Cloning and Characterization of Cytokeratins 8 and 19 in Adult Rat Striated Muscle

We used degenerate primers for the amino- and carboxyl-terminal ends of the rod domains of intermediate filament proteins in reverse transcriptase-PCR experiments to identify and clone cytokeratins 8 and 19 (K8 and K19) from cardiac muscle of the adult rat. Northern blots showed that K8 has a 2.2-kb transcript and K19 has a 1.9-kb transcript in both adult cardiac and skeletal muscles. Immunolocalization of the cytokeratins in adult cardiac muscle with isoform-specific antibodies for K8 and K19 showed labeling at Z-lines within the muscle fibers and at Z-line and M-line domains at costameres at the sarcolemmal membrane. Dystrophin and K19 could be co-immunoprecipitated and co-purified from extracts of cardiac muscle, suggesting a link between the cytokeratins and the dystrophin-based cytoskeleton at the sarcolemma. Furthermore, transfection experiments indicate that K8 and K19 may associate with dystrophin through a specific interaction with its actin-binding domain. Consistent with this observation, the cytokeratins are disrupted at the sarcolemmal membrane of skeletal muscle of the mdx mouse that lacks dystrophin. Together these results indicate that at least two cytokeratins are expressed in adult striated muscle, where they may contribute to the organization of both the myoplasm and sarcolemma.

Nearly two decades ago, Craig, Lazarides and their colleagues (1–4) first identified structures at the sarcolemmal membrane of striated muscle enriched in cytoskeletal proteins that mirrored the regular organization of Z-lines in the contractile apparatus. Because of the “rib-like” appearance of these structures at the sarcolemmal membrane, Craig (1, 2) named them “costameres.” We later expanded the definition of costameres to include, in addition to the structures overlying Z-lines, two additional structures that we identified by immunofluorescence techniques and confocal microscopy (5). One overlies the M-lines of superficial myofibrils, consistent with ultrastructural findings of connections between the sarcolemma and M-lines (6–8). The other costameric domain is oriented parallel to the long axis of the muscle cells and has not yet been correlated with sarcomeric structures. These three “costameric” domains, which we refer to as “Z-line domains,” “M-line domains” and “L domains,” form an extensive rectilinear lattice at the sarcolemma of rodent muscle (5, 9–11).

The cytoskeletal proteins that are organized into costameres at the sarcolemma of skeletal muscle include dystrophin and the dystrophin-associated protein complex, βII2-spectrin, αII-spectrin, ankyrin 3, vinculin, and actin (1–5, 11–13). The specific factors that align these and other proteins in the costameres with the underlying contractile apparatus are still unknown. Ultrastructural studies have shown that the sarcolemmal regions lying between the costameres can bulge outwards during muscle contractions, whereas the costameres remain tightly connected to the Z disks of the nearby myofibrils (6–8). Although the nature of these connections remains unknown, biochemical evidence suggests that they may be composed of actin microfilaments, whereas ultrastructural images reveal similarities to intermediate filaments (6–8, 14).

Intermediate filaments, defined as filaments that are 10 nm in diameter (Ref. 15 and reviewed in Refs. 16 and 17), constitute a large superfamily of proteins that has six major branches (Types I–VI). All intermediate filament (IF) proteins have a central α-helical rod domain that is flanked by non-helical head and tail domains. Overall sequence identity between members of the IF superfamily varies widely, but a key feature of all IFs is the high homology near the beginning and end of the central rod domain.

Desmin is the major IF protein of striated muscle (16). It is found together with other type III intermediate filament proteins, including syncoilin, paranemin, and synemin (also known as desmuslin) at the Z-lines of mature skeletal muscle (18–23). Other IF proteins, including vimentin, nestin, and cytotkeratins, are expressed in developing muscle but are thought to be down-regulated as the muscle matures (24–26). The keratins, which form the largest group of IFs, are categorized as Type I (acidic, K9–K23) and Type II (basic, K1–K8) IF proteins. Although desmin can self-assemble to form IFs, the keratins only form filaments as heterodimers comprised of Type I and Type II subunits. They are not thought to co-polymerize with other types of IF proteins, such as desmin (27, 28). Like other IFs, cytokeratin filaments function primarily to provide mechanical support to cells, but recent evidence also implicates the cytokeratins in such roles as cell signaling and apoptosis (29).
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In striated muscle, the desmin-based IFs at the Z-line may play a role in aligning the myofilaments in the sarcomere and may also link the Z disks of the superficial myofilaments to the sarcolemma, at the Z-line domains of costameres (14, 30–32). Recent work by our laboratory (33) suggests that desmin preferentially stabilizes Z-line domains of costameres but that it is not necessary for the stability of M-line and L domains. These studies examined costameres in skeletal muscle from mice lacking desmin because of homologous recombination and found that many of the M-line and L domains, as well as some Z-line domains, were still intact (33). As our preliminary cloning data (34) indicated that the cytokeratins were potential candidates for the role of organizing the costameres in adult striated muscle, O’Neill et al. (33) immunolabeled skeletal muscle with Pan-specific cytokeratin antibodies. The results showed that the cytokeratins were present at all domains of costameres. However, the identity of those cytokeratins and the mode of their association with the sarcolemmal membrane remain unknown.

To identify additional IFs that play a role in organizing costameres at the sarcolemmal membrane of striated muscle, we designed degenerate oligonucleotide primers to the regions of high identity located at both ends of the central rod domain, for use in RT-PCR experiments. Using this information in combination with the results of Northern blots and immunofluorescence studies, we have identified cytokeratins 8 and 19 (K8 and K19, respectively) as the two major cytokeratins expressed in skeletal and cardiac muscles of the adult rat. Immunoprecipitation experiments suggest that the cytokeratins associate directly or indirectly with the costameric protein, dystrophin. Cellular transfection experiments indicate that, when assembled into heteropolymeric filaments, K8 and K19 interact specifically with the amino-terminal actin-binding domain of dystrophin. We propose that K8 and K19 at costameres work in conjunction with desmin to stabilize the Z-line domains and alone to stabilize the M-line and L domains, where they may interact directly with dystrophin.

EXPERIMENTAL PROCEDURES

Tissue Preparation—The heart and gastrocnemius muscles were collected from adult Sprague-Dawley rats (Zivic-Miller, Zelienople, PA) and adult CD-1 mice. In addition, the tibialis anterior muscles were collected from dystrophin-deficient mdx mice. Animals were anesthetized and perfused through the left ventricle with either phosphate-buffered saline (PBS) containing protease inhibitors (Complete Protease Inhibitor Mixture Tablets, Roche Diagnostics Corp.) or 2% paraformaldehyde in PBS (for mdx tissue only). The muscle tissue was excised and frozen in one of two ways. Tissue to be used for RNA purification or homogenization was immediately plunged frozen in liquid nitrogen. Tissue to be used for frozen sections was snap frozen in a slush of liquid nitrogen, to reduce freeze damage. Dissociated mouse cells were prepared as described previously (35) and the heart cells were fixed with ice-cold ethanol for at least 1 h prior to immunolabeling.

Antibodies—Antibodies against purified bovine α-fodrin (all-spectrin) were prepared in rabbits and antibodies against human erythroid β-spectrin were prepared in chickens. The antibodies were affinity purified as previously described (36) and were used at a concentration of 2 μg/ml for immunofluorescence experiments. Antibodies against γ-actin were prepared in rabbits and used at a dilution of 1:200 for Western blots (kindly provided by Dr. J. C. Bulinski, Columbia University Medical Center, New York). Subunit-specific antibodies to the sheep antibodies to cytokeratins 8, 18, and 19 were purchased from the Binding Site (Birmingham, United Kingdom). These antibodies were used at dilutions of 1:50 for immunofluorescence experiments and 1:500 for Western blots. Monoclonal mouse antibodies to cytokeratin 19 (clone Ks19.1, purchased from Progen, Heidelberg, Germany), dystrophin (Clone NCL-DYS2, purchased from Novocastra Laboratories Ltd, New Castle upon Tyne, UK), and a control mouse IgG1 (MOPC21, purchased from Sigma) were used in immunoprecipitation experiments, as described below. Pan-specific antibodies to the cytokeratins, which recognize multiple isoforms but are specific for the cytokeratins, were purchased from Biogenex (San Ramon, CA). All were used at dilutions of 1:500 for Western blots. Two additional antibodies were employed in transfection experiments: a monoclonal antibody specific for human cytokeratin 19 (Sigma), used at a dilution of 1:50, and a monoclonal antibody specific for the myc epitope tag (Invitrogen), used at a dilution of 1:500. Purified non-immune rabbit and sheep IgGs were from Sigma and Jackson ImmunoResearch Laboratories, Inc (West Grove, PA), respectively. Secondary antibodies included donkey anti-chicken IgG conjugated to fluorescein isothiocyanate, donkey anti-sheep IgG conjugated to tetramethylrhodamine, and goat anti-mouse conjugated to tetramethylrhodamine (Jackson ImmunoResearch) as well as Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 488 or 568 goat anti-mouse IgG (Molecular Probes, Eugene, OR). Alkaline phosphatase-conjugated donkey anti-sheep and goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch.

Immunofluorescence Labeling—Dissociated cardiomyocytes were prepared from adult mouse or rat hearts and immunolabeled in suspension. Cells were allowed to sediment at unit gravity between steps in the labeling procedure. After fixation in ice-cold ethanol, the cells were washed and incubated in a solution containing 5% normal goat serum and 3% bovine serum albumin in PBS for a minimum of 30 min, to block nonspecific labeling prior to adding the primary antibody. Samples were incubated with the primary antibodies overnight at 4 °C with gentle mixing, washed with 1 mg/ml bovine serum albumin in PBS, and incubated with either secondary antibodies with 1-2 h at room temperature. Frozen sections of mdx skeletal muscle were cut at 20 μm on a Reichert-Jung cryostat (Cambridge Instruments, Deerfield, IL), collected on slides, and double labeled with chicken antibodies to β-spectrin and pan-specific mouse antibodies to cytokeratins. Blocking and labeling solutions and times were as described above. All samples were mounted on slides with Vectashield anti-fade (Vector Laboratories, Inc., Burlingame, CA) to reduce photobleaching. Slides were examined with a Zeiss 410 confocal laser scanning microscope (Carl Zeiss, Inc., Tarrytown, NY) with pinholes set at 18 and a resolution in the x and y directions of 0.124 μm. Figures were prepared with Corel Draw 10 (Corel Corporation Limited, Ottawa, Ontario, Canada).

Immunoblotting—We tested the specificity of the cytokeratin antibodies by immunoblotting. Purified recombinant human cytokeratins 8, 18, and 19 (Research Diagnostics, Inc., Flanders, NJ) were heated for 10 min at 65 °C in SDS-PAGE sample buffer (37) and loaded onto a 4–12% gradient polyacrylamide gel. The proteins were transferred electrophoretically to nitrocellulose (38) and the blots were incubated in Blotto (3% dry milk solids in PBS containing 0.5% Tween 20 and 10 mM sodium azide) for 2 h. The blots were probed with the subunit-specific antibodies to the cytokeratins (see above), followed by incubation with donkey anti-sheep IgG conjugated to alkaline phosphatase. The bound antibody was detected by chemiluminescence (Western Light Detection, Tropix Laboratories, Bedford, MA).

Immunoprecipitation—Immunoprecipitations, gastrocnemius or cardiac muscle from adult rats were homogenized with a Brinkmann Polytron homogenizer (Switzerland) at a w/v ratio of 0.05 in 2% Empigen (39) in PBS, pH 7.2, with protease inhibitors (Complete Protease Inhibitor Mixture Tablets, Roche Diagnostics). The homogenates were incubated on ice for 1 h before centrifugation at 12,000 × g and collection of the supernatant. Immunoprecipitations were performed from 1 mg of this homogenate with goat anti-mouse M450 Dynabeads (Dynal, Lake Success, NY), according to the manufacturer’s instructions, and monoclonal antibodies to cytokeratin 19, dystrophin, or a monoclonal control antibody (see above). After separation on a 4–12% polyacrylamide gradient gel, proteins in the immunoprecipitates were blotted as described above.

Full Partial Purification of the Dystrophin-associated Glycoprotein Complex—The dystrophin-associated glycoprotein complex was partially purified using the procedure reported by Ervasti et al. (40) with several modifications. In brief, homogenates of adult cardiac muscle were prepared as above in 1% digitonin, 2% Empigen, or 1% Nonidet P-40 in 140 mM NaCl, 5 mM Tris-HCl, pH 7.2, with added protease inhibitors. The homogenates were mixed overnight at 4 °C with 1 ml aliquots of WGA-agrose (Vector Laboratories, Burlingame, CA). The samples were washed first with buffer containing 0.1% of the appropriate detergent in phosphate-buffered saline, pH 7.4, followed by extensive washing with phosphate-buffered saline. In control samples, the WGA-agrose was washed with 1 ml of 0.5 M N-acetylglucosamine in phos- phate-buffered saline prior to addition of the homogenate. In control samples, the WGA-agrose was washed with 1 ml of 0.5 M N-acetylglucosamine was added to the homogenate and was present in all subsequent washing steps. After washing, the beads were heated for 10 min at 65 °C in SDS-PAGE sample buffer (37) and loaded...
onto either 3–8 or 4–12% polyacrylamide gradient gels. The proteins were immunoblotted as described above.

**RT-PCR**—RNA was purified from adult rat heart using TRIzol (Sigma) and following the manufacturer’s directions. Aliquots containing 5 μg of RNA were used in a First Strand cDNA synthesis (Amer-sham Biosciences). Primers and Platinum Taq polymerase (Invitrogen) were added to the first strand reaction and placed in the thermocycler to generate the PCR products. Initially, degenerate primers were designed to the regions of high homology found at the amino- and carbox-yl-terminal ends of the rod domains of intermediate filaments. The first set of degenerate primers that successfully yielded cytokeratin products included: sense, 5’-TTCCGTGAG(C/G)(C/G)AGAA(C/T)-3’ and antisense, 5’-(C/T)TCCAGCAG(C/T)GTTAGGT-3’. Subsequent primers were either designed to match cloned regions of the cytokeratins or to match sequences obtained from the GenBank data base (rat cytokeratin 8 under GenBank accession number M63482; mouse cytokeratin 19 under GenBank accession number M28698).

**Northern Blots**—Northern blots were prepared with the NorthernMax™ Kit (Ambion, Inc., Austin, TX) according to the manufacturer’s instructions, with minor modifications. Briefly, 5 μg of poly(A)+ RNA aliquots from adult rat brain, heart, skeletal muscle, and skin (OriGene Technologies, Inc., Rockville, MD) were separated on a 1% agarose gel and transferred to BrightStar-Plus nylon transfer membrane (Ambion, Inc.). The resulting Northern blot was blocked and probed at high stringency with portions of cDNA encoding cytokeratins 8 and 19, as indicated in the legend to Fig. 1, using QuikHyb (Stratagene). Probes that had less than 90% identity failed to produce a signal under these stringency conditions. Labeled blots were exposed to film under an intensifier screen at −80 °C. Size markers, in kb, are shown in the center of the Northern blots of Fig. 1.

**Transfections**—cDNA encoding the actin-binding domain of dystrophin (Dys-ABD) was amplified by PCR from plasmid pDys246, kindly provided by Dr. J. Ervasti (University of Wisconsin, Madison, WI). The cDNA, encoding the Dys-ABD in the NH2-terminal 246 amino acids of dystrophin (41, 42), was subcloned into the XhoI and EcoRI sites of the plasmid pEGFP vector (Clontech) to yield plasmid Dys-ABD-GFP. Primers were designed according to the dystrophin cDNA sequence obtained from the GenBank data base (GenBank accession number M18533).

**Cytokeratin 8** (K8) was amplified from plasmid pH1.1 (ATCC, Manassas, VA) with primers designed according to the sequence obtained from the GenBank data base (GenBank accession number NM02273). The coding sequence of K8 was subcloned into the EcoRI and KpnI sites of the pCMV-Myc vector to yield Myc-K8. The human cytokeratin 19 plasmid (PPA125) was kindly provided by Dr. P. Coulombe (Johns Hopkins University, Baltimore, MD). PPA125 contains K19 in a GW1 vector under the control of the cytomegalovirus promoter. DH5α cells were transformed with all of the resulting plasmids. For transfections, COS-7 cells were grown on 13-mm coverslips in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in an humidified atmosphere of 95% air and 5% CO2. Cells were transfected when ∼30% confluent. Transient transfections utilized the calcium phosphate method and were performed with 1 μg each of the appropriate plasmids. After transfection, cultures were incubated for 24 h before they were prepared for immunofluorescence. Cells were then fixed for 15 min in 2% paraformaldehyde, washed with PBS, and permeabilized with 1% Triton X-100 in PBS for 10 min. Additional steps for immunofluorescence or fluorescence imaging were as above. Primary antibodies were either anti-myc (1:500) or anti-human K19 (1:50, Sigma), followed by incubation with Alexa Fluor 568-labeled goat anti-mouse antibodies. Images were collected by confocal microscopy, as described above.

**Materials**—Unless otherwise stated, all materials were the highest grade available from Sigma.

**RESULTS**

Previous work from our laboratory (33) that examined skeletal muscle from desmin-deficient mice suggested that additional IF proteins, possibly cytokeratins, might help to connect the contractile apparatus to costameres at the sarcolemma membrane. We therefore designed degenerate oligonucleotide primers for use in RT-PCR experiments to identify other, possibly novel IF proteins in adult striated muscle.

Cloning of Cytokeratins 8 and 19 from Rat Cardiac Muscle—We first designed oligonucleotide primers to the highly conserved regions at the 5’ and 3’ ends of the rod domain (17) of both desmin and paranemin. These primers were used in RT-PCR experiments with mRNA from adult rat cardiac muscle. The first clone we identified using this method encoded 100 amino acids of the rod domain of cytokeratin 8 (K8). We used primers that exactly matched the sequence for K8 (accession number M63482) to clone the entire open reading frame (see Fig. 2).
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Fig. 1A and portions of the 5′ and 3′ untranslated regions from the myocardium of the adult rat (GenBank™ accession number AY464139). Below the diagrams in Fig. 1, bars indicate the individual clones obtained and sequenced to compile the full-length sequences. An asterisk indicates the specific clone used in Northern blot experiments (see below). The translated sequence for K8 from cardiac muscle is 100% identical to the recently reported amino acid sequence for rat cytokeratin 8 and 99% identical to the nucleotide sequence (GenBank™ accession number XM343335), although we did identify a single clone in which Trp-123 was replaced with a Gly. The open reading frame contains 1449 nucleotides encoding 483 amino acids with a calculated molecular mass of 54,019 Da.

Because the cytokeratins form heteromeric filaments composed of acidic and basic filaments, we performed additional RT-PCR experiments to identify a second cytokeratin in adult striated muscle. K8 is a type II, or basic, IF subunit that can exist of acidic and basic filaments, we performed additional RT-PCR experiments to identify a second cytokeratin in adult heart muscle (Fig. 1). Cytokeratin 8 typically has a transcript size of 1.8 kb (see lane labeled “brain”; additional data not shown) but the most prominent form in heart, skeletal muscle, and skin has a transcript size of 2.2 kb. Based on our results, we believe that an additional 0.4 kb of sequence is present in the 5′ untranslated region. Cytokeratin 19 has a major transcript size of ~1.9 kb in all tissues examined.

Fig. 2. Multiple tissue Northern blots for cytokeratins 8 and 19. A Northern blot was prepared with 5 µg each of mRNA from rat brain, heart, skeletal muscle, and skin. The blots were probed at high stringency using QuikHyb (Stratagene) following the manufacturer’s suggestions. The DNA probes for cytokeratins 8 and 19 were those labeled by asterisks in Fig. 1. Cytokeratin 8 typically has a transcript size of 1.8 kb (see lane labeled “brain”; additional data not shown) but the most prominent form in heart, skeletal muscle, and skin has a transcript size of 2.2 kb. Based on our results, we believe that an additional 0.4 kb of sequence is present in the 5′ untranslated region. Cytokeratin 19 has a major transcript size of ~1.9 kb in all tissues examined.

Our laboratory previously used pan-specific antibodies to the cytokeratins in immunofluorescence experiments to localize cytokeratins at costameres in adult rat skeletal muscle (33). Because the above cloning data indicated that K8 and K19 were the specific isoforms of cytokeratins in adult rat cardiac tissue, we wanted to confirm their presence at costameres in the heart. We therefore used the isotype-specific antibodies, described above, to label dissociated mouse cardiocytes by immunofluorescence. Each sample was double labeled with antibodies to αII-spectrin, which is present in cardiac muscle at

![Immunoblot of subunit-specific antibodies to cytokeratins 8, 18, and 19](Image 74x597 to 290x738)

**Fig. 3. Immunoblot of subunit-specific antibodies to cytokeratins 8, 18, and 19.** Purified cytokeratins 8, 18, and 19 were separated on 4–12% polyacrylamide gels and transferred to nitrocellulose paper. The blots were probed with antibodies to cytokeratin 8, cytokeratin 18, and cytokeratin 19. Lanes 1, K8; lanes 2, K18; lanes 3, K19. The results show that each antibody is specific for the appropriate cytokeratin subunit. Size markers are indicated on the left of the figure, in kDa.

Because K18 is also a potential binding partner for K8, we tried to identify the presence of this transcript in adult heart muscle. Using the same RT-PCR technique described above and primers designed according to the previously published sequence (GenBank™ accession number M26326), we were able to identify the presence of K18 in hearts from day-16 rat embryos, but not from adults (data not shown). Therefore, it seems that while K18 is down-regulated with cardiac development, both K8 and K19 persist in adult heart muscle.

We used portions of the cloned K8 and K19 sequences in multiple tissue Northern blots to confirm the presence of transcripts for these cytokeratins in adult heart and skeletal muscle. The clones used for hybridization in the Northern blot procedures are labeled by asterisks in Fig. 1. In addition to heart and skeletal muscle, brain and skin were also probed. We found that K19 had a 1.9-kb transcript in all tissues examined (Fig. 2). In contrast, we found that the transcript size for K8 varied within this group of tissues. Brain (e.g. Fig. 2) had a transcript size of 1.8 kb for K8, but the most prominent form in heart, skeletal muscle, and skin was 2.2 kb. We also examined a variety of other tissues by Northern blot, including thymus, liver, kidney, stomach, small intestine, spleen, and testis, which yielded results similar to brain in every case (data not shown). Therefore, the larger transcript size for K8 in adult heart, skeletal muscle, and skin may be unique to these tissues. Although we sequenced the entire coding region of K8, we did not find significant changes or any alternatively spliced regions that could account for the larger transcript size. We also sequenced the entire 3′ end of this transcript, including the poly(A) tail, and a short segment of the 5′ untranslated region. Neither region contained sequence that could account for the additional 0.4 kb of the larger K8 transcript. It is therefore most likely that this additional sequence is further upstream in the 5′ untranslated region.

**Immunofluorescence**—Although the Northern blot results suggest that heart and skeletal muscle contain significant levels of K8 and K19 mRNA transcripts, these proteins may be expressed in the connective tissue or blood vessels rather than within the muscle cells. We used immunofluorescence to exclude this possibility. Because there are regions of high homology between the intermediate filament proteins, we first wanted to ensure that the antibodies we used were specific for cytokeratins 8, 18, and 19. To test the specificity, we separated purified cytokeratin proteins by SDS-PAGE and transferred them to nitrocellulose membrane. These blots were probed with the isotype-specific antibodies. The results show that each antibody is specific for the appropriate cytokeratin (Fig. 3). Similar experiments confirmed that they also did not react with desmin (data not shown).

Our laboratory previously used pan-specific antibodies to the cytokeratins in immunofluorescence experiments to localize cytokeratins at costameres in adult rat skeletal muscle (33). Because the above cloning data indicated that K8 and K19 were the specific isoforms of cytokeratins in adult rat cardiac tissue, we wanted to confirm their presence at costameres in the heart. We therefore used the isotype-specific antibodies, described above, to label dissociated mouse cardiocytes by immunofluorescence. Each sample was double labeled with antibodies to αII-spectrin, which is present in cardiac muscle at

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† J. A. Ursitti, P. C. Lee, M. M. McNally, and R. J. Bloch, unpublished observations.
both the sarcolemmal and t-tubule membranes. The labeling for both cytokeratins 8 and 19 was localized at the sarcolemma and within the muscle fibers, similar to that observed in skeletal muscle with antibodies that recognize multiple isoforms of cytokeratin (33). Confocal images taken through the middle of the cells show that both K8 and K19 are located along the Z-lines throughout the cells, with occasional weak labeling at the M-lines (Fig. 4, \( \text{E and K} \), respectively). At the sarcolemmal membrane, however, confocal images taken at more superficial planes show strong labeling in regions overlying both the Z- and M-lines, with labeling at the level of the M-line visible only a short distance into the cell (Fig. 4, \( \text{B and H} \), for K8 and K19, respectively). The third domain of costameres, the longitudinal or L domains, are not as prominent in cardiac muscle as they are in skeletal muscle and are not seen clearly in these examples. Consistent with the results of our RT-PCR experiments, which failed to identify transcripts of cytokeratin 18 in adult heart, our immunofluorescence experiments also found no label for this protein in dissociated cardiocytes (data not shown). In addition, controls labeled with non-immune antibodies (see “Experimental Procedures”) showed no significant labeling of Z-lines or M-lines either within the cardiocytes or at the level of the costameres (data not shown). Dissociated rat heart cells were also labeled with control and cytokeratin-specific antibodies, with similar results (data not shown).

Immunoprecipitations—The immunofluorescent labeling shown in Fig. 4 indicated that the cytokeratins are enriched at...
the Z- and M-line domains of costameres in cardiocytes. In addition to αII-spectrin, these structures are also enriched in a number of other cytoskeletal proteins, including dystrophin and proteins of the dystrophin-associated protein complex (5).

We performed immunoprecipitation experiments with antibodies to K19 and dystrophin, as well as with a non-immune control antibody, to learn if the cytokeratins associated directly or indirectly with dystrophin or related proteins. We examined homogenates of gastrocnemius muscle from adult rats for these experiments, in an attempt to extend our observations to skeletal muscle. In the first set of experiments, we performed immunoprecipitation experiments with antibodies to dystrophin (Dys), K19, and a control monoclonal antibody (lanes 1–3, respectively). The immunoprecipitates were separated by SDS-PAGE on a 4–12% gel and transferred to nitrocellulose paper. The blots were blocked in 5% milk before immunolabeling with anti-dystrophin and anti-K19 (as indicated at the left). The results show that both anti-dystrophin and anti-K19 co-immunoprecipitate the two proteins, whereas control antibodies do not precipitate either protein. We prepared homogenates of heart using either digitonin (lanes 1 and 2) or Empigen (lanes 3 and 4) as the detergent. The dystrophin-glycoprotein complex was then partially purified on a WGA-agarose column. The WGA-agarose was either not blocked (lanes 1 and 3) or blocked (lanes 2 and 4) with N-acetylglucosamine prior to application of the homogenates. Bound samples were separated by SDS-PAGE on either 3% or 4% gels and transferred to nitrocellulose paper and blocked with milk, as above. Blots were immunolabeled with anti-dystrophin, anti-β-dystroglycan (β-DG), anti-K8, anti-K19, anti-γ-actin, or anti-connexin 43 (as indicated to the left). The results show that both anti-dystrophin and anti-K19 co-immunoprecipitate the two proteins, whereas control antibodies do not precipitate either protein. 

In additional experiments, we partially purified the dystrophin-associated glycoprotein complex from homogenates of adult rat gastrocnemius muscle and heart. A, immunoprecipitations from adult rat gastrocnemius muscle were performed using monoclonal mouse antibodies to dystrophin (Dys), K19, and a control monoclonal antibody (lanes 1–3, respectively). The immunoprecipitates were separated by SDS-PAGE on a 4–12% gel and transferred to nitrocellulose paper. The blots were blocked in 5% milk before immunolabeling with anti-dystrophin and anti-K19 (as indicated at the left). The results show that both anti-dystrophin and anti-K19 co-immunoprecipitate the two proteins, whereas control antibodies do not precipitate either protein. B, we prepared homogenates of heart using either digitonin (lanes 1 and 2) or Empigen (lanes 3 and 4) as the detergent. The dystrophin-glycoprotein complex was then partially purified on a WGA-agarose column. The WGA-agarose was either not blocked (lanes 1 and 3) or blocked (lanes 2 and 4) with N-acetylglucosamine prior to application of the homogenates. Bound samples were separated by SDS-PAGE on either 3% or 4% gels and transferred to nitrocellulose paper and blocked with milk, as above. Blots were immunolabeled with anti-dystrophin, anti-β-dystroglycan (β-DG), anti-K8, anti-K19, anti-γ-actin, or anti-connexin 43 (as indicated to the left). The control lanes (lanes 2 and 4, + GlcNAc) are virtually devoid of any protein, whereas the experimental lanes (lanes 1 and 3, − GlcNAc) show significant co-purification of dystrophin, β-dystroglycan, K8, K19, and γ-actin. Connexin 43 does not appear to co-purify with the dystrophin-glycoprotein complex with either homogenate.

In additional experiments, we partially purified the dystrophin-associated glycoprotein complex from homogenates of adult rat cardiac muscle on a WGA-agarose column and examined these samples to see if the cytokeratins co-purified with the dystrophin complex (Fig. 5B, lanes 1 and 3). The homogenates were prepared with either Empigen (Fig. 5B, lanes 1 and 3), or digitonin (Fig. 5B, lanes 2 and 4). In control samples, we inhibited binding of the dystrophin complex to the WGA-agarose with N-acetylglucosamine (Fig. 5B, lanes 2 and 4), to show the specificity of the interactions. As expected, dystrophin, β-dystroglycan, and γ-actin were concentrated in the experimental samples (13, 40) along with K8 and K19. This indicates that the Z- and M-line domains of costameres in cardiocytes. In addition to αII-spectrin, these structures are also enriched in a number of other cytoskeletal proteins, including dystrophin and proteins of the dystrophin-associated protein complex (5).

We performed immunoprecipitation experiments with antibodies to K19 and dystrophin, as well as with a non-immune control antibody, to learn if the cytokeratins associated directly or indirectly with dystrophin or related proteins. We examined homogenates of gastrocnemius muscle from adult rats for these experiments, in an attempt to extend our observations to skeletal muscle, in which we first observed cytokeratins associated with costameres (33). The immunoprecipitation studies were difficult because less than 20% of the cytokeratins is present in skeletal muscle, either directly or indirectly. The immunoprecipitation experiments show that antibodies to K19 immunoprecipitate both K19 and dystrophin (Fig. 5, lane 1), whereas antibodies to dystrophin co-immunoprecipitate dystrophin and K19 (Fig. 5, lane 2). Control antibodies did not precipitate either protein (Fig. 5, lane 3). These experiments indicate that the cytokeratins and dystrophin associate in adult skeletal muscle, either directly or indirectly.

In additional experiments, we partially purified the dystrophin-associated glycoprotein complex from homogenates of adult rat cardiac muscle on a WGA-agarose column and examined these samples to see if the cytokeratins co-purified with the dystrophin complex (Fig. 5B, lanes 1 and 3). The homogenates were prepared with either Empigen (Fig. 5B, lanes 1 and 3), or digitonin (Fig. 5B, lanes 2 and 4). In control samples, we inhibited binding of the dystrophin complex to the WGA-agarose with N-acetylglucosamine (Fig. 5B, lanes 2 and 4), to show the specificity of the interactions. As expected, dystrophin, β-dystroglycan, and γ-actin were concentrated in the experimental samples (13, 40) along with K8 and K19. This indicates...
again that the cytokeratins are bound in some way, either directly or indirectly, to dystrophin and its associated proteins. None of these proteins were present in significant amounts in the N-acetylglucosamine-blocked control samples. As a negative control, we also probed for connexin 43 in all preparations. This protein is present in the gap junctions of heart and is not believed to associate with the dystrophin complex. Despite large amounts of connexin 43 in the homogenates (not shown), this protein did not bind to the WGA-agarose column, indicating that the other proteins bound specifically.

Cellular Transfections—We used cellular transfection techniques to explore the association of the cytokeratins with dystrophin. Because previous studies of neurofilaments (44) indicated that these members of the IF family could bind to the actin-binding domain of β-fodrin (βII-spectrin), we focused our initial studies on the homologous region of dystrophin. The sequence encoding the actin-binding domain of dystrophin (Dys-ABD) was inserted into the multiple cloning site of the pEGFP vector under control of a cytomegalovirus promoter (see “Experimental Procedures”). The protein was expressed as a fusion protein with the GFP fluorescent tag and was transfected concurrently into COS-7 cells with both cytokeratins 8 and 19 using K8-MYC and PPA125. (K8 and K19 invariably co-distribute in co-transfected cells: data not shown.) The results (Fig. 6) show that the Dys-ABD associates with the network of filaments formed by K8 and K19 in the cytoplasm of COS-7 cells (Fig. 6, A–D). Immunolabeling of K8 (Fig. 6B) in the presence of the Dys-ABD (Fig. 6A) shows a complex mesh of filaments that exhibit considerable amounts of overlap (see arrows). Likewise, when the same experiment is performed and K19 immunolabeling is compared with that of the Dys-ABD, the same network of filaments is seen in both the dystrophin and K19 panels (Fig. 6, C and D, respectively). Notably, the Dys-ABD fails to label filamentous structures other than the cytokeratin filaments, suggesting that it does not associate avidly with actin filaments, at least when cytokeratin filaments are present. The specificity of its association with cytokeratins is further supported by the observation that the Dys-ABD does not co-distribute with GFP alone in co-transfection experiments (Fig. 6, E and F). Additional preliminary controls (data not shown) indicate that the association of the Dys-ABD with cytokeratins is specific, as an homologous ABD from βI-spectrin fails to do so, and the Dys-ABD fails to associate with desmin IFs.3 These results indicate that the Dys-ABD associates selectively and specifically with cytokeratins. This supports a function for the cytokeratins distinct from that of desmin at the costameres of striated muscle.

Frozen sections of tibialis anterior muscle, collected from an mdx mouse, were co-labeled with antibodies to β-spectrin and Pan-specific antibodies to cytokeratin. The image in Fig. 6H shows that in these samples the organization of β-spectrin is completely disrupted from its more regular striated pattern at the sarcolemmal membrane (published previously by Porter et al. (5), and Williams and Bloch (9)). Similarly, the normally striated pattern of cytokeratins at the sarcolemmal membrane (see Fig. 4) is partially disrupted. This suggests that both β-spectrin and the cytokeratins are linked to dystrophin at the sarcolemmal membrane and that the absence of dystrophin results in these proteins becoming disorganized. This is consistent with our conclusion that the cytokeratins associate with dystrophin in healthy muscle.

DISCUSSION

Cardiac and skeletal muscle fibers have a highly regular organization of proteins that starts with the nearly crystalline structure of the contractile apparatus and continues to the sarcolemmal membrane, with its ordered array of peripheral and integral membrane proteins concentrated at costameres. As seen in the model in Fig. 7, the organization of proteins within the sarcolemmal membrane largely mirrors the organization of the contractile apparatus within the cell. Whereas there has been much speculation about how these highly ordered structures are formed at the membrane, the specific mechanism has not been revealed. In this paper, we present evidence that cytokeratins 8 and 19, present in adult striated muscle, create heteropolymers filaments that may link the contractile apparatus to each of the distinct domains of costameres at the sarcolemmal membrane (see Fig. 7).

Over the last several years, a number of new intermediate filament proteins have been identified and studied, including syncoilin and synemin (also known as desmin). Both of these proteins have been localized to the Z-lines in striated muscle and are believed to aid in aligning the myofibers with costameres in the sarcolemmal membrane (20–22). Syncoilin (22) and synemin (21) bind to α-dystrobrevin, which forms a part of the dystrophin-associated protein complex at the sarcolemmal membrane. This interaction, coupled with further interactions of synemin with α-actinin, desmin, vimentin, and vinculin (20, 21, 23) and of syncoilin with desmin (22), solidifies the role of these proteins in linking the Z-line of the myofibrils with the costameres overlying Z-lines. However, these and other IF proteins identified in striated muscle have only been localized to the Z-lines. Most of the peripheral and integral membrane proteins at the Z-line domains of costameres are also present at the M-line and L domains, yet the structures that align these domains with the underlying myofibrils remain to be identified. Furthermore, our studies of the desmin null mouse indicate that desmin is not required for the formation or maintenance of intact costameres in many myofibers (33). Our immunofluorescence data strongly suggest that K8 and K19 are present in these structures and, consistent with our earlier results (33), can account for the presence and stability of costameres when desmin is absent.

Northern blot analysis showed that K8 had a unique transcript size in adult rat cardiac and skeletal muscle, as well as in skin (Fig. 2). This transcript was 0.4 kb larger than the typical transcript size found for every other tissue tested. Although the origin of the difference was not identified, we believe it is most likely in the 5′ untranslated region of the K8 gene, possibly related to its regulation and expression. The amino acid sequence of this protein is identical to the recently published sequence prediction for rat K8 (GenBank™ acces-
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The present studies are consistent with the hypothesis that K8 and K19 are responsible for linking the myofibrils in striated muscle to the sarcolemmal membrane at both the Z- and M-line domains (and, in rodent skeletal muscle, at L domains). It seems likely that dystrophin is at least one of the proteins with which the cytokeratins interact at the sarcolemmal membrane. The fact that the cytokeratins also associate with myofilament structures at the levels of the Z- and M-lines further suggests that they may also interact with sarcomeric proteins, but the nature of their role in the contractile apparatus remains to be determined. These studies are currently in progress.

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Cloning and Characterization of Cytokeratins 8 and 19 in Adult Rat Striated Muscle: INTERACTION WITH THE DYSTROPHIN GLYCOPROTEIN COMPLEX
Jeanine A. Ursitti, Pervis C. Lee, Wendy G. Resneck, Minda M. McNally, Amber L. Bowman, Andrea O'Neill, Michele R. Stone and Robert J. Bloch

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