Skelemins: Cytoskeletal Proteins Located at the Periphery of M-Discs in Mammalian Striated Muscle

Maureen G. Price

Research Institute of Scripps Clinic, Department of Molecular Biology, La Jolla, California 92037

Abstract. The cytoskeletons of mammalian striated and smooth muscles contain a pair of high molecular weight (HMW) polypeptides of 220,000 and 200,000 mol wt, each with isoelectric points of about 5 (Price, M. G., 1984, Am. J. Physiol., 246:H566–572) in a molar ratio of 1:1:20 with desmin. The HMW polypeptides of mammalian muscle have been named "skelemins," because they are in the insoluble cytoskeletons of striated muscle and are at the M-discs. I have used two-dimensional peptide mapping to show that the two skelemin polypeptides are closely related to each other. Polyclonal antibodies directed against skelemins were used to demonstrate that they are immunologically distinct from talin, fodrin, myosin heavy chain, synemin, microtubule-associated proteins, and numerous other proteins of similar molecular weight, and are not oligomers of other muscle proteins. Skelemins appear not to be proteolytic products of larger proteins, as shown by immunoautoradiography on 3% polyacrylamide gels. Skelemins are predominately cytoskeletal, with little extractable from myofibrils by various salt solutions. Human, bovine, and rat cardiac, skeletal, and smooth muscles, but not chicken muscles, contain proteins cross-reacting with anti-skelemin antibodies. Skelemins are localized by immunofluorescence at the M-lines of cardiac and skeletal muscle, in 0.4-µm-wide smooth striations. Cross sections reveal that skelemins are located at the periphery of the M-discs. Skelemins are seen in threads linking isolated myofibrils at the M-discs. There is sufficient skelemin in striated muscle to wrap around the M-disc about three times, if the skelemin molecules are laid end to end, assuming a length-to-weight ratio similar to M-line protein and other elongated proteins. The results indicate that skelemins form linked rings around the periphery of the myofibrillar M-discs. These cytoskeletal rings may play a role in the maintenance of the structural integrity of striated muscle throughout cycles of contraction and relaxation.

Muscle cells contain a cytoskeleton of desmin-containing intermediate filaments that link organelles (reviews, Lazarides, 1980; Price and Sanger, 1983). In striated muscle cells, longitudinal intermediate filaments course parallel to myofibrils, linking Z-discs of individual myofibrils (Price and Sanger, 1979, 1984; Tokuyasu, 1983; Thornell et al., 1985). The most prominent features of the intermediate filament cytoskeleton are bundles of transverse filaments encircling the Z-discs and joining adjacent myofibrils as well as linking the myofibrils to the sarcotela and the nuclear membrane (Price and Sanger, 1983 for review; Chiesi et al., 1981; Pierobon-Bormioli, 1981; Street, 1983; Tokuyasu et al., 1983a, 1983b; Wang and Ramirez-Mitchell, 1983). The intermediate filaments are strongly associated with the Z-discs, so that myofibrils remain linked together at the levels of the Z-discs by parallel and transverse arrays after extensive extraction of the contractile filaments (Granger and Lazarides, 1978; Price and Sanger, 1979, 1983; Wang and Ramirez-Mitchell, 1983). In addition, ultrastructural studies of striated muscle have occasionally demonstrated a cytoskeleton at the level of the M-line or M-disc, in the form of transverse filaments linking neighboring myofibrils together or connecting myofibrils with the sarcotela (Pierobon-Bormioli, 1981; Street, 1983; Wang and Ramirez-Mitchell, 1983; Thornell et al., 1985).

Several proteins that may contribute to anchoring the desmin filaments of the myofibrillar cytoskeleton have been found at the Z-discs of striated muscles, including synemin, paranemin, and zeugmatin in avian muscles (Granger and Lazarides, 1980; Breckler and Lazarides, 1982; Maher et al., 1985), and plectin and 210,000–220,000-mol-wt proteins in vertebrate muscles (Lin, 1981; Muguruma et al., 1981; Wiche et al., 1983). The avian proteins synemin and paranemin are distributed in filamentous arrays in some nonstriated muscle cells, and therefore seem to be specifically intermediate filament-associated proteins of chicken skeletal and cardiac muscle, respectively (Price and Lazarides, 1983). Their mammalian counterparts are unknown. Immunohistochemical studies (Nelson and Lazarides, 1983, 1984) and in vitro binding experiments (Georgatos and Marchesi, 1985; Mangeat and Burridge, 1984; Langley and Cohen, 1986) suggest that ankryin and spectrin may play a role in the association of intermediate filaments with membranes.
In a previous study I found two high molecular weight (HMW) proteins, of 220,000 and 200,000 mol wt, that are enriched in cytoskeletons of mammalian muscle (Price, 1984). The HMW polypeptides are co-enriched with desmin and are copurified with desmin and vimentin through sequential steps of column chromatography. Intermediate filaments reconstituted in the presence of the HMW polypeptides have diameters 30% greater than filaments reconstituted from pure desmin, strongly suggesting that the HMW polypeptides can associate with the cytoskeletal filaments. The HMW polypeptides are generally expressed in mature bovine muscle, being present in smooth muscle and skeletal muscle in addition to myocardium. To compare the two HMW polypeptides with other cytoskeletal proteins and with each other, I have performed two-dimensional peptide mapping studies. Specific polyclonal antibodies were prepared for use in further comparison of the HMW polypeptides with other cytoskeletal proteins, and for characterizing the extraction properties, cell-type and species specificity, and subcellular localization of the HMW polypeptides in striated muscle. I find that the HMW polypeptides are two closely related proteins that are immunologically distinguishable from numerous other cytoskeletal proteins. The HMW polypeptides are found almost exclusively in the cytoskeletal fraction of muscle. They are expressed in several mammalian species, including human, but not in chicken. Biochemical and immunocytochemical studies indicate that the HMW polypeptides are not the mammalian analogue of the avian intermediate filament-associated protein, synemin. In both skeletal and cardiac muscle, the HMW polypeptides are localized in rings at the periphery of the M-lines. This distribution suggests that the HMW polypeptides may be anchorage proteins for the longitudinal and transverse intermediate filaments of the mammalian muscle cytoskeleton. The HMW polypeptides are designated "skelemins," because they are cytoskeletal polypeptides present at the level of the M-disc in striated muscle.

**Materials and Methods**

**Preparation of Cytoskeletons and Tissue Samples**

Bovine myocardium, skeletal (masseter) muscle, and smooth muscle from large intestine were obtained from a local slaughterhouse and kept on ice for 1-2 h. Cytoskeletal residues of each muscle sample were prepared as previously described (Price, 1984) except that 0.1% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO) was substituted for Triton X-100, and the deproteinization was performed by Deutone homogenization and boiling for 5 min in 0.1% SDS, 25 mM Tris-HCl, pH 7.5, to reduce subsequent proteolysis. After the samples cooled, ultrapure urea (Schwartz-Mann, Inc., Spring Valley, NY) was added to 9.5 M, 2-mercaptoethanol to 5%, and Nonidet P-40 to 2% final concentration. Two-dimensional polyacrylamide gel electrophoresis (O’Farrell, 1975) was performed as described (Price, 1984). Isoelectric focusing gels were 0.2 cm diam by 1.2 cm in length, containing 2% ampholines 5.7-9.6% ampholines 4.6-6.7% ampholines 3-10 (Bio-Rad Laboratories, Richmond, CA). SDS resolving gels contained 11% polyacrylamide and 0.11% N,N'-methylene-bisacrylamide.

**Two-Dimensional Peptide Mapping**

Peptide mapping was performed as previously described (Price and Lazarides, 1983) according to established methods (Elder et al., 1977) modified for HMW proteins (Gomer and Lazarides, 1983). Proteins, and blank pieces of polyacrylamide gel serving as controls, were excised from two-dimensional gels of muscle cytoskeletal residues. Approximately 0.25-0.50 μg of radiolabeled protein was radiolabeled with 100 μCi of 125I by the chloramine-T method (Greenwood et al., 1963). Excess iodine was removed by dialysis against 1 M NaCl and the control blank gel was treated for 24-30 h at 37°C with α-chymotrypsin or trypsin-nas-α-toy-l-phenylalanine chloromethyl ketone (Worthington Diagnostics, Freehold, NJ) at 0.05 mg/ml. Peptide aliquots containing 350,000 dpm were used for each map. Peptides were spotted onto 20 × 20 cm cellulose chromatograms (Eastman Kodak Co., Rochester, NY) and separated in the first dimension by high-voltage electrophoresis (800 V) in an acidic solution. The second dimension of separation was by thin-layer chromatography in butanol/pyridine/water/acetic acid (5.3:3.3:3:1). For direct comparison of two proteins' peptide maps, the three chromatograms containing the two individual digests and a mixture of the two digests were treated simultaneously in the 70 × 28 cm apparatus. A chromatogram containing an individual protein digest and the mixture of digests were then placed in the same tank for thin-layer chromatography. Maps were exposed to Kodak XAR-5 film, with Chronex Lightening-Plus intensifying screens (Dupont Co., Wilmington, DE), at -70°C for 1-3 d.

**Antibody Production**

The 220,000- and 200,000-mol wt skelemins were partially purified from bovine ventricular myocardium according to previous methods (Price, 1984) based on those of O'Shea et al. (1981). The yield was ~54 μg of skelemins/g of wet weight of myofibrils. The hydrolyxlapatite fractions eluted between 20 and 55 mM potassium phosphate were solubilized by boiling in SDS sample buffer and electrophoresed on 11% polyacrylamide slab gels. The gels were briefly stained with Coomassie Blue and destained. The two bands of the appropriate molecular weights were excised with a razor blade and neutralized in water. Proteins were electroeluted and concentrated according to the method of Hunkapiller et al. (1983), using ammonium bicarbonate-SDS buffer, and were lyophilized.

Lyophilized proteins were solubilized in sterile water and emulsified with equal volumes of Freund's complete (for the first injection) or incomplete (for the second injection) adjuvant, and injected according to a modification of the methods of Goudie et al. (1966). Two adult female New Zealand White rabbits were anesthetized with Diobutal. 20 μg of protein was used for the first injection of each rabbit, with approximately 3 μg directly injected into each of two popliteal lymph nodes (per rabbit) and the remaining 14 μg injected intradermally. 1 mo later, 100 μg of protein was injected subcutaneously on the back and intramuscularly in the hindquarters. Blood was drawn from the medial ear artery 1 wk later, and at 1-3-wk intervals for several months. Both rabbits produced high-titer, specific antibodies within 1 wk of the second injection and were not boosted again.

The IgG fractions of the preimmune and immune sera were prepared by 50% ammonium sulfate precipitation (Garvey et al., 1977), and for some experiments, by subsequent DEAE-affinity chromatography using DEAE-Sepharose (Ibiod). For double-label immunofluorescence, anti-skelemim antibodies were coupled to biotin-33,33'-diaminobenzidine ester (Bethesda Research Laboratories, Gaithersburg, MD) following the manufacturer's instructions.

**Polyacrylamide Gel Electrophoresis**

One-dimensional electrophoresis was performed according to modified methods of Laemmli (1970), as described (Price, 1984), except that 11% rather than 12.5% polyacrylamide was used for all but the 3% polyacrylamide gel of Fig. 2. Densitometry was performed by scanning at 570 nm (Helena Laboratories, Beaumont, TX). Samples for two-dimensional electrophoresis were prepared by Deutone homogenization and boiling for 3 min in 12.5% SDS, 25 mM Tris-HCl, pH 7.5, to reduce subsequent proteolysis.

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1. *Abbreviation used in this paper: HMW, high molecular weight.*
Immunohistochemistry

Immunohistochemistry was performed by a variation of standard techniques (Towbin et al., 1979). Gels were transferred to nitrocellulose in Laemmli running buffer with 20% methanol, at 80 V for 14 h, with water-cooling. All subsequent procedures were at room temperature. Blots were briefly washed in PBS with 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma Chemical Co.), and incubated with 1:20,000 dilutions of ammonium sulfate-pretreated or anti-skelemin sera in PBS-Tween for 1 h. After three 5-min washes in PBS-Tween, the blots were incubated with 5 x 10^6 dpm ^125I-Protein A/ml for 1 h and washed as above. Protein A (Sigma Chemical Co.) was radiolabeled by the chloramine-T method (Greenwood et al., 1963) and used within 2 wk. Blots were exposed to Kodak XR-5 film with Cronex Lightning-Plus intensifying screens at -70°C for 4-100 h. Fodrin (see Fig. 3) from bovine brain and anti-fodrin antibodies were generous gifts of Dr. John Glenney (Glenney and Glenney, 1984). Talin (see Fig. 3) was donated by Dr. Keith Burridge (Burridge and Connell, 1983). Blots were stained with 0.1% India ink in PBS-Tween (Hancock and Tsang, 1983).

Immunofluorescence

Thin pieces of skeletal muscle or myocardium were stretched between forces and plunged directly in liquid nitrogen. On a block of dry ice, small pieces of the stretched muscle tissues were chopped off and immediately embedded in OCT medium (Tissue-Tek, Miles Laboratories, Inc., Irvine, CA) and then immersed in liquid nitrogen. Only the very surface of the tissue thawed when the procedure is used. Frozen sections of between 6 and 12 μm were cut, picked up on uncoated coverslips, and fixed at room temperature in either 95% ethanol for 10 min or in freshly made 3% paraformaldehyde in PBS, pH 7.4, for 20 min. Myofibrils were allowed to adhere to uncoated coverslips for 30 min at room temperature and were used both unfixed or fixed as were the tissue sections. Coverslips were briefly rinsed in PBS with 0.1% Nonidet P-40 and incubated with anti-skelemin antibodies diluted 40-200 times from the serum concentration. Fluorescein-labeled goat anti-rabbit IgG (Miles/ICN Immunobiologicals, Irvine, CA) was used at 200 times dilution for the second antibody. Commercial polyclonal anti-actin and anti-α-actinin (Miles/ICN Immunobiologicals), and polyclonal anti-filamin and anti-myosin (gifts of Dr. Richard Gomer and Dr. S. John Singer [University of California, San Diego], respectively), as well as polyclonal antibodies against chicken myocardin desmin (Price, M. G., unpublished results) were used at 10-40 times dilutions. In double-label immunofluorescence experiments, the biotinylated anti-skelemin antibodies were detected with (4 μg/ml) streptavidin-Texas Red (Bethesda Research Laboratories). Coverslips were mounted in 90% glycerol in PBS, pH 8, containing 1 mg of phenylenediamine/ml to reduce the quenching of fluorescein (Johnson and Araujo, 1981). Samples were observed with a Microphot FX phase-contrast/epifluorescence microscope (Nikon, Inc., Garden City, NY). Photographs taken on Kodak Tri-X film at ASA 1600 were developed in Diafine (Acufine Corp., Chicago, IL). Measurements from negatives were made by projecting 20x enlarged images with a Nikon VN-2 projector equipped with a CM 65 digital counter.

Results

Skelemins Are Related to Each Other but Not to Vimentin and Desmin

The cytoskeletal residue of bovine myocardium contains the intermediate filament proteins desmin and vimentin, and two HMW polypeptides with apparent molecular weights of 220,000 and 200,000 and isoelectric points between pH 5.0 and 5.1. The HMW polypeptides will be called "skelemins" herein because this study showed that they are cytoskeletal proteins located at the M-line. Skelemins are eluted together with vimentin and a subpopulation of desmin, when urea-solubilized cytoskeletal proteins are fractionated on hydroxylapatite (Fig. 1 A; see Price, 1984). The skelemins of bovine myocardium comigrate with skelemins of other bovine muscle types (Fig. 1 B). The molar ratios of desmin/220,000/200,000-mol-wt skelemins are about 20:1:1 in the cytoskeletal electrophoresis (HVE) and thin-layer chromatography (TLC) are indicated. (B) Coomassie-stained two-dimensional gel of a mixture of proteins from cytoskeletal residues of bovine myocardium and visceral smooth muscle to demonstrate the comigration of the HMW polypeptides from the two muscle types. Identical results are obtained with skeletal muscle. The α- (skeletal) and γ- (smooth muscle) actin isoforms are seen. (C) Two-dimensional map of ^125I-labeled chymotryptic peptides from desmin from bovine muscle. Several peptides unique to desmin, not seen in maps of the other cytoskeletal proteins, are indicated (arrowheads). The directions of high-voltage electrophoresis (HVE) and thin-layer chromatography (TLC) are indicated. (D) Chymotryptic peptide map of vimentin from bovine muscle. Some characteristic peptides, absent in maps of desmin and skelemins are indicated (arrowheads). (E) Chymotryptic map of the 200,000-mol wt (200 K) skelemin from myocardial cytoskeleton. A minor peptide that is absent in the 220,000-mol-wt HMW polypeptide is indicated (double arrowhead). (F) Map of a mixture of chymotryptic peptides from the 200,000- and 220,000-mol-wt myocardial skelemins. The major background spot from labeled polyacrylamide is marked (*); see Fig. 4. (G) Chymotryptic map of the 220,000-mol-wt (200 K) skelemin from bovine myocardial cytoskeleton, with a peptide absent in the smaller HMW polypeptide (double arrowhead). A few peptides that comigrate with peptides from the smaller skelemin but vary in intensity of labeling are indicated with single arrowheads.
Figure 2. Immunoautoradiography to demonstrate specificity of skelemin antibodies. (A) India ink-stained nitrocellulose blot of an 11% polyacrylamide gel of whole bovine myocardium. Myosin (m) and actin (a) are indicated. (B) Corresponding immunoautoradiogram of bovine myocardium stained with preimmune (PI) antibodies. (C) Immune (IM) or anti-skelemin antibody staining of myocardial proteins, showing two bands (arrowheads) at 220,000 and 200,000 mol wt. (D) Anti-skelemin antibody staining of blot of bovine myocardial proteins separated on a 3% polyacrylamide gel, with two major bands labeled (arrowheads). The left edge of this lane is the cut edge of a bisected gel; aggregates of 125I-Protein A stick nonspecifically to the edge, causing a large blot below the D and other small dots. (E) Immunoautoradiogram of a two-dimensional gel of bovine myocardium, labeled with anti-skelemin antibodies. The two spots have isoelectric points near pH 5 and migrate with apparent chain weights of 220,000 and 200,000 mol wt. (F) Preimmune staining of a gel identical to that in the previous panel. No proteins are labeled.

Specificity of Anti-Skelemin Antibodies

Because the 220,000- and 200,000-mol-wt skelemins are highly homologous by two-dimensional peptide mapping, they were combined for use as antigens to generate polyclonal antibodies in rabbits. The anti-skelemin antibodies were characterized by immunoautoradiography on blots of one- and two-dimensional polyacrylamide gels. Preimmune serum is unreactive with proteins of whole bovine myocardium (Fig. 2, A and B) or with many other tissue samples (see Fig. 6 B, data not shown). The anti-skelemin antibodies are specific for the 220,000- and 200,000-HMW polypeptides, as indicated by their reactivity with only these two bands in a sample of whole myocardium (Fig. 2 C). The 200,000-mol-wt band is enlarged and distorted, most likely due to its comigration with the heavy chain of myosin. This conclusion is supported by the lack of reactivity of the anti-skelemin antibodies with partially purified myosin (Fig. 3, lane 2). The immunoblot was overexposed to demonstrate that the antibodies detect a few very minor bands of about 20,000 mol wt, seen below the actin band (a in Fig. 2 A). These minor bands could result from proteolysis of the larger skelemin polypeptide to 200,000 mol wt, consistent with the observed relative increase of the smaller skelemin in samples repeatedly frozen and thawed. This proteolysis would also contribute to the disparity in amounts of the two skelemins detected in the immunoblot (Fig. 2 C). Apparent proteolysis of the larger skelemin seems more pronounced in SDS-sample buffer than in the urea-Nonidet P-40 solution used for isoelectric focusing.

To determine that skelemins are distinct entities rather than proteolytic products of larger polypeptides, immunoautoradiography of proteins separated on a 3% polyacrylamide gel was performed. As shown in Fig. 2 D, the anti-skelemin
antibodies label two major bands corresponding to 220,000 and 200,000 mol wt, and do not bind to the upper third of the gel that would contain cytoskeletal polypeptides of up to $1 \times 10^6$ molecular weight, such as titin (Wang et al., 1979) and zeugmatin (Maher et al., 1985).

In blots of two-dimensional gels of whole myocardium, the skelemin antibodies react only with polypeptides of 220,000 and 200,000 mol wt, having isoelectric points near pH 5 (Fig. 2 E). The smaller anti-skelemin-labeled polypeptide is slightly more acidic than the larger one, consistent with the isoelectric separation of skelemin seen in Coomassie-stained two-dimensional gels of muscle cytoskeletons (Fig. 1, A and B and Fig. 4 B). The anti-skelemin antibodies do not react with other proteins. No labeling is obtained with the preimmune antibodies (Fig. 2 F), even in blots exposed for five times longer than immune blots.

**Skelemins Are Unrelated to Other HMW Polypeptides**

To determine whether the skelemins are immunologically related to known cytoskeletal proteins of similar molecular weight, including synemin, the avian muscle desmin-associated protein (Granger and Lazarides, 1980; Sandoval et al., 1983), tissues, extracts, and purified proteins were screened on blots with the anti-skelemin antibodies. Tissues were also screened by immunofluorescence of frozen sections. Fig. 3 shows that the anti-skelemin antibodies react only with the 220,000- and 200,000-mol-wt bands of skelemins, and fail to react with chicken gizzard (giz, lane 1), which contains appreciable amounts of the 230,000-mol-wt intermediate filament-associated protein, synemin (Granger and Lazarides, 1980). The anti-skelemin antibodies are unreactive with myosin heavy chain (myo, lane 2), present in a myosin-enriched extract of bovine myocardium. Skelemin antibodies also fail to label the chicken myosin in the gizzard sample or rabbit myosin in a commercially available mixture of molecular weight standards (st). The skelemin antibodies fail to recognize purified mammalian (bovine) fodrin (fod, lanes 3 and 4), a prominent HMW (235,000) component of the membrane cytoskeleton of most cells including striated muscle (Glenney and Glenney, 1984; Nelson and Lazarides, 1983), or its major proteolytic products. Fodrin antibodies do not react with skelemins in immunoautoradiography (data not shown). Purified chicken talin (tal, lanes 5 and 6) has a molecular weight (215,000) close to those of the skelemins (Burridge and Connell, 1983). Neither talin nor its 190,000-mol-wt breakdown product are recognized by the anti-skelemin antibodies. Mammalian brain samples were stained to test whether the anti-skelemin antibodies cross-react with neuronal or glial components, especially the 180,000-200,000-mol-wt neurofilament-associated protein (Willard and Simon, 1981) and the microtubule-associated proteins, MAP-1 and MAP-2; no reactivity was seen with any component of a mixture of gray and white matter from rat cerebral cortex and cerebellum (bra, lane 8) or bovine cortex (data not shown). In summary, the anti-skelemin antibodies are specific for the mammalian muscle cytoskeletal proteins that were the antigens, and do not react with myosin or with a variety of other known cytoskeletal proteins.

**Skelemins Are Not Related to Synemin**

Two-dimensional polyacrylamide gel electrophoresis and chymotryptic peptide mapping were used to further compare the skelemins with synemin, because they are cytoskeletal proteins of similar molecular weight and extraction properties and are both associated with the cytoskeletons of mammalian and avian muscle, respectively. Synemin from chicken gizzard cytoskeletons is focused in two to four elongated

Figure 3. Specificity of anti-skelemin antibodies. (A) India ink-stained blot of tissues and extracts. The end lanes contain protein standards (st); the molecular weights of which are indicated on the left. Lane 1 contains chicken gizzard proteins (giz). Lane 2 contains a crude preparation of bovine cardiac myosin (myo). Lane 3 contains bovine brain fodrin (fod). Lane 4 (+skel) contains a mixture of fodrin and a preparation of bovine myocardial cytoskeletal proteins, including skelemins and desmin, the major band migrating as 55,000 mol wt. Lane 5 contains talin (tal) from chicken gizzard. Lane 6 contains a mixture of talin and the skelemin-enriched preparation. Lane 7 contains only the skelemin-enriched preparation of bovine myocardial cytoskeletal proteins (skel). Lane 8 contains brain (bra) proteins from the grey and white matter of rat brain cerebral cortex. (B) Corresponding immunoautoradiogram of the blot, stained with anti-skelemin antibodies.
Table 1. Cross-reactivity of the Skelemin Antibodies Directed against Bovine Myocardial Skelemins with Bovine Muscles and Muscles from Other Species

| Muscle                               | Reactivity |
|--------------------------------------|------------|
| Bovine myocardium                    | ++         |
| Bovine skeletal muscle               | ++         |
| Bovine visceral smooth muscle        | ++         |
| Human myocardium                     | ++         |
| Human skeletal muscle                | ++         |
| Human visceral smooth muscle         | ++         |
| Rat myocardium                       | +          |
| Rat skeletal muscle                  | ++         |
| Rat visceral smooth muscle           | +          |
| Chicken myocardium                   | –          |
| Chicken skeletal muscle              | –          |
| Chicken visceral smooth muscle       | –          |

Data were compiled from results of immunoautoradiography on blots and immunofluorescence on frozen sections. The approximate relative intensity of reactivity is indicated. See text for discussion.

Figure 4. Comparison of avian muscle synemin and bovine skelemins. (A) Coomassie-stained two-dimensional polyacrylamide gel of cytoskeletal proteins of chicken gizzard, including synemin variants (Syn, arrowheads), vimentin (V), and desmin isozymes (D). The vertical line separates two gizzard proteins of 48,000 mol wt, passing through approximately pH 5.25. Notice that the most acidic synemin is on the basic side of this reference line. Basic and acidic ends and several isoelectric points are indicated. (B) Coomassie-stained two-dimensional gel of a mixture of cytoskeletal proteins from chicken gizzard and bovine intestinal smooth muscle. The vertical line demarcates pH 5.25, as before. The most acidic synemin variant streaks into the 220,000-mol-wt skelemin. Bovine desmin (d) and vimentin (v), and chicken desmin (D) are indicated. (C) Chymotryptic map of skelemins, several peptides absent in synemin maps are indicated (arrowheads). A background spot is marked (*). (D) Map of mixture of skelemins and synemin chymotryptic peptides, including the major background spot (*). (E) Chymotryptic map of synemin, with several peptides absent in the skelemin maps indicated (arrowheads). (F) Background spots attributable to the blank polyacrylamide. The darkest spot (*) is also seen in some peptide maps of skelemins, such as Fig. 1 F.

spots (Fig. 4 A), at approximately pH 5.35, which is quite basic compared with skelemins focused at pH 5–5.1 (Fig. 4 B). Synemin, with a molecular weight of 230,000, migrates 3–4 % less distance into the SDS gel than does the 220,000-mol-wt skelemin. In heavily loaded gels such as that in Fig. 4 B, a minority of the total synemin, the most acidic synemin variant, streaks and partially merges with the skelemins.

Comparison of the chymotryptic peptide maps of the 220,000-mol wt skelemin HMW polypeptide (Fig. 4 C) and the pl 5.35 synemin (Fig. 4 E) clearly demonstrates that they are different proteins. Subtracting spots arising from blank polyacrylamide (Fig. 4 F), there are 37 skelemin and 33 synemin chymotryptic peptides. Only 11 peptides comigrate (Fig. 4 D), corresponding to 30% of the skelemin peptides and 33% of the synemin peptides.

Skelemins Are Found in Mammalian but Not Avian Muscles

Immunoautoradiography and immunofluorescence on samples of chicken, bovine, human, and rat muscle types were performed to determine whether they express proteins cross-reacting with the bovine skelemins. Table I summarizes these results, showing that anti–skelemin antibodies react strongly with bovine heart, skeletal, and smooth muscle. Proteins cross-reacting with the anti–skelemin antibodies are present in human heart, skeletal, and smooth muscle. The anti–skelemin antibodies react less strongly with rat than with human proteins. The greatest reactivity is with rat skeletal muscle, and less is found with rat heart and smooth muscle.

Skelemins Are Cytoskeletal Proteins

Immunoautoradiography of a variety of serial extracts and residues of bovine myocardium was performed in order to examine the extraction properties of skelemins. As shown in Fig. 5, skelemins are detectable in whole bovine myocardium. Sequential extraction of ventricular myocardium was performed as described previously (Price, 1984) except that Nonidet P-40 was substituted for Triton X-100 in extraction buffer 2. The Coomassie Blue-stained profile of these extracts and subsequent residues is shown in Fig. 1 in Price (1984). Skelemins are not extracted from homogenized myocardium by either the first physiological salt solution (Fig. 5, e-1) or by a similar salt solution containing detergent (e-2). Both extractions were repeated twice. Skelemins were not extracted from the membrane-depleted myofibrils by a subsequent low-salt solution (e-3) that primarily solubilizes actin. As the myofibrils ghosts further disintegrate in the next extraction solution, a very low-salt buffer that mainly further extracts actin and many actin-associated proteins, <10% of the total skelemins are released (e-4). This lane was heavily overloaded to demonstrate the presence of skelemins; with normal loads such as that shown previously (Price, 1984),
skelemins are barely detectable. No skelemins are extracted with the myosin and additional actin solubilized by 0.5 M KCl buffer (KCl-e), and a extremely small amount is extracted by subsequent treatment with a 0.6 M KI buffer that extensively extracts not only myosin and actin but also the α-actinin of the Z-discs (KI-e). Washing the cytoskeletal residue in physiological saline (wash) to restore normal osmolarity does not extract skelemins. In the course of the serial extractions of myocardial tissue, the skelemins are therefore fractionated almost exclusively into the insoluble residues (r-1 to KI-r) and are thereby enriched in the final cytoskeletal residue that resists extraction by 0.6 M KI buffer, as can be seen by comparing the density of the anti-skelemin-labeled bands in the whole heart sample (which was exposed three times longer) with a similar protein load of the final KI residue. The small disparity in apparent mobility of the skelemins bands is probably due to the vast differences in protein content of the various samples and the distorting effects of large amounts of myosin heavy chain in some. Densitometry of the cytoskeletal residue of bovine myocardium show that there are 54 μg skelemins/g of original weight of myofibrils. Because immunooautoradiography shows that very little skelein is lost in the preparation of the cytoskeletal residue, this is roughly the correct value for the amount of skelein in myofibrils.

Skelemins Form a Ring around the M-Disc

Immunofluorescence on frozen sections of various mammalian muscles, including bovine masseter muscle, and rat and human thigh muscle, was done to localize skelemins. The preimmune antibodies do not significantly stain muscle cells or fibroblasts (Fig. 6, A and B) or any other cells (data not shown), although some faint fluorescence of the dense structures of Z-discs and intercalated discs is sometimes noticed (Fig. 6 G). Phase-contrast microscopy of well-stretched bovine skeletal muscle clearly demonstrates the alternating pattern of phase-dark, myosin-containing A-bands and phase-light actin-containing I-bands that are bisected by very phase-dark Z-discs (Fig. 6 C). An M-disc is in the middle of each phase-dark A-band (M, Fig. 6 C); M-discs are not well differentiated by phase-contrast microscopy of unstained sections.

The anti-skelemin antibodies stain the M-discs, giving a regular striated pattern of 0.4-μm-thick stripes that are spaced about 2.5 μm, or one sarcomere length, apart. The stained bands can be traced laterally from one myofibril to another throughout the body of a muscle fiber. There is no staining of the connective tissue cells between neighboring muscle fibers, or of the muscle nuclei. Whereas the anti-skelemin-stained bands are relatively smooth rather than punctate, the intensity of staining varies along the fiber circumference, suggesting that the depth or concentration of the skelemin-containing regions varies through a muscle section. When only anti-skelemin antibodies are used, their M-disc staining is confirmed by combining phase-contrast and epifluorescence microscopy, i.e., by illuminating the sample simultaneously with transmitted white light and ultraviolet fluorescence-excitation light. A resultant image is seen in Fig. 6 E, clearly showing that labeling with anti-skelemin antibodies causes distinct bright lines coursing over each M-disc. These lines are represented as white in black-and-white photographs. Comparing this figure with the phase-contrast image of Fig. 6 C, it is obvious that the relative density of the Z-discs is unchanged while the M-discs are dramatically lighter.

The M-disc localization of skelemins is also observed in mammalian myocardium, as demonstrated by the stained bands within ventricular myofibrils being offset a half-sarcomere from the intercalated discs (Fig. 6, F and G). The intercalated discs themselves are very faintly stained by anti-skelemin antibodies, as are the cardiac Z-discs, but this staining is comparable to the nonimmune staining and does not represent significant levels of skelemins.

In cross sections of muscle, the anti-skelemin antibodies stain narrow ovals or rings (Fig. 6, H and J). This pattern of ringlike staining peripheral to myofibrils is consistent with the varying intensity of staining observed along the myofiber circumference in longitudinal sections. The narrow cuffs of fluorescence would appear more or less intense depending on the plane of section and plane of focus.

To demonstrate more convincingly the M-disc distribution of skelemins, immunofluorescence with anti-skelemin antibodies was performed in conjunction with antibodies specific for other myofibrillar elements. Fig. 7, A and B shows that when sections of mammalian muscle are labeled with both anti-skelemin and anti-actin antibodies, both the M-lines and the actin filaments of the I-bands are stained, yielding a striking pattern of alternating thin and thick fluorescent bands. Anti-actin antibodies alone stain only actin filaments, as seen in Fig. 7 C. A combination of anti-skelemin antibodies and anti-filamin antibodies results in fluorescent striations that are spaced a half-sarcomere apart, corresponding to alternating M-discs and Z-discs (Fig. 7 D). Anti-filamin antibodies stain only the Z-discs of fast skeletal muscle (Gomer and Lazarides, 1981). Identical results were obtained by double-label immunofluorescence with biotinylated anti-skelemin antibodies and indirectly la-
Figure 6. Localization of skel-eminins in striated muscle, as shown by immunofluorescence staining of frozen sections. (A) Phase-contrast micrograph of a longitudinal section of bovine skeletal muscle. (B) Pre-immune staining in the corresponding fluorescence image of the section shown in A demonstrates the lack of staining: a few dots along the sarcolemma of one fiber are present in the lower left. (C) Phase-contrast micrograph of a longitudinal section of bovine skeletal muscle stained with anti-skel-emin antibodies, showing the phase-light I-bands, bisected by black Z-discs (Z), and the phase-dark A-bands, which are bisected by M-discs (M). M-discs sometimes appear slightly lighter than the rest of the A-bands in an unstained preparation such as this. (D) Corresponding fluorescence image obtained with anti-skel-emin antibodies, demonstrating the skel-eminins in regular narrow striations passing from myofibril to myofibril across the muscle fibers. (E) The same area, viewed by fluorescence microscopy combined with transmitted white light, showing that the stained regions are the M-discs. There are white (fluorescent) lines passing through the middle of each dark A-band, which are not prominent in the phase-contrast image. The relative density of the Z-discs is unchanged from that in the phase-contrast image. (F) Phase-contrast micrograph of a longitudinal section of bovine ventricular myocardium, with several myofibers joined by phase-dark intercalated discs (id). Arrows indicate M-discs on either side of an intercalated disc (arrowhead). (G) Corresponding fluorescence image, showing skel-eminins localized in the M-discs and offset by a half-sarcomere from the intercalated disc. (H, I) Phase-contrast micrograph of a cross section of bovine skeletal muscle. (I) Corresponding immunofluorescence image, showing anti-skel-emin staining primarily in narrow, closely packed rings. Bars in A and F, 5 μm; bar in H, 10 μm.

beled anti-actin, anti-filamin, and anti-desmin antibodies (data not shown). Further substantiation of the M-disc localization comes from double-label immunofluorescence with anti-myosin antibodies (data not shown).

Several interesting details are seen with isolated myofibrils stained with anti-skel-emin antibodies. The myofibrils do not lie exactly flat on the coverslips, and because skel-eminins are present in ringlike structures around the myofibrils (Fig. 6, H and I), the stained areas may appear to be straight lines or, in tilted regions, irregular discs (Fig. 7 E). Pairs of myofibrils apparently linked by skel-emin-containing strands are often observed (Fig. 7 E).
Skelemins Are Related to Each Other

Skelemins are 220,000- and 200,000-mol-wt polypeptides that are enriched with the intermediate filament proteins, desmin and vimentin, throughout the preparation of cytoskeletal residues of bovine muscle, and subsequent partial purification of the intermediate filament proteins (Price, 1984). Skelemins are relatively scarce compared with desmin, in all bovine muscle types examined so far. The comparative two-dimensional peptide mapping and immunochemical experiments described here conclusively demonstrate that skelemins are distinct from desmin and vimentin, and thus are not aggregates of these proteins.

Skelemins are initially found in muscle cytoskeletons in a 1:1 ratio. The smaller polypeptide is relatively enriched as the larger one is diminished, in samples that are frozen and thawed, suggesting that the smaller one may be a product of proteolysis of the larger one. The two skelemins are highly homologous to one another, with maps that are 97% identical, each providing only one unique chymotryptic peptide while sharing 36. The relationship between the two skelemins may be of a precursor-product or a proteolytic nature, with proteolysis arising by intracellular mechanisms or simply artefactually, despite the presence of multiple protease inhibitors.

Skelemins Are Unrelated to Other Known Cytoskeletal Proteins

Polyclonal antibodies were directed against both of the skelemins because they are virtually identical as determined by two-dimensional peptide mapping. Although skelemins and myosin heavy chain have similar molecular weights, the anti-skelemin antibodies do not label myosin heavy chain as shown by immunoblotting of crude myosin or muscle samples (Figs. 2 and 3). Furthermore, the anti-skelemin antibodies stain the periphery of the M-disc rather than the core of the A band, where the myosin filaments are (Figs. 6 and 7). The lack of reactivity of the anti-skelemin antibodies with myosin heavy chain indicates that the smearing of the immunolabeling of the 200,000-mol-wt skelemin, especially in samples of unfractionated muscle (Fig. 2), is the result of its distortion by the large amount of myosin heavy chain comigrating with it in one-dimensional gels, and possibly also to continuing breakdown of the larger skelemin in sample buffer. Proteolysis continuing after SDS-sample preparation is seen for other HMW proteins, including pararemin (Brecelder and Lazarides, 1982), synemin (Granger and Lazarides, 1980), talin (Burridge and Connell, 1983), and fodrin (Glenney and Glenney, 1984; see Fig. 3).

Immunohistochemical analysis of the extraction properties of skelemins supports the hypothesis that they are virtually exclusively cytoskeletal, with roughly 10% soluble in a very low-salt buffer (1 mM Tris, 1 mM EGTA, pH 7.5) used at the point in the sequential extraction process when the membrane-depleted myofibrils are disintegrating and some desmin filaments are solubilized (Hubbard and Lazarides, 1979). Slightly more alkaline buffers of similarly low osmotic strength are known to remove the electron density of the muscle M-discs, solubilizing MM-creatine kinase and the majority of two other M-disc components (Eppenberger et al., 1979).
al., 1982; Grove et al., 1984). Dissolution of these intramyofibrillar M-disc elements could in turn cause some loss of the skelemins that are associated with the M-disc periphery. The extraction of some skelemins in the low-salt buffer will be useful for future studies with isolated proteins.

The bovine skelemins described here are not the mammalian analogue of synemin, an avian muscle intermediate filament-associated protein (Granger and Lazarides, 1980; Price and Lazarides, 1983). The definite dissimilarity of these cytoskeletal proteins is shown by a combination of two-dimensional gel electrophoresis, immunoaautoradiography, two-dimensional peptide mapping, and immunofluorescence. The peptide maps are distinctly different, showing at most 30% homology between synemin and skelemins. The anti-skelemn antibodies do not react with any chicken muscle proteins, either by immunoaautoradiography (Fig. 5) or immunoautoradiography (Fig. 6) or immunofluorescence on tissue sections (data not shown), demonstrating that skelemins are immunologically distinct from synemin. Furthermore, these two cytoskeletal elements are located at disparate regions of the myofibril and are expressed in different muscle types. Synemin is located at the periphery of skeletal muscle Z-discs (Granger and Lazarides, 1980), and is absent in myocardial cells (Price and Lazarides, 1983), whereas skelemins are localized in rings surrounding the M-discs of both skeletal and ventricular myocardial myofibrils in several mammalian species. The other avian muscle cytoskeletal protein, paranemin (Breckler and Lazarides, 1982; Price and Lazarides, 1983), is so dissimilar in chain size and charge (280,000 mol wt, pI 4.5) from skelemins and so limited in tissue distribution, being absent in skeletal muscle and visceral smooth muscle, that the additional fact of nonreactivity of anti-skelemn antibodies with paranemin-rich chicken heart (Fig. 5) was sufficient to rule out possible homology between paranemin and skelemins.

The combined immunoautoradiography and immunohistochemistry results presented here strongly suggest that skelemins are unrelated to other known HMW proteins of the cytoskeleton, including fodrin (Nelson and Lazarides, 1983; Glenney and Glenney, 1984), talin (Burridge and Connell, 1983; Sealock et al., 1986), filamin (Gomer and Lazarides, 1981), zeugmatin (Maher et al., 1985), ankyrin (Bennett, 1979; Nelson and Lazarides, 1984), plectin (Wiche et al., 1983), neurofilament- or glial filament-associated proteins (Czeneck et al., 1980; Willard and Simon, 1981), microtubule-associated proteins including a 210,000-mol-wt microtubule-associated protein (Bulinski and Borisy, 1980) and a 215,000-mol-wt microtubule-associated protein of lymphoid and nervous tissues (Olimsted and Lyon, 1981; Parysek et al., 1984), a 200,000-mol-wt fascia adherens protein (Maher and Singer, 1983) or a 300,000-mol-wt cytoskeletal protein (Yang et al., 1985), a 210,000-mol-wt Z-disc/M-line/cytoskeletal protein (Lin, 1981), a 220,000-mol-wt Z-disc protein (Muguruma et al., 1981) or M-protein or myomesin (Grove et al., 1984, 1985).

**Structure of the M-Disc**

Immunofluorescence studies of myofibrils and frozen sections of myocardium and skeletal muscle reveal that skelemins are localized at the M-disc in mammalian striated muscle, and not at the desmin-rich Z-disc (Figs. 6 and 7). Skelemins proteins seem to form single smooth lines at the M-line, not splitting into doublets as the desmin-containing Z-disc often does when muscle is stretched (Price, unpublished observation). From cross sections, it is clear that skelemins form narrow rings at the periphery of the M-discs. This distribution has not previously been reported for a cytoskeletal protein; most cytoskeletal proteins are found at the desmosomes, or at the Z-discs and intercalated discs, where they are associated with intermediate filaments (Maier et al., 1985; Price and Lazarides, 1983; Wiche et al., 1983). There are 54-μg skelemins/g of myofibrils. Assuming that a sarcomere is a cylinder of 0.8-μm diam and 2.5 μm in length, there would be 194 skelemn molecules per sarcomere. If skelemins form a single continuous ring around the M-disc, a skelemn molecule would have to be 13 nm long; if skelemins have length-to-mass ratios similar to M-protein (36-nm long, 165,000-170,000 mol wt, Woodhead and Lowey, 1982), a molecule would be 45-nm long, and if laid end to end, skelemns could wrap around the M-disc 3.5 times.

The M-line or M-disc (from the German Mittellinie, middle line) is the middle portion of the A-band. The A-band consists mainly of myosin filaments, whereas the 75-nm-wide M-line is the region where there are no myosin heads, only bare zones of myosin heavy chains. The structure of the M-disc is still debated. There are up to nine electron-dense substriations in the M-disc, of which three to five are major densities (Sjostrom and Squire, 1977). The exact number and relative density of the major substriations depend on the developmental stage of the muscle, the fiber type, and the species (Thornell and Carlsson, 1984; Sjostrom and Squire, 1977). The major substriations are due to 4-nm-wide M-bridges that link adjacent myosin filaments transversely within the interior of the myofibril (Knappes and Carlsen, 1968; Pepe, 1975). Secondary M-bridges that interconnect the M-bridges, and M-filaments that run parallel to the myofilaments, have been described in frog muscle (Knappes and Carlsen, 1968; Luther and Squire, 1978) but not in any mammalian muscle. There are at least three known nonmyosin proteins present in the M-disc, aside from skelemins. The main component of M-bridges is MM-creatine kinase (Strehler et al., 1983 and references therein). Two other M-line proteins, 165,000-mol-wt M-protein and 185,000-mol-wt myomesin (Grove et al., 1984, 1985) are closely associated with the bare zone of myosin filaments (Herasymowycz et al., 1980; Bahler et al., 1985) and can be considered integral M-disc proteins. It is not known what structures are formed by myomesin and M-protein, but it seems unlikely that they contribute to the M-bridges (Bahler et al., 1985). There may be a linkage of the M-bridge network and/or the other M-disc-associated proteins to the peripheral ring of skelemins.

Previous work indicated that skelemins may be associated with intermediate filaments (Price, 1984). The M-disc/cytoskeletal distribution of skelemins suggests that they may be part of the anchorage system for the transverse intermediate filaments linking myofibrils at the level of the M-discs (Pierobon-Bormioli, 1981; Street, 1983; Wang and Ramirez-Mitchell, 1983) and possibly for the longitudinal intermediate filaments as they pass through the M-disc (Price and Sanger, 1979, 1983; Wang and Ramirez-Mitchell, 1983). The transverse filaments at the level of the M-discs seem to be more fragile than the desmin filaments at the Z-discs (Pierobon-Bormioli, 1981), which may explain why they are often not seen.
The existence of an insoluble cytoskeleton at the M-disc indicates that skelemins may function to align adjacent myofibrils at the level of the A-bands. The fact that skelemins form a ring around the myofibril, as desmin does at the Z-discs (Granger and Lazarides, 1978), leads to several hypotheses. The cytoskeletal rings at the Z- and M-discs, partially composed of desmin and skelemins, respectively, may serve to prevent lateral expansion of the myofibrils during contraction. The skelein rings could also conceivably form a ring around the myofibril, as desmin does at the M-band proteins represent constituents of native thick filaments, frayed filaments and bare zone assemblies. J. Muscle Res. Cell Motil. 4:137-167.

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