Petrof et al., 1993). A mechanically strong attachment of dystrophin with the sarcolemmal membrane has already been demonstrated (Straub et al., 1992). Regarding possible interaction with the cortical cytoskeleton, dystrophin is located adjacent to the cytoplasmic face of the sarcolemmal membrane in a discrete, rib-like lattice termed costameres (Porter et al., 1992; Straub et al., 1992), a cytoskeletal protein assembly that links the force-generating sarcomeric apparatus to the sarcolemmal membrane (Pardo et al., 1983a; Craig and Pardo, 1983). Costameres transmit contractile forces laterally through the sarcolemmal membrane to the basal lamina (Dannowski et al., 1992), which may be important for maintaining uniform sarcomere length between active and resting muscle fibers of different motor units. While dystrophin is not required for the assembly of several proteins comprising costamere-like structures, the absence of dystrophin in humans and mice leads to an altered costameric lattice (Minetti et al., 1992, 1994; Porter et al., 1992; Ehmer et al.,

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1997; Williams and Bloch, 1999). These data suggest that dystrophin plays an important role in organizing or stabilizing costameres, possibly via direct binding to actin filaments. Biochemical studies have demonstrated that purified dystrophin complex can bind actin filaments with an apparent dissociation constant in the sub-micromolar range (Rybakova et al., 1996). However, it has been difficult to assess whether the affinity of the dystrophin/F-actin interaction measured *in vitro* is sufficient to support a mechanically strong interaction *in vivo*.

To address this question, we isolated inside-out sarcolemmal membranes by mechanically peeling the membranes from single myofibers teased from normal mouse hindlimb muscles. We used confocal immunofluorescence microscopy to visualize the cytoskeletal proteins retained on the sarcolemma without interference from sarcomeric proteins (Straub et al., 1992). In brief, we have obtained the first evidence demonstrating that the dystrophin complex is necessary for a mechanically strong physical linkage between the sarcolemmal membrane and γ -actin of the costameric cytoskeleton. Furthermore, our data point toward destabilization of γ -actin filaments as a possible intermediate between dystrophin deficiency and global disorganization of the costameric apparatus (Minetti et al., 1992, 1994; Porter et al., 1992; Ehmer et al., 1997; Williams and Bloch, 1999). Finally, our approach should be broadly applicable to assessing the contributions made by other costameric proteins toward the macromolecular organization and mechanical integrity of the membrane cytoskeleton in skeletal muscle.

Materials and Methods

Antibodies and Fluorescent Probes

Rabbit 2 polyclonal antiserum against dystrophin was obtained from an animal immunized with a recombinant protein encoding the first 246 amino acids of human dystrophin (DYS246), which was expressed and purified as previously described (Rybakova et al., 1996). Rabbit 56 polyclonal antiserum against a peptide corresponding to the last 12 amino acids of utrophin (Ohlendieck et al., 1991) was the kind gift of Dr. Kevin Campbell (University of Iowa, Iowa City, IA). The pan-actin monoclonal antibody C4 was purchased from ICN. Monoclonal antibodies specific for α -actinin, α -sarcomeric actin, β -actin, and vinculin were purchased from Sigma-Aldrich. The preparation and characterization of γ -actin polyclonal antibodies was previously described (Otey and Bulinski, 1988). The β -dystroglycan monoclonal antibody was purchased from Vector Laboratories. FITC- and TRITC-conjugates of anti-rabbit IgG and anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. Alexa488- and Alexa568-conjugates of phalloidin, anti-rabbit IgG and anti-mouse IgG were purchased from Molecular Probes.

Mechanical Isolation of Single Myofibers and Sarcolemma

Age matched, or littermate control, C57BL/10ScSn-DMD^{mdx}/J, or C57BL/6J-Lama2^{dy} mice were obtained from Jackson ImmunoResearch Laboratories. The vastus lateralis was dissected from tendon to tendon in ice-cold Ringers solution and transferred to a 0.5-ml drop of relaxing solution (100 mM BES, 15 mM creatine phosphate, 5 mM DTT, 4.74 mM ATP, 5.43 mM MgCl₂, 7 mM EGTA, and 0.02 mM CaCl₂ with the ionic strength adjusted to 180 mM with potassium propionate, pH 7.0) surrounded by silicone oil. To isolate sarcolemma membranes, one end of a single myofiber was secured with a very fine forceps, the sarcolemma was hooked near the forceps with a stainless steel micro-needle (<100 μ m diameter filed to a fine point) and rolled down and off the free end of the myofiber. Isolated sarcolemma and mechanically peeled or intact single myofibers were transferred with fine-tipped glass micropipets to a 0.02-ml drop of relaxing

solution stationed on a gelatin-coated glass slide. Isolated sarcolemma and mechanically peeled myofibers were fixed for 5 min with 4% paraformaldehyde in PBS. To assess the location of γ -actin *in situ*, intact myofibers from the vastus lateralis were fixed and permeabilized for 5 min with 4% paraformaldehyde and 0.1% Triton X-100 in PBS.

Immunofluorescence Analysis

Isolated myofibers and sarcolemmal membranes were blocked for 30 min at room temperature with 5% goat serum in PBS and incubated with primary antibodies overnight at 4°C. The samples were washed with PBS, incubated with the appropriate fluorescently tagged secondary antibodies for 30 min at 37°C, rinsed, and sealed under coverslips in an anti-fade solution. Confocal microscopy was performed with a Bio-Rad MRC1000 scan head mounted transversely to an inverted Nikon Diaphot 200 microscope in the Keck Neural Imaging Lab at the University of Wisconsin. The krypton/argon mixed gas air-cooled laser was set to allow only the 488- and 568-nm excitation lines, while the green and red emission signals were directed to separate photomultiplier tubes. Images were collected during a sequential scan to reduce the possibility of fluorescence bleed-through. To further verify that bleed-through of the green signal was not contaminating the red signal, serial optical sections (z series) were collected in some experiments using both single channel and double channel modes. Digital images were cropped using Adobe Photoshop 5.0 and figure montages prepared with Adobe Illustrator 8.0.

Miscellanea

The dystrophin/utrophin-glycoprotein complexes were amplified for immunoblot analysis from control and *mdx* total skeletal muscle membranes (Ohlendieck and Campbell, 1991) using digitonin extraction and WGA-Sepharose chromatography (Ervasti et al., 1990). Immunofluorescence analysis of frozen cryostat sections from control and *mdx* muscle was performed as previously described (Ervasti and Campbell, 1991).

Results and Discussion

A Population of Actin Filaments Is Tightly Associated with Costameres on Isolated Sarcolemma

Costameric proteins are typically visualized by immunofluorescence analysis of glancing longitudinal cryosections (Craig and Pardo, 1983; Porter et al., 1992), or in permeabilized single myofibers from mature skeletal muscle (Straub et al., 1992; Ehmer et al., 1997). If used in combination with widely available actin probes, analysis of dystrophin/actin colocalization by either of these methods is greatly complicated by the intense and ubiquitous signal provided by sarcomeric actin (Rybakova, I.N., and J.M. Ervasti, unpublished results). Therefore, we adopted a method (Straub et al., 1992) that would enable us to visualize the costameres without interference from the sarcomeric cytoskeleton. We isolated inside-out sarcolemmal membranes by mechanical peeling of single myofibers teased from normal mouse hindlimb muscles. Sarcolemmal membranes double stained with rabbit polyclonal antibodies to dystrophin and a fluorescent conjugate of phalloidin were examined by confocal immunofluorescence microscopy, which revealed closely overlapping costameric staining patterns consisting of alternately bright and dark transverse bands (Fig. 1 a) with an average periodicity of $2.8 \pm 0.3 \mu\text{m}$ ($n = 7$). However, only phalloidin stained the mechanically peeled myofibers (2.5 μm) while no dystrophin staining was detected (Fig. 1 b). Analysis of sarcolemma stained with other, better-characterized antibodies to dystrophin yielded similar results. However, we found that the rabbit 2 polyclonal antiserum to dystrophin raised in our laboratory yielded the greatest signal-to-

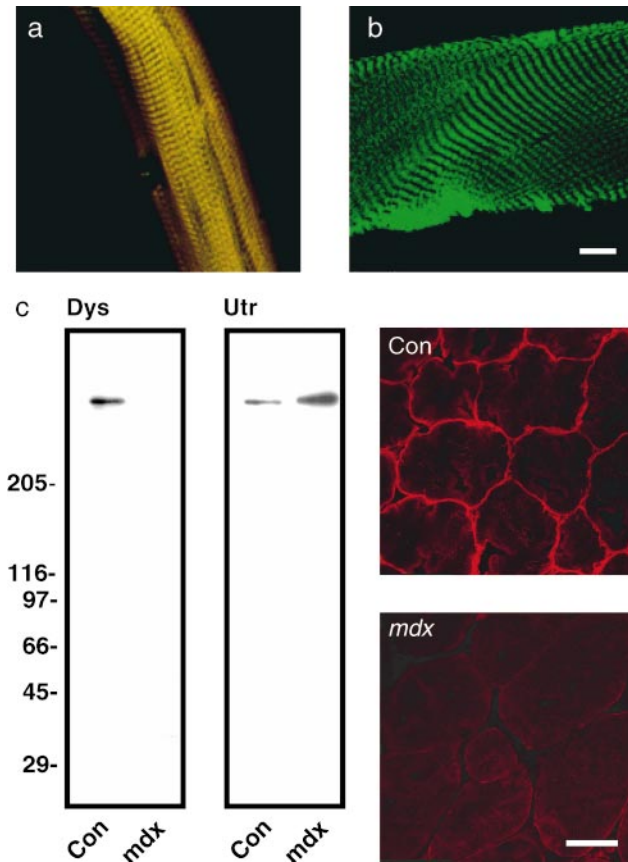


Figure 1. Dystrophin and F-actin colocalize on mechanically isolated sarcolemma in a costameric pattern. Shown is a mechanically isolated sarcolemma (a), or a skinned myofiber (b) both stained with Alexa488-phalloidin (green) and rabbit 2 antiserum to dystrophin (red). Red and green channels were collected simultaneously and areas of coincidence appear yellow. Shown on the left in (c) are immunoblots containing WGA-Sepharose eluates from detergent solubilized control and *mdx* skeletal muscle membranes stained with rabbit 2 antiserum to dystrophin (Dys), or rabbit 56 antiserum to utrophin (Utr). Shown on the right in c are transverse cryosections of control and *mdx* skeletal muscle stained with rabbit 2 antiserum to dystrophin. Bars: (b) 10 μm ; (c) 50 μm .

noise. Since the rabbit 2 antiserum was used throughout this study and had not been previously characterized, we have included evidence documenting its specificity for dystrophin in Fig. 1 c. To confirm that phalloidin was appropriately reporting the presence of actin, we double stained sarcolemma with rabbit 2 dystrophin antibodies and a well documented pan-actin monoclonal antibody (C4) reactive with all mammalian actin isoforms (Lessard, 1988). Again, dystrophin and actin staining exhibited closely overlapping staining patterns suggestive of costameres (Fig. 2, a–c). As an additional control, similar staining patterns were observed when sarcolemma were single-stained for dystrophin or actin. Finally, no staining was observed when fluorescent secondary antibodies were incubated alone with sarcolemma, nor did the secondary antibodies exhibit any inappropriate species cross-reactivity that could potentially explain the closely overlapping patterns obtained for dystrophin and actin. Thus, we conclude that a population of actin filaments and dystrophin are tightly associated with

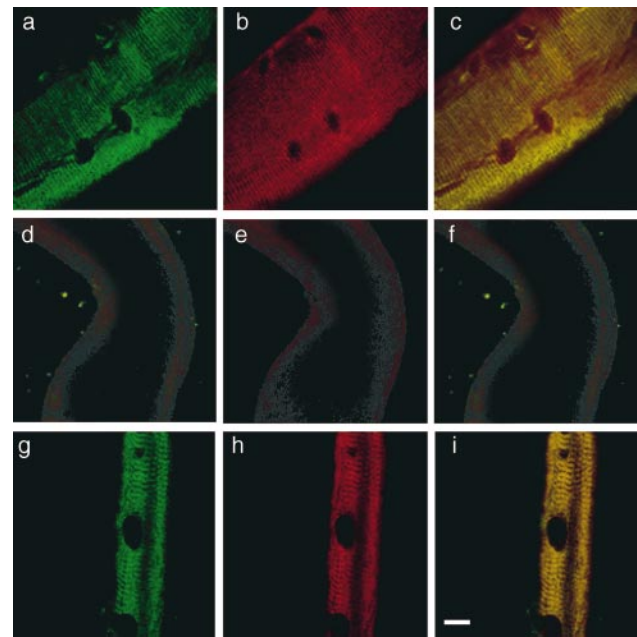


Figure 2. Costameric actin is absent from dystrophin-deficient *mdx* sarcolemma. Shown are representative images of sarcolemma isolated from control (a–c), dystrophin-deficient *mdx* (d–f), or laminin-2-deficient *dy/dy* (g–i) muscle stained with rabbit 2 antiserum to dystrophin (green) and monoclonal antibody C4 to actin (red). Green (a, d, and g) and red (b, e, and h) channels were collected separately, or simultaneously (c, f, and i) where areas of coincidence appear yellow. Dark ovoid shaped areas are nuclear lacunae, which mark the location of peripheral myonuclei before skinning (Straub et al., 1992). Bar, 10 μm .

the costameric cytoskeleton of normal skeletal muscle such that both can withstand the rigors of mechanical peeling.

Costameric Actin Is Specifically Absent from Dystrophin-deficient Sarcolemma

Because dystrophin binds actin in vitro (Rybakova et al., 1996) and the costameric cytoskeleton is altered in dystrophin-deficient muscle (Minetti et al., 1992, 1994; Porter et al., 1992; Ehmer et al., 1997; Williams and Bloch, 1999), we hypothesized that costameric actin should display an abnormal staining pattern on sarcolemma isolated from dystrophin-deficient *mdx* mice. To our surprise, we observed that costameric actin was completely absent from *mdx* sarcolemma as reported by monoclonal antibody C4 (Fig. 2, d–f), or phalloidin (Fig. 3, a–c). The absence of actin from *mdx* sarcolemma can be most directly and simply explained through an actin stabilizing function normally performed by dystrophin. Alternatively, the loss of actin from *mdx* sarcolemma might not be specifically due to the absence of dystrophin but rather to some other aspect of the dystrophic pathomechanism. However, we have detected costameric actin in all 24 sarcolemma isolated from 8 different control mice while costameric actin was absent from 21 different sarcolemma obtained from 7 different *mdx* mice. The animals used ranged from 2 to 6 months of age with the isolated sarcolemmal diameters varying from 18–60 μm for controls and 20–43 μm for *mdx* mice. Furthermore, we examined costameric actin distribution in sarco-

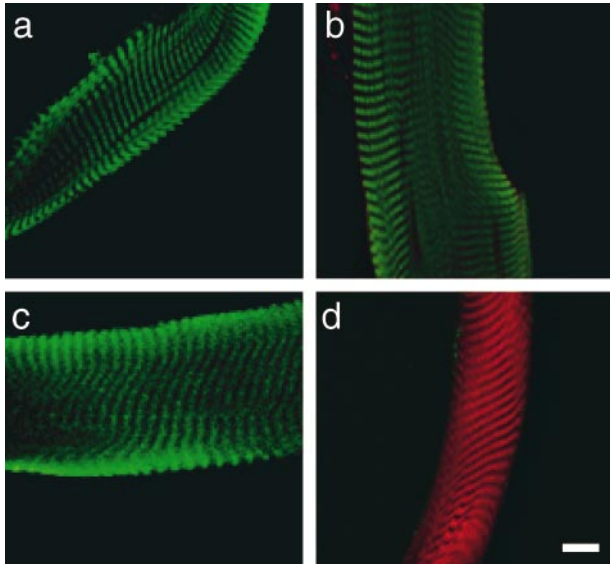


Figure 3. Retention of vinculin, β -dystroglycan and utrophin on *mdx* Sarcolemma. Shown are images of mechanically isolated sarcolemma from *mdx* (a–c) or control muscle (d) stained with Alexa568-phalloidin (a–d) in red, and antibodies to vinculin (a), β -dystroglycan (b), or utrophin (c and d) in green. Bar, 10 μ m.

lemma isolated from the hindlimb muscles of severely dystrophic *dy/dy* mice. The *dy/dy* mouse is deficient in laminin-2, the major extracellular matrix ligand for α -dystroglycan in the dystrophin-glycoprotein complex (Campbell, 1995). In contrast to *mdx* muscle, the dystrophin-glycoprotein complex is normally expressed at the sarcolemma of *dy/dy* muscle (Ohlendieck and Campbell, 1991) and shows little evidence of sarcolemmal membrane damage (Straub et al., 1997). Sarcolemma isolated from *dy/dy* muscle retained both dystrophin and actin in an overlapping pattern (Fig. 2, g–i) similar to the costameric distribution observed in non-dystrophic control sarcolemma (Fig. 2, a–c). These results suggest that in the absence of dystrophin, costameric actin is not stably associated with the sarcolemma membrane.

Because sarcolemmal integrity is compromised in dystrophin-deficient muscle (Straub et al., 1997), the absence of costameric actin might simply reflect a nonspecific loss of peripheral sarcolemmal proteins as a consequence of the stresses imposed during mechanical peeling. However, we found that both vinculin (Fig. 3 a) and α -actinin (Fig. 4 e) were retained on sarcolemma isolated from *mdx* muscle. Recently, both residual β -dystroglycan and upregulated utrophin in *mdx* muscle were shown to exhibit a costameric staining pattern in situ (Williams and Bloch, 1999). Consistent with this study, we observed that β -dystroglycan and utrophin were also retained on *mdx* sarcolemma and exhibited a costameric staining pattern (Fig. 3, b–d). Importantly, these data indicate that the loss of costameric actin was not due to generalized weakness of the sarcolemma membrane but is instead a specific consequence of dystrophin deficiency. Furthermore, our data suggest that utrophin either lacks the actin filament stabilizing activity of dystrophin (Amann et al., 1999), or that the amount of costameric utrophin in *mdx* muscle was not

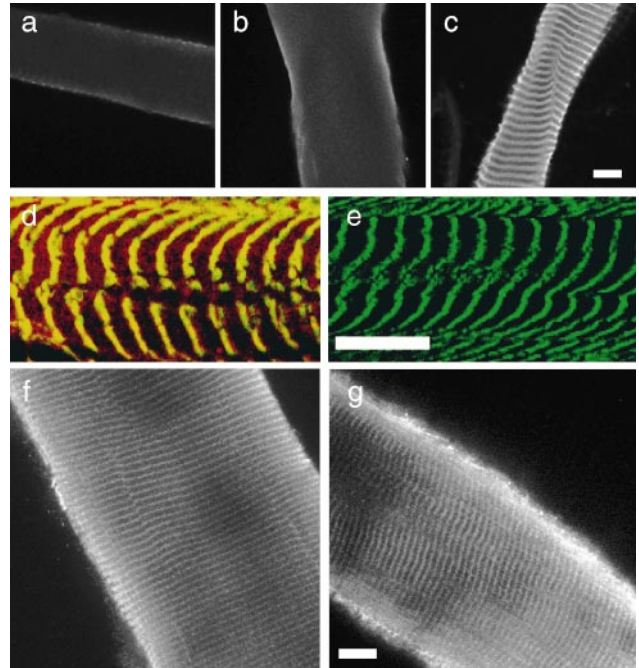


Figure 4. Costameric γ -actin is appropriately expressed and localized in *mdx* muscle in situ. Shown in a–c are images of sarcolemma from normal mouse muscle stained with antibodies specific to α -sarcomeric (a), β -nonmuscle (b), or γ -actin (c). Shown in d and e are images of control (d), or *mdx* sarcolemma (e) double stained with monoclonal antibodies to α -actinin (green) and polyclonal antibodies to γ -actin (red). Areas of coincidence between the two probes appear yellow. Shown in f and g are images of fixed and permeabilized myofibers from control (f) and *mdx* (g) muscle stained with antibodies specific for γ -actin. Bars, 10 μ m.

sufficient to retain costameric actin on mechanically isolated sarcolemma.

Costameric Actin Is Appropriately Expressed and Localized in *mdx* Muscle In Situ

The γ -actin isoform was previously shown to colocalize with vinculin at costameres in chick skeletal muscle (Craig and Pardo, 1983). However, the polyclonal antibody used in this study (Craig and Pardo, 1983) did not significantly stain the sarcolemma of mouse diaphragm (Pardo et al., 1983b). Thus, it remained unclear which actin isoform(s) may populate costameres of mammalian muscle. Therefore, we stained normal sarcolemma with monoclonal antibodies specific for α -sarcomeric actin, or β -actin, or a polyclonal antibody raised against a peptide corresponding to the unique amino-terminus of γ -actin (Otey and Bulinski, 1988). Only the γ -isoform antibody yielded a bright, costameric pattern of staining while α - and β -specific antibodies yielded only weak and diffuse background labeling (Fig. 4, a–c). As was found using monoclonal antibody C4 (Fig. 2), or phalloidin (Fig. 3), the γ -actin staining pattern evident on normal sarcolemma (Fig. 4, c and d) was absent from dystrophin-deficient *mdx* sarcolemma (Fig. 4 e). These data indicate that costameric actin appears to be exclusively comprised of the γ -isoform.

It remained to be determined whether costameric actin was absent from *mdx* muscle before mechanical peeling,

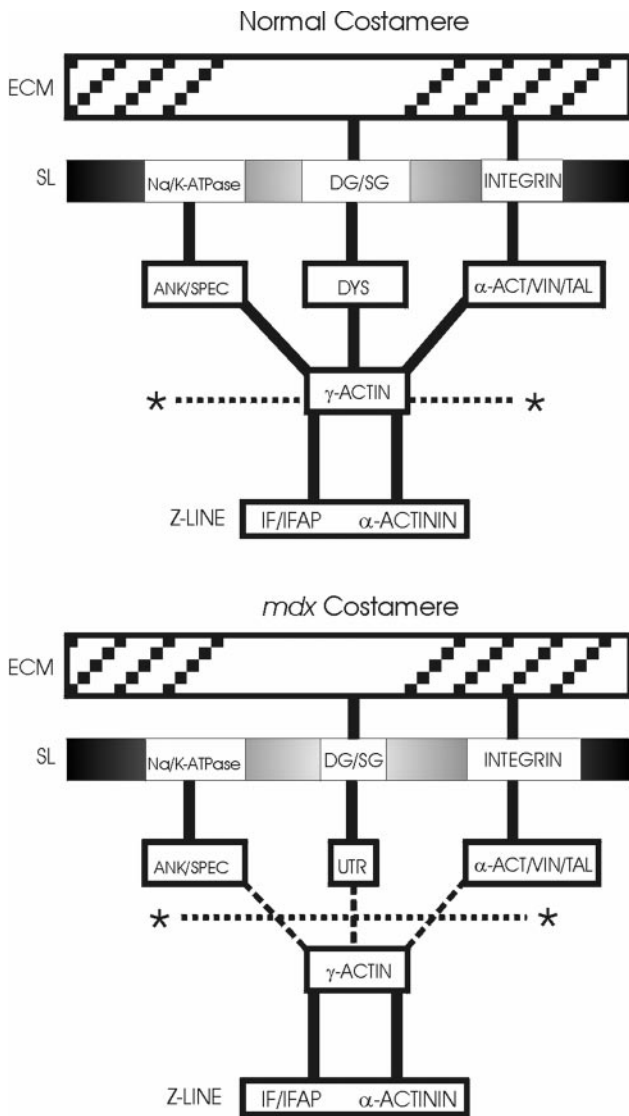


Figure 5. Costameric actin as a common nexus for mechanical coupling between the contractile apparatus and extracellular matrix of striated muscle. Shown is a schematic model depicting the molecular organization of costameres in normal (top) and dystrophin-deficient *mdx* mouse muscle (bottom). Based on evidence presented in the current report, the fracture plane effected by mechanical peeling is also indicated (*.....*).

or was lost as a result of the peeling procedure. Therefore, we examined γ -actin staining in single fibers from control and *mdx* muscle that were fixed and permeabilized, but not mechanically peeled. Both control and *mdx* myofibers exhibited peripheral γ -actin staining patterns marked by regularly spaced transverse fluorescent bands of similar intensities (Fig. 4, f and g). Finally, the γ -actin antibodies also labeled the periphery of Z-lines in mechanically peeled myofibers from both control and *mdx* muscle, however, the γ -actin signal appeared brighter on *mdx* myofibers compared with controls (not shown). Taken together, our results indicate that γ -actin is appropriately expressed and assembled into costameres in *mdx* muscle, but that its stable association with the sarcolemmal membrane is severely compromised.

Based on its structure, protein interactions, and membrane defects associated with its absence or abnormality in dystrophic muscle, the dystrophin complex has long been hypothesized to mechanically stabilize the sarcolemmal membrane against the stresses imposed during muscle contraction or stretch (Petrof et al., 1993; Campbell, 1995). Whereas dystrophin is strongly anchored to the sarcolemmal membrane (Straub et al., 1992), no study has demonstrated a mechanically strong linkage between dystrophin and any component of the costameric cytoskeleton. Thus, ours are the first data demonstrating that the dystrophin complex is necessary for a mechanically strong physical linkage between the sarcolemmal membrane and costameric γ -actin. Furthermore, our data point toward destabilization of γ -actin filaments as a possible intermediate between dystrophin deficiency and global disorganization of the costameric apparatus (Porter et al., 1992; Minetti et al., 1992, 1994; Ehmer et al., 1997; Williams and Bloch, 1999). Complexes comprised of ankyrin/spectrin (Williams and Bloch, 1999), vinculin/talin/ α -actinin (Williams and Bloch, 1999) and intermediate filament/intermediate filament associated proteins (Milner et al., 1996; Andra et al., 1997; Dalpe et al., 1999) are also localized to costameres and are capable of binding actin (Fig. 5). Therefore, it is possible that multiple, distinct costameric protein complexes act synergistically to form a strong mechanical linkage between the sarcolemmal membrane and the force-generating sarcomeric apparatus. In support of this possibility, targeted inactivation of desmin (Milner et al., 1996), plectin (Andra et al., 1997), or BPAGn1/dystonin (Dalpe et al., 1999) also cause muscular dystrophy and sarcolemmal instability. Our methods should be valuable in further assessing the macromolecular organization and mechanical integrity of the membrane cytoskeleton in dystrophic animal models lacking other components of costameres.

Alternatively, dystrophin may be specifically designed to mechanically stabilize costameric actin filaments. We previously demonstrated that dystrophin contains two distinct and spatially separated actin binding sites located at the amino terminus and middle rod domains (Rybakova et al., 1996; Amann et al., 1998), which enables dystrophin to form an extended lateral association with F-actin and protect actin filaments from depolymerization in vitro (Rybakova et al., 1996; Rybakova and Ervasti, 1997). Studies by others suggest that the dystrophin carboxy-terminal domain may also participate in binding actin, either directly (Howard et al., 1998), or indirectly through α -syntrophin (Iwata et al., 1998). Analysis of sarcolemma from *mdx* mice transgenically expressing dystrophin constructs deleted in different domains (Chamberlain et al., 1997), or mice lacking α -syntrophin (Kameya et al., 1999) will resolve which elements in the dystrophin complex are necessary for strong actin filament association with the sarcolemmal membrane. Finally, the methods used in this study will be valuable in assessing whether utrophin overexpression (Tinsley et al., 1998) can rescue the mechanically strong linkage between costameric actin and the sarcolemmal membrane observed in normal muscle.

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