

Zeugmatin: A New High Molecular Weight Protein Associated with Z Lines in Adult and Early Embryonic Striated Muscle

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ABSTRACT Monoclonal antibodies were generated to a purified preparation of the fascia adherens domains of the intercalated discs of chicken cardiac cell membranes. One of these antibodies, McAb 20, immunofluorescently labeled the Z lines of adult skeletal muscle, the Z lines and intercalated discs of adult cardiac muscle, and the dense bodies and dense plaques of adult gizzard smooth muscle. In addition, McAb 20 was found to label regenerating muscle cells in a cross-striated pattern much like that of Z lines in 24-h muscle cell cultures before the appearance of Z lines was detectable by phase or Nomarski optics and before the concentration of α -actinin occurred at the Z lines. Thus, McAb 20 appears to be directed against an antigen involved in early myofibrillar organization. Preliminary biochemical characterization of the antigen recognized by McAb 20 indicates that it is a high molecular weight doublet of over 5×10^5 kD that is highly susceptible to proteolysis. By virtue of its presence in Z lines, and its possible role in the end-on attachment of microfilaments to Z lines and membranes, we have named this protein zeugmatin ($\xi\epsilon\nu\gamma\mu\alpha \equiv$ yoking).

Although the linkages of actin-containing microfilaments to membranes in a variety of cell types have been found to play important roles in cell motility, cell shape, and cell-cell and cell-substrate adhesion, the array of molecular components that comprises these linkages has not yet been determined. Two proteins, α -actinin and vinculin, have been localized to a number of different sites of end-on microfilament-membrane attachment (16, 17, 44), suggesting that the various sites are homologous in structure and function. In an attempt to define more completely the protein components involved in end-on microfilament-membrane interactions, we embarked on a systematic analysis of the fascia adherens domains of the intercalated disc membranes of cardiac muscle cells. The fascia adherens is a stable membrane domain at which microfilament bundles terminate, and where vinculin and α -actinin have been localized (44). We isolated fascia adherens junctions from chicken cardiac muscle and, in an earlier study (28), reported on a previously unidentified 200-kD protein of the fascia adherens. Polyclonal antibodies to this protein cross-reacted with an intracellular component of fibroblasts and immunolabeled the fibroblast focal adhesions, another site where microfilaments terminate at the membrane. More recently, a 135-kD protein of the fascia adherens has been

identified using monoclonal antibodies to the fascia adherens (18). This protein is also localized to adherens-type junctions in epithelial cells, further supporting the proposition that a number of different sites of end-on microfilament-membrane interaction are homologous.

As a continuation of our earlier work we generated and examined a battery of monoclonal antibodies (McAb)¹ to the fascia adherens. Among these, we identified by immunofluorescence experiments several antibodies that labeled the Z line of adult striated muscle. One of these antibodies, McAb 20, has the unusual property of labeling regenerating muscle cells in a cross-striated pattern much like that of Z lines in 24-h muscle cell cultures, and as a result, we investigated it in further detail. Most previous studies on regenerating embryonic skeletal muscle have not clearly detected cross-striations by immunofluorescence, phase, or Nomarski optics until skeletal muscle cells have been in culture for several days. Thus, McAb 20 appears to be directed against an antigen involved in the early organization of the myofibril. This paper

¹ *Abbreviations used in this paper:* ALD, anterior latissimus dorsi; DME, Dulbecco's modified Eagle's medium; McAb, monoclonal antibody; PLD, posterior latissimus dorsi; TBS, Tris-buffered saline.

reports on the staining of regenerating muscle cells with both McAb 20 and polyclonal antibodies to α -actinin, another Z line protein thought to play a critical role in myofibrillar organization. A second monoclonal antibody, McAb 284, showed similar staining characteristics to McAb 20. In addition, we present a detailed analysis of the immunofluorescent localization of McAb 20 in adult cardiac, skeletal, and gizzard smooth muscle. A preliminary biochemical characterization of the antigen recognized by McAb 20 and McAb 284 has been carried out. We have named this protein antigen zeugmatin, because of its presence in Z lines and its possible role in the end-on attachment of actin microfilaments to Z lines and membranes. A preliminary account of this work has been published (29).

MATERIALS AND METHODS

Immunogen: Purified fascia adherens preparations from chicken cardiac muscle were isolated as described previously (28).

Monoclonal Antibody Production: Mouse monoclonal antibodies to the fascia adherens were produced following the procedure of Köhler et al. (26). BALB/c mice were immunized with 600 μ g protein per injection as follows: day 1, subcutaneous and intramuscular in complete Freund's adjuvant; days 21 and 35, subcutaneous and intramuscular in incomplete Freund's adjuvant; day 49, intraperitoneal in 10 mM Tris, pH 9.0. 3 d after the final injection the spleens were removed from two mice and fused with mouse NS-1 myeloma cells at a ratio of 3.3:1 (spleen cells/myeloma cells) using 0.5 ml 50% polyethylene glycol 4000 (Merck & Co., Inc., Rahway, NJ) in Dulbecco's modified Eagle's medium (DME) for 90 s at 37°C. After diluting the polyethylene glycol with 10 ml DME over 10 min, the cells were diluted into HAT medium (DME containing 15% fetal calf serum, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine), and $\sim 1 \times 10^5$ cells in 0.05 ml were added to each well of 23 96-well microtiter plates already containing 1 ml HAT medium and 1×10^5 spleen cells as a feeder layer. Samples of supernatant were collected from the wells beginning on day 11 (as the cultures became visible) and screened for antibodies to the fascia adherens by an ELISA technique and immunofluorescence microscopy (see following sections). Antibody-producing hybrids were cloned two times by limiting dilution into 96-well microtiter plates with a feeder layer. Selected clones were grown up in mass culture and injected intraperitoneally into pristane-primed mice to produce ascites culture. The antibody subclass was determined using an ELISA method and antibodies specific to each mouse immunoglobulin class (Boehringer-Mannheim Diagnostics, Inc., Houston, TX). IgG was purified from the ascites fluids by ammonium sulfate precipitation followed by DEAE-cellulose chromatography, and was stored in 50% glycerol in phosphate-buffered saline (PBS) at -20°C .

Hybridoma Screening by ELISA: Aliquots (100 μ l/well) of a fascia adherens preparation solubilized in urea buffer (10 μ g protein/ml in 7 M urea, 5 mM EDTA, 10 mM dithiothreitol, 0.05 M Tris-HCl, pH 8.0) were added to 96-well microtiter plates for 2 h at room temperature. Plates were blocked by incubation for 2 h with 3% bovine serum albumin (BSA) in PBS, rinsed and incubated 2 h at room temperature with 100 μ l supernatant (diluted 1:4 in 3% BSA, 0.02% NaN_3 in PBS). After rinsing thoroughly, β -galactosidase-conjugated sheep anti-mouse (1/500 dilution in PBS, 10 mM MgCl_2 , 10 mM β -mercaptoethanol; Zymed, S., San Francisco, CA) was added for 90 min at room temperature. After washing, the plates were developed using 4-methyl umbelliferyl-galactose (Sigma Chemical Co., St. Louis, MO) (0.02 mg/ml in Tris-buffered saline [TBS], 10 mM MgCl_2 , 0.1 M β -mercaptoethanol) as substrate. The plates were examined under ultraviolet light and photographed.

Indirect Immunofluorescence Microscopy: Secondary cultures of trypsinized 8-d chick embryos were grown on round glass coverslips in 24-well Costar (Costar Co., Cambridge, MA) plates for 18–24 h. The coverslips were fixed for 5 min in 3% formaldehyde in PBS at room temperature, rinsed in PBS, permeabilized 5 min in cytoskeletal buffer (10 mM HEPES, 300 mM sucrose, 3 mM MgCl_2 , 50 mM NaCl, 0.5% Triton X-100, pH 7.4) at room temperature, and rinsed again. The coverslips were inverted onto 50- μ l drops of hybridoma supernatant on a sheet of parafilm and incubated 30 min at room temperature. They were rinsed by dipping in PBS, then incubated 10 min with rhodamine-conjugated rabbit anti-mouse IgG (10 μ g/ml), rinsed again, incubated 10 min with rhodamine-conjugated goat anti-rabbit IgG (10 μ g/ml), rinsed and mounted in 90% glycerol, 10 mM Tris-HCl, pH 7.4. The coverslips were examined with a Zeiss photomicroscope III (Carl Zeiss, Inc.,

Thornwood, NY) equipped with epifluorescent illumination, a 63 \times Nomarski and a 63 \times phase contrast lens. Pictures were taken on Tri-X film (ASA 400) with 10–60-s exposures and developed in D19.

Cell Culture: Primary cultures of 8-d chick embryos were prepared by trypsinization of decapitated, minced embryos. The cells were cultured in DME/Coon's F-12 (1:1) containing 10% fetal calf serum. Primary myogenic cultures were prepared from 12-d embryonic chicken thigh muscle by mechanical dissociation as described by Konigsberg (27). After 18–24 h in culture these cells were suspended by trypsinization (27) and plated onto gelatin-coated coverslips in DME containing 10% fetal calf serum and 5% chick embryo extract. Overproliferation of fibroblasts was prevented by treating the cells on day 3 with 10 mM cytosine arabinofuranoside.

Isolated Myofibrils: Isolated skeletal and cardiac muscle myofibrils were prepared by homogenization of strips of glycerinated muscle (35). Isolated gizzard myofibrils were prepared from fresh, cleaned gizzard as described (23) including washes in Triton X-100. All preparations were stored in buffer with 50% glycerol at -20°C . For immunofluorescent labeling experiments, aliquots of the isolated myofibrils were spread on coverslips, allowed to attach for 10 min at room temperature, incubated 10 min in 1% gelatin in PBS, and then immunostained as described below.

Preparation of Frozen Sections: Adult white leghorn chickens (1–2 kg) were anesthetized with sodium pentobarbital (10 mg/100 g body weight). ALD and PLD muscles were cut into thin longitudinal strips with a sharp scalpel, stretched to various lengths, tied at their ends onto wooden sticks with thread, excised from the animal, and immersed in fresh 3% formaldehyde in 0.1 M HEPES, pH 7.4. The muscles were fixed for 30 min at room temperature with constant agitation and then stored in 0.5% formaldehyde, 0.1 M HEPES, pH 7.4 at 4°C for up to 1 wk. Cardiac papillary muscle was obtained by nicking the femoral artery and perfusing the heart under pressure with the above fixative containing 2 mM CaCl_2 . After 5 min the heart was excised, cut in half, and immersed in fixative for an additional 25 min at room temperature with constant agitation. The papillary muscles were dissected out and stored at 4°C as for skeletal muscle. Tissue blocks were prepared for cryosectioning, and sections of 0.5–1.0 μ m thickness were cut, according to the method of Tokuyasu (42, 43, 45).

Various methods of sample preparation were used to try to obtain immunostaining with McAb 20 on frozen sections of gizzard muscle. The method that worked best is described below, while other methods that were tried are discussed in Results. Gizzard strips were directly immersed into glycerination solution (50% glycerol, 1 mM EGTA in PBS) and maintained overnight at 4°C. Then the strips were transferred to -20°C for >1 mo. Next, a portion of the glycerinated strip was brought to room temperature, cut into 0.25–0.5-mm cubes, and taken through a series of increasing dilute glycerol solutions in PBS/1 mM EGTA (25%, 10%, 5%, 0% glycerol for 5 min each). Subsequently, the cubes were infused with 2.3 M sucrose/1 mM EGTA for 30 min and cryosectioned. Before immunostaining, the sections were treated with Triton X-100 as follows. The sections were washed in gizzard myofibril homogenization buffer (20 mM imidazole, 60 mM KCl, 10 mM β -mercaptoethanol, 5 mM EDTA, 0.02% NaN_3 , pH 7.0) for 10 min to remove the sucrose. Next they were incubated for 10 min at a time in the above buffer containing 1, 0.5, and 0.25% Triton X-100, in that order. Finally, the sections were washed in PBS, 0.01 M glycine for 10 min, and prepared for immunostaining.

Immunofluorescent Labeling of Cells and Isolated Myofibrils: Cells on coverslips were fixed and permeabilized as described above and then incubated with either McAb 20 (100 μ g/ml IgG) or McAb 284 (100 μ g/ml IgG) in single labeling experiments; or with a mixture of McAb 20 and either rabbit anti-chicken gizzard α -actinin (10 μ g/ml) or desmin (10 μ g/ml) for double labeling experiments. Incubations were carried out in PBS for 30 min at room temperature. After rinsing by dipping in PBS, the coverslips were treated for 10 min at room temperature in either the fluorescein-conjugated F(ab')₂ fragment of goat anti-mouse IgG (100 μ g/ml; Cappel Laboratories, Cochranville, PA) or a mixture of this conjugate and rhodamine-conjugated goat anti-rabbit IgG (10 μ g/ml), and then processed for microscopy as discussed earlier.

Immunofluorescent Labeling of Frozen Sections: Frozen sections were immunolabeled as described by Tokuyasu et al. (45) with the following modifications. Sections were incubated with McAb 20 (25 μ g/ml IgG in PBS/0.01 M glycine) for 30 min at room temperature. A double layer of rhodamine-conjugated secondary antibodies was used as described earlier for the immunofluorescence microscopy screening of hybridoma supernatants. Use of only a single layer of rhodamine-conjugated secondary antibodies did not provide a strong enough signal for clear visualization of the labeling pattern on the frozen sections.

SDS PAGE and Immunoblotting: SDS PAGE followed by transfer to nitrocellulose was carried out as described previously (45) except for the

myofibrils and KCl-extracted myofibrils which were washed and solubilized as recommended (49). Blots were blocked for 2 h at room temperature with 3% BSA in TBS, rinsed and incubated with hybridoma supernatants (200–400 μ l/5 ml TBS/BSA), McAb 20 (25 μ g/ml IgG in TBS/BSA), or McAb 284 (25 μ g/ml IgG in TBS/BSA), overnight at room temperature. Excess antibody was washed out and the blots were incubated for 2 h at room temperature with rabbit anti-mouse IgG (1 μ g/ml in TBS/BSA), washed, treated with peroxidase-conjugated goat anti-rabbit IgG (1/1,000 dilution; Cappel Laboratories) 30 min at room temperature, washed again, and developed with diaminobenzidine (Sigma Chemical Co.) (0.5 mg/ml in 50 mM Tris, pH 7.5; 0.15% H₂O₂).

Peptide Mapping: Two-dimensional mapping of ¹²⁵I-labeled tryptic peptides from proteins separated by SDS PAGE was carried out as described previously (45, 51).

Zeugmatin Isolation: (All volumes are with respect to initial wet weight.) Myofibrils were prepared from fresh chicken pectoralis muscle as described (49), including 5 mM EGTA. They were extracted twice for 30 min by stirring at 4°C in 8 vol 0.5 M KCl, 2 mM MgCl₂, 2 mM Na₄P₂O₇, 0.1 M NaH₂PO₄, 5 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 6.8 and collected by centrifugation at 4,000 *g* for 15 min. The myofibril pellet was washed twice with distilled water containing 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0 and extracted overnight with stirring at 4°C in 4 vol 0.6 M KI, 20 mM Na₂S₂O₃, 20 mM Tris-HCl, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5. Insoluble material was removed by centrifugation at 100,000 *g* for 1 h. The supernatant was applied to a 2-ml column of McAb 20 IgG (3.12 mg/ml) coupled to glutaraldehyde-activated Ultrogel ACA 22 (LKB Instruments, Inc., Gaithersburg, MD) by a standard technique (41). The column was washed with PBS containing 5 mM EGTA until the absorbance at 280 nm of the effluent fell below 0.01. Specifically bound material was then eluted with 0.2 M HCl-glycine, pH 2.8 into 1.5 M Tris base.

Amino Acid Analysis: Purified zeugmatin was dialyzed extensively against H₂O at 4°C, and amino acid analysis was performed as described (4).

Miscellaneous Reagents: The preparation and affinity purification of rabbit anti-chicken gizzard α -actinin and desmin, rhodamine-conjugated goat anti-rabbit IgG and rabbit anti-mouse IgG, and unmodified rabbit anti-mouse IgG were as described (15, 45). Sarcoplasmic reticulum vesicles were prepared from chicken pectoralis muscle by modification of a standard procedure (30) for their preparation from rabbit back and thigh muscles. Fascia adherens was isolated as described (28). A control immunoaffinity column was prepared by coupling a monoclonal IgG directed against the N protein of vesicular stomatitis virus (3.74 mg/ml) to glutaraldehyde-activated Ultrogel ACA 22 as described above.

RESULTS

Production and Isolation of Monoclonal Antibodies

The spleen cells from two BALB/c mice immunized with a fascia adherens preparation from chicken cardiac muscle were fused with NS-1 myeloma cells. From this fusion 950 wells were screened by an ELISA method for antibodies that bound to the fascia adherens preparation as described in Materials and Methods. Seventy-one positive supernatants were identified and further screened by indirect immunofluorescence microscopy on secondary cultures of trypsinized 8-d chick embryos. These cultures contained not only large numbers of fibroblasts but also many immature myotubes. Six supernatants showed interesting staining patterns and also gave positive results when immunoblotted against the fascia adherens as described in Materials and Methods. These hybridomas were cloned twice by limiting dilution and grown as ascitic tumors in mice. Two of these antibodies, McAb 20 and McAb 284, were found to be of the IgG2a class. Results with McAb 20 will be described in detail below. In addition, some work with McAb 284, which we demonstrated was directed against the same antigen, will also be discussed.

Microscopic Localization of Zeugmatin

CELL CULTURES: Indirect immunofluorescent staining

of fixed, permeabilized primary cultures of chick embryo fibroblasts with McAb 20 showed labeling only in elongated, multinucleate cells that were apparently developing myotubes (Fig. 1, *a* and *b*). Mononucleated myoblasts, identified by labeling with rabbit anti-desmin (3), were not specifically labeled with McAb 20 (Fig. 1, *c* and *d*). Unpermeabilized cells did not stain, which lead us to conclude that the epitope recognized is intracellular. Later passages of fibroblasts, which were devoid of myotubes, were also not labeled. The labeling of the myotubes was usually at least partially cross-striated even after only 18 h in culture. McAb 20 also labeled cultures of chicken cardiac muscle cells in a striated pattern (not shown). Epithelial cells from a variety of sources were not labeled (not shown).

ISOLATED MYOFIBRILS: To determine whether the antigen was present in adult muscle tissue, isolated myofibrils were prepared from glycerinated cardiac and skeletal muscle and fresh gizzard and indirectly immunostained with McAb 20. As shown in Fig. 2, *a*, *c*, and *e*, the antigen was localized to the Z line (arrows) in cardiac, and slow (ALD) and fast (PLD) skeletal muscle. A single strong uniform band of labeling was always visible, suggesting that the antigen was located throughout the Z band since those proteins thought to be localized to the periphery of the Z band (e.g., desmin) gave patchy Z line labeling under the same conditions (Fig. 2*j*). In addition, in isolated cardiac myofibrils the intercalated disc could clearly be seen to be labeled as a pair of narrowly spaced stripes (arrowheads in Fig. 2, *a* and *b*). The myofibrils isolated from gizzard were not striated but instead consisted of bundles of filaments joined together by the dense bodies, which were revealed by immunolabeling with anti- α -actinin (Fig. 2*g*). As shown in Fig. 2*h*, the staining pattern seen with McAb 20 was very similar to that observed with anti- α -actinin.

TISSUE SECTIONS: To characterize further the localization of zeugmatin in adult chicken muscle, semithin frozen sections were prepared from cardiac, fast (PLD) and slow (ALD) skeletal, and smooth muscle (gizzard). In cardiac papillary muscle, McAb 20 exclusively stained regions corresponding to the Z lines and intercalated discs (Fig. 3, *a* and *b*). The Z line staining appears as a single \sim 0.6- μ m wide fluorescent stripe (arrows). This staining is present at all levels and uniformly across the entire widths of the sectioned myofibrils, agreeing with the results from the isolated cardiac myofibrils which suggest that the antigen is located throughout the Z line and is not only present at its periphery. In areas exhibiting slightly separated and nonaligned myofibrils, little or no staining of the intermyofibrillar spaces was observed. At the intercalated discs (arrowheads), McAb 20 immunostaining appeared as a narrowly separated pair of stripes. All the discs were similarly labeled although the intensity of the stain varied considerably from disc to disc, probably due to variations in the exposure of the disc in the section. McAb 20 did not label the plasmalemma (asterisk, top of Fig. 3*b*) or the vasculature. Using immunoelectron microscopy techniques, we have observed labeling of McAb 20 only at the Z band and in the fascia adherens region of the intercalated disc (unpublished observations).

As seen by immunofluorescence microscopy, McAb 20 labeled the Z line of skeletal muscle but in a somewhat different manner from cardiac muscle. In semithin frozen sections of both fast and slow skeletal muscle, the Z line was labeled as a very closely spaced pair of stripes \sim 1.0–1.2- μ m wide (Fig. 4*b* and 5, *d–f*, arrows). Often the staining over-

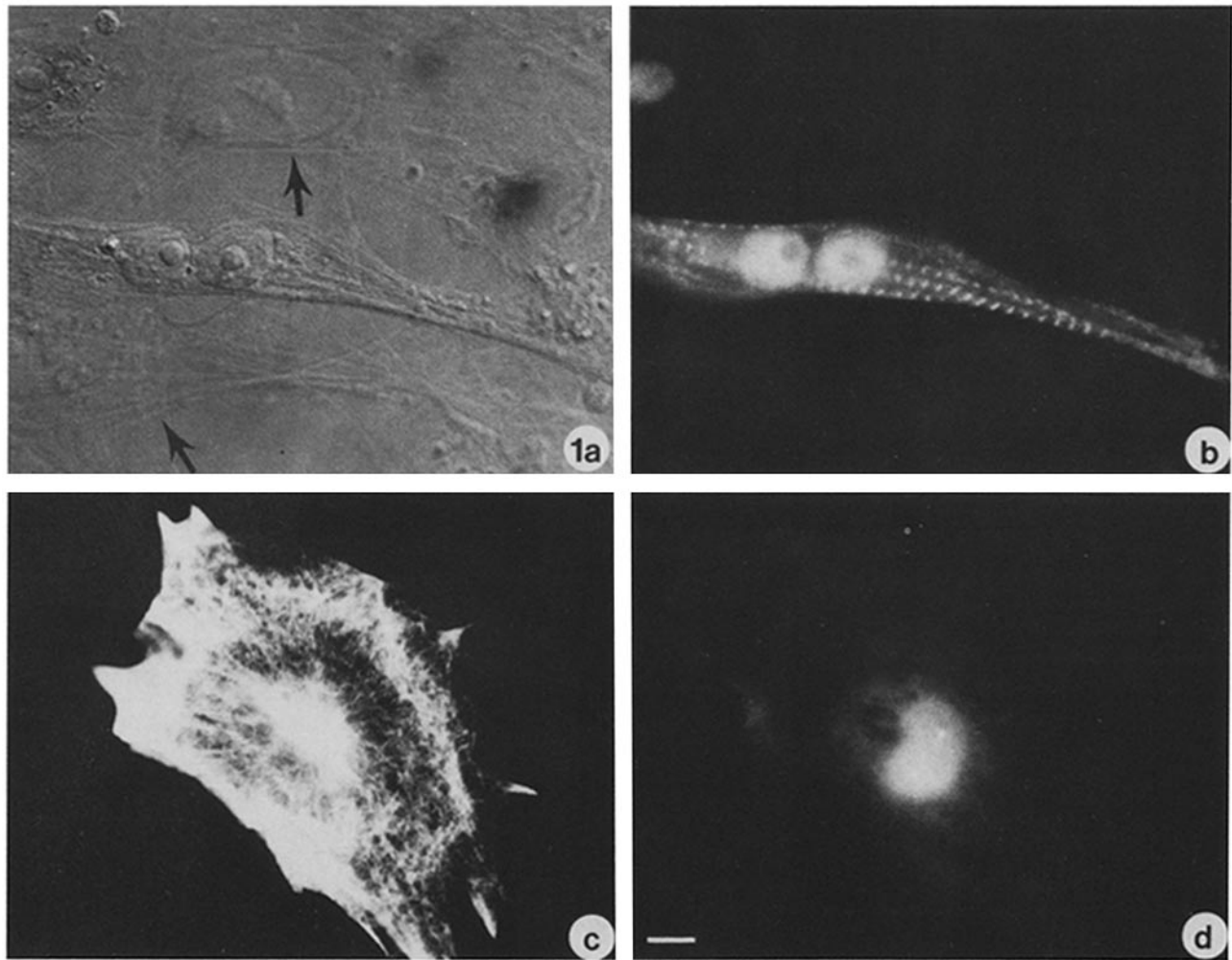


FIGURE 1 1-d muscle cell cultures indirectly immunostained with McAb 20 (a, Nomarski and b, immunofluorescence) and indirectly double immunostained with desmin (c) and McAb 20 (d). Arrows in a indicate fibroblasts that are not labeled in b. Nuclear staining with McAb 20 in d is due to the secondary antibody since it is seen in the absence of primary antibody. A weak staining of fibroblast nuclei is also observed but is not apparent in these photographs. Bar, 5 μm . $\times 1,250$.

lapped to produce a single band at the Z line. The paired stripe staining was more easily and frequently observed in semithin sections of ALD than of PLD, perhaps owing to the fact that the Z line is wider in slow skeletal muscle than in fast skeletal muscle (36). To determine whether this pattern changed with alterations in sarcomere length, we systematically stretched samples of skeletal muscle to different lengths before fixation (Fig. 5, ALD; PLD not shown). In both PLD and ALD, stretching the muscle to sarcomere lengths of ~ 3.0 , 4.0 , and $5.0 \mu\text{m}$ did not appreciably alter the width of the fluorescently labeled pair of stripes, which remained 1.0 – $1.2 \mu\text{m}$, nor did it change the position of the immunostaining relative to the Z line. In all cases, the fluorescent pair of stripes was directly superimposable upon the Z line. At the level of resolution of immunofluorescence microscopy, one cannot unequivocally determine whether the paired staining pattern represents the edges of the Z line or some domain in the I band close to the Z line. At the electron microscope level of resolution, however, we have observed the immunolabeling to be clustered along both edges of the Z disc with little or no labeling in the I band (unpublished observations). As in cardiac muscle, neither the intermyofibrillar spaces nor the plasmalemma was stained in skeletal muscle. The nuclear

staining in Fig. 4b is apparently due to nonspecific staining by the secondary rhodamine conjugates since control sections treated only with these antibody conjugates also displayed nuclear labeling.

Using the same fixation conditions as for skeletal muscle, we observed no immunolabeling with McAb 20 of semithin frozen sections of gizzard smooth muscle. Treating fixed sections with acetone (100%, 30 s, room temperature) or Triton X-100 (0.5%, 5–30 min, room temperature) did not permit any immunostaining, nor did incubating freshly excised tissue in EGTA (5 mM in PBS, 5–30 min, room temperature) before fixation. However, the glycerination procedure described in the Materials and Methods did permit immunostaining by McAb 20 (Fig. 6). Those samples that had been glycerinated, sectioned, and washed in Triton X-100 without any fixation produced the strongest labeling (Fig. 6). In particular, McAb 20 labeled structures that appeared to be cytoplasmic dense bodies (arrows) and possibly membranous dense plaques (arrowheads). Double labeling of the same gizzard cross-section with α -actinin (Fig. 6b) and McAb 20 (Fig. 6c) produced almost identical labeling patterns as would be expected if McAb 20 labeled the aforementioned structures exclusively. Single labeling of sections for each protein pro-

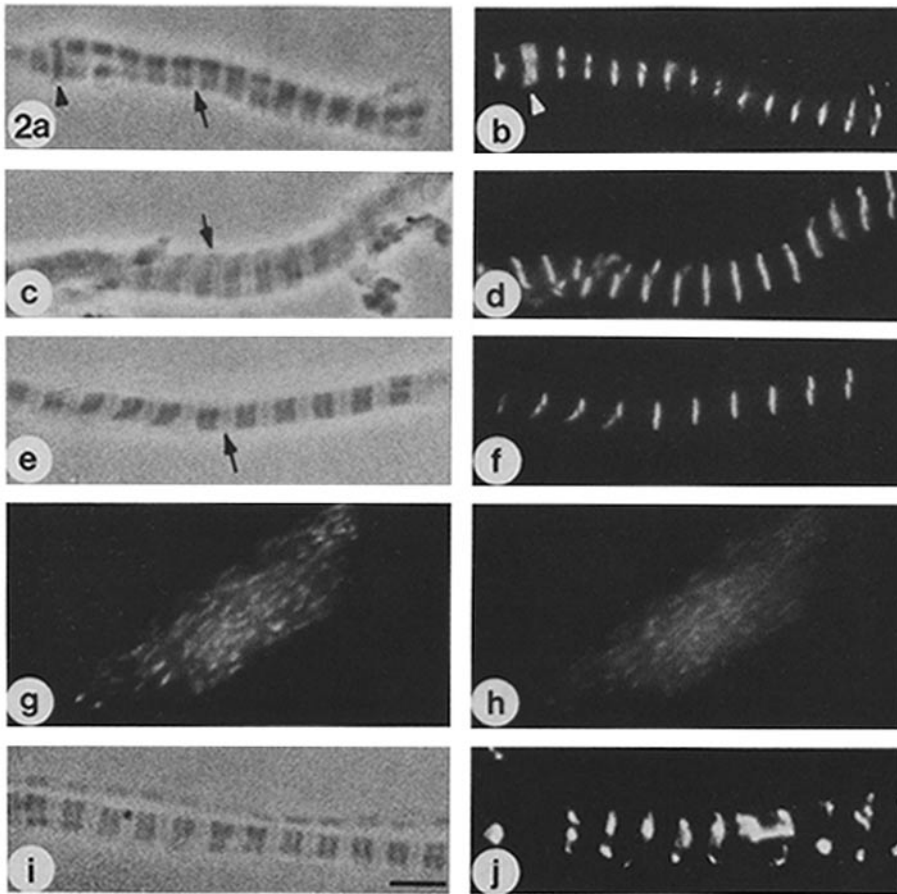


FIGURE 2 Indirect immunofluorescent labeling of isolated myofibrils with McAb 20. *a, c, e, and i* are phase images, and *b, d, f, and j* are the corresponding fluorescence images. *g* and *h* are a pair of doubly immunolabeled fluorescent images. (*a* and *b*) cardiac; (*c* and *d*) ALD; (*e* and *f*) PLD; (*g* and *h*) gizzard doubly immunolabeled with rabbit anti-chicken gizzard α -actinin (*g*) and McAb 20 (*h*); (*i* and *j*) PLD labeled with rabbit anti-chicken gizzard desmin. Arrows in *a, c,* and *e* indicate the Z line. Arrowheads in *a* and *b* mark an intercalated disc. Bar, 5 μm . $\times 1,400$.

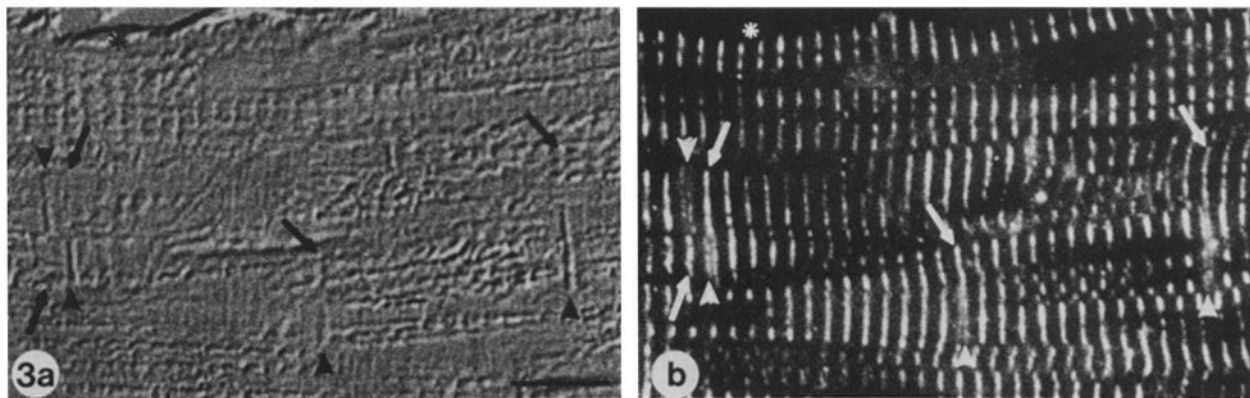


FIGURE 3 Semithin frozen sections of adult cardiac papillary muscle immunostained with McAb 20. (*a*) Nomarski; (*b*) immunofluorescence. Z lines (arrows) are labeled as a single fluorescent band, whereas intercalated discs (arrowheads) are labeled as a narrowly separated pair of fluorescent stripes. No staining of the plasmalemma (*) is observed. Bar, 10 μm . $\times 1,300$.

duced the same results so that coincident patterns were not due to fluorescence filter leakthrough. The plasma membrane labeling for both proteins is not well demarcated as linear dense plaques but is either continuous or punctate. This may be a result of the glycerination procedure. Gizzard samples that had been glycerinated and sectioned as above but without the Triton X-100 washes were also immunolabeled by McAb 20 at the cytoplasmic dense bodies, but no labeling was observed at the plasma membrane (not shown). Glycerinated gizzard samples that had been fixed before sectioning revealed a labeling pattern similar to the unfixed samples, but increasing the fixation time up to 30 min significantly decreased the overall intensity of the signal. It should be noted that whereas

the immunolabeling pattern produced by McAb 20 was very sensitive to the specimen and section preparation conditions, that for α -actinin was relatively unaffected, with positive immunostaining for dense bodies and dense plaques under all conditions.

REGENERATING EMBRYONIC CELL CULTURES: The location of zeugmatin on the Z line in adult striated muscle and the observance of a cross-striated staining pattern in 18-h embryonic muscle cell cultures suggested that this protein might play a role in the organization of actin filaments into myofibrils in the myotube. In these preliminary studies, we compared the staining pattern of McAb 20 with that of α -actinin in developing myotubes since α -actinin had already

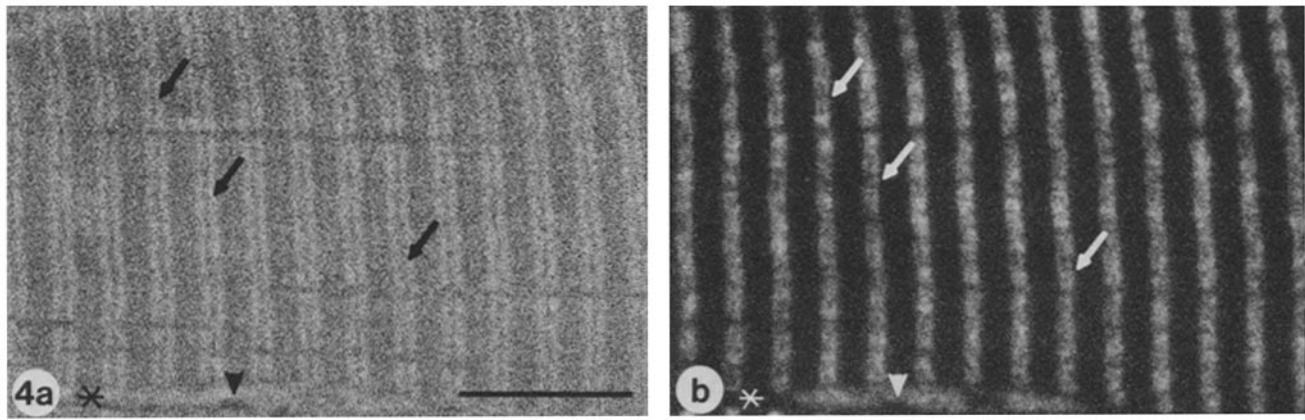


FIGURE 4 Semithin frozen sections of adult fast skeletal muscle (PLD) immunostained with McAb 20. (a) Phase; (b) immunofluorescence. Note the paired stripe labeling at the Z lines (arrows). The dark area of the myonucleus (arrowhead) directly beneath an A band serves as a reference point. The plasmalemma (*) is not stained. The myonucleus is nonspecifically stained by the rhodamine antibody conjugate. The sarcomere length from Z line to Z line is $2.9 \mu\text{m}$. Bar, $10 \mu\text{m}$. $\times 2,300$.

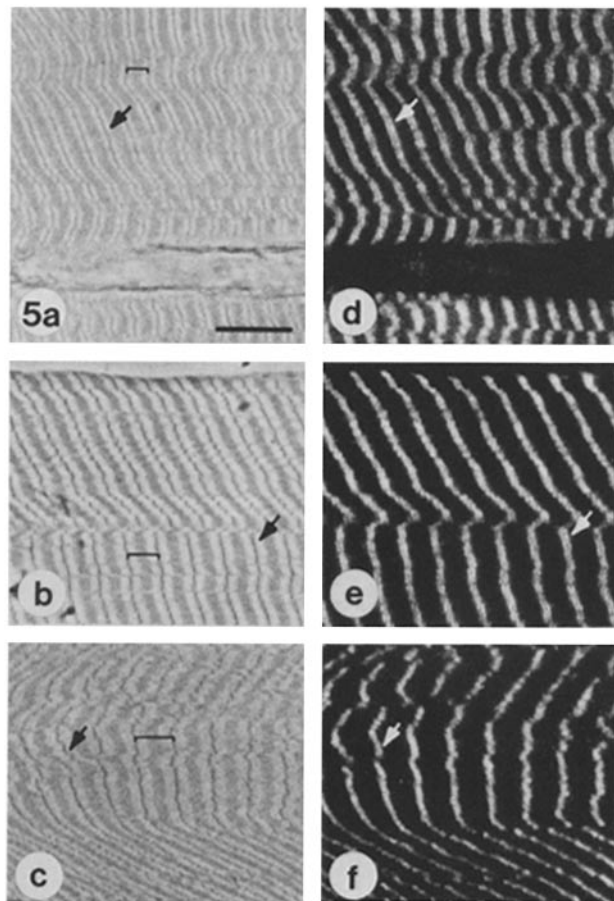


FIGURE 5 Semithin frozen sections of adult slow skeletal muscle (ALD) immunostained with McAb 20. (a-c) Phase optics; (d-f) immunofluorescence. Sarcomere lengths as marked by the brackets are $3 \mu\text{m}$ (a and d), $4 \mu\text{m}$ (b and e), and $5 \mu\text{m}$ (c and f). Note the paired stripe staining at the Z line (arrows). The width of the fluorescent stripes does not increase with increasing sarcomere lengths. The staining is always coincident with the Z line. Bar, $10 \mu\text{m}$. $\times 1,000$.

been shown to be cross-striated during myotube development (14, 20, 24). Cultures of muscle cells were prepared from 10–12-d chick embryos, passaged 18–24 h later, and then double immunolabeled with McAb 20 and rabbit anti- α -actinin 1–6

d later. As shown in Fig. 7, c and f, McAb 20 revealed a fairly extensive pattern of fluorescent cross-striations on many myotubes within 18–24 h after plating, whereas at the same time α -actinin (Fig. 7, b and e, respectively) was generally quite patchily distributed throughout the myotube and especially at the periphery. Fibroblasts in the same culture were strongly and specifically labeled with the anti- α -actinin antibodies (Fig. 7, b and e). The cross-striated staining pattern observed at this stage with McAb 20 was apparent in at least 50% of the myotubes on a coverslip. When the same cell was examined by Nomarski optics, cross-striations were generally not seen. However, fibrils were apparent (e.g., Fig. 7, a and d). By 3 d after plating, the staining for α -actinin in the myotubes was also beginning to be cross-striated (not shown) and by 5–6 d in culture the staining patterns of McAb 20 and anti- α -actinin became essentially identical (Fig. 8, b and c). By this last time, the fluorescent cross-striations generally corresponded to cross-striations visible with Nomarski optics (Fig. 8a).

STAINING WITH McAB 284: The staining of 24-h myotubes with McAb 284 and anti- α -actinin is shown in Fig. 9. The pattern of labeling appears identical to that observed with McAb 20.

Antigen Identification

In our initial experiments, the fascia adherens was separated into its components by SDS PAGE, and immunoblotting with McAb 20 was carried out; typical results are shown in Fig. 10a. The antibody reacted with several polypeptide bands between 100 and 200 kD as well as some material over 200 kD. When myofibrils, prepared and solubilized as described (49), were separated by SDS PAGE and immunoblotted with McAb 20, a doublet of high molecular weight bands was always observed (Fig. 10c). On 4, 5, or 6% SDS polyacrylamide gels this doublet migrated slightly less than nebulin but more than titin and so has an estimated molecular weight between 5×10^5 and 1×10^6 kD (Fig. 10, b, c, d, and e). To facilitate further studies with zeugmatin we decided to purify the protein. This was accomplished by using the antibody as both a probe and a specific adsorbant. It was found that zeugmatin was enriched in 0.6 M KCl-extracted myofibrils relative to intact myofibrils (Fig. 10, d and e). A further extraction of these KCl-extracted myofibrils with 0.6 M KI resulted in partial solubilization of zeugmatin. This extract

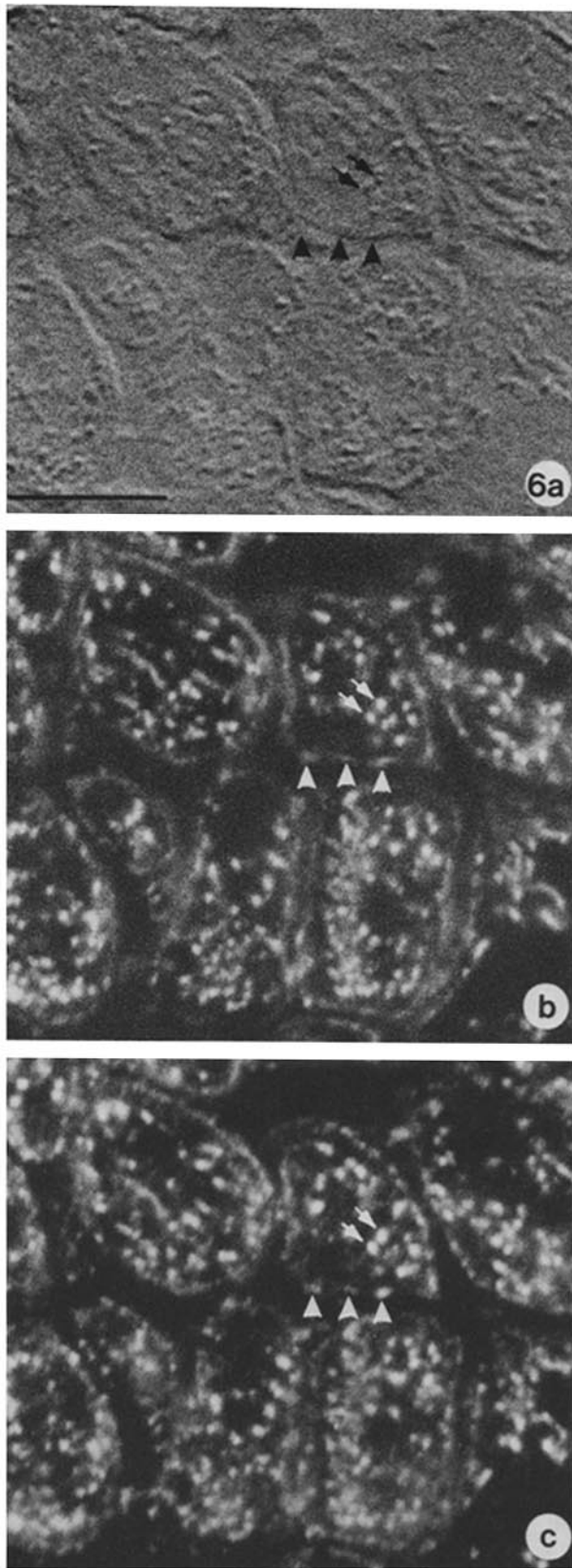


FIGURE 6 Semithin frozen sections of adult gizzard smooth muscle, (a) Nomarski; and doubly labeled with (b) guinea pig anti-chicken gizzard α -actinin and (c) McAb 20. The two immunolabeling patterns are very similar. Generally, dense bodies (arrows) and dense plaques (arrowheads) are labeled with both antibodies. Bar, 10 μ m. \times 2,200.

was passed over an affinity column prepared by coupling the McAb 20 to glutaraldehyde-activated ACA 22. Specifically bound protein was eluted with 0.2 M HCl-glycine, pH 2.8 and analyzed by SDS PAGE and immunoblotting. The major and largest Coomassie Blue-staining band appearing in the eluant was a doublet with a molecular weight over 5×10^5 kD and corresponding to the doublet seen in immunoblots of myofibrils (Fig. 10, *f* and *g*). This doublet as well as several lower molecular weight bands were stained by McAb 20 on immunoblots (Fig. 10 *g*). When a similar 0.6 M KI extract was passed over an affinity column prepared with an unrelated IgG, the high molecular weight doublet was not observed either by Coomassie Blue staining (Fig. 10 *h*) or immunoblotting with McAb 20 (Fig. 10 *i*).

Immunoblotting of zeugmatin with McAb 284 is shown in Fig. 10 *j*. This McAb weakly blotted the high molecular weight doublet as well as several lower molecular weight bands in a pattern similar to that seen with McAb 20.

SARCOPLASMIC RETICULUM: To examine whether zeugmatin was a protein of the sarcoplasmic reticulum, sarcoplasmic reticulum was purified from chicken pectoralis muscle, solubilized in SDS, separated by SDS PAGE on 5% gels, and immunoblotted with McAb 20. The results are shown in Fig. 10 *k*. No reaction is seen.

PEPTIDE MAPS: To determine if the two polypeptides of the high molecular weight doublet were due to closely related proteins, two-dimensional 125 I-labeled peptide maps of trypsin digests of each of the two bands were obtained. The results are shown in Fig. 11. Many of the peptides appear to be common to both of the polypeptides although there are some spots unique to each one. These similarities and differences are summarized in Fig. 11 *c*.

AMINO ACID ANALYSIS: The results of the amino acid analysis of zeugmatin are shown in Table I.

DISCUSSION

Using monoclonal antibody technology we have identified a new high molecular weight protein component of the Z line of striated muscle whose appearance in the early stages of myofibrillogenesis suggests it plays an important role in the organization of myofibrils. This protein, which we have named zeugmatin, is also found in the intercalated disc of cardiac muscle and both dense plaques and dense bodies of gizzard smooth muscle. However, unlike the major Z line component, α -actinin (21, 32), which is also located at the intercalated disc (44) and in the dense plaques and dense bodies, zeugmatin is not found in fibroblasts or epithelial cells as shown by both immunofluorescence microscopy (Fig. 1) and immunoblotting. Thus, it appears to be a muscle-specific protein.

Molecular Properties of Zeugmatin

Although immunoblots of the fascia adherens did not give clear cut results as to the molecular characteristics of zeugmatin, immunoblots of myofibrils prepared from fresh pectoralis muscle in the presence of a high concentration of EGTA did. These immunoblots (Fig. 10) consistently showed only a high molecular weight doublet that migrated slightly less than nebulin. The ambiguous results obtained using the fascia adherens are probably due to extensive breakdown of zeugmatin during the fascia preparation since the procedure

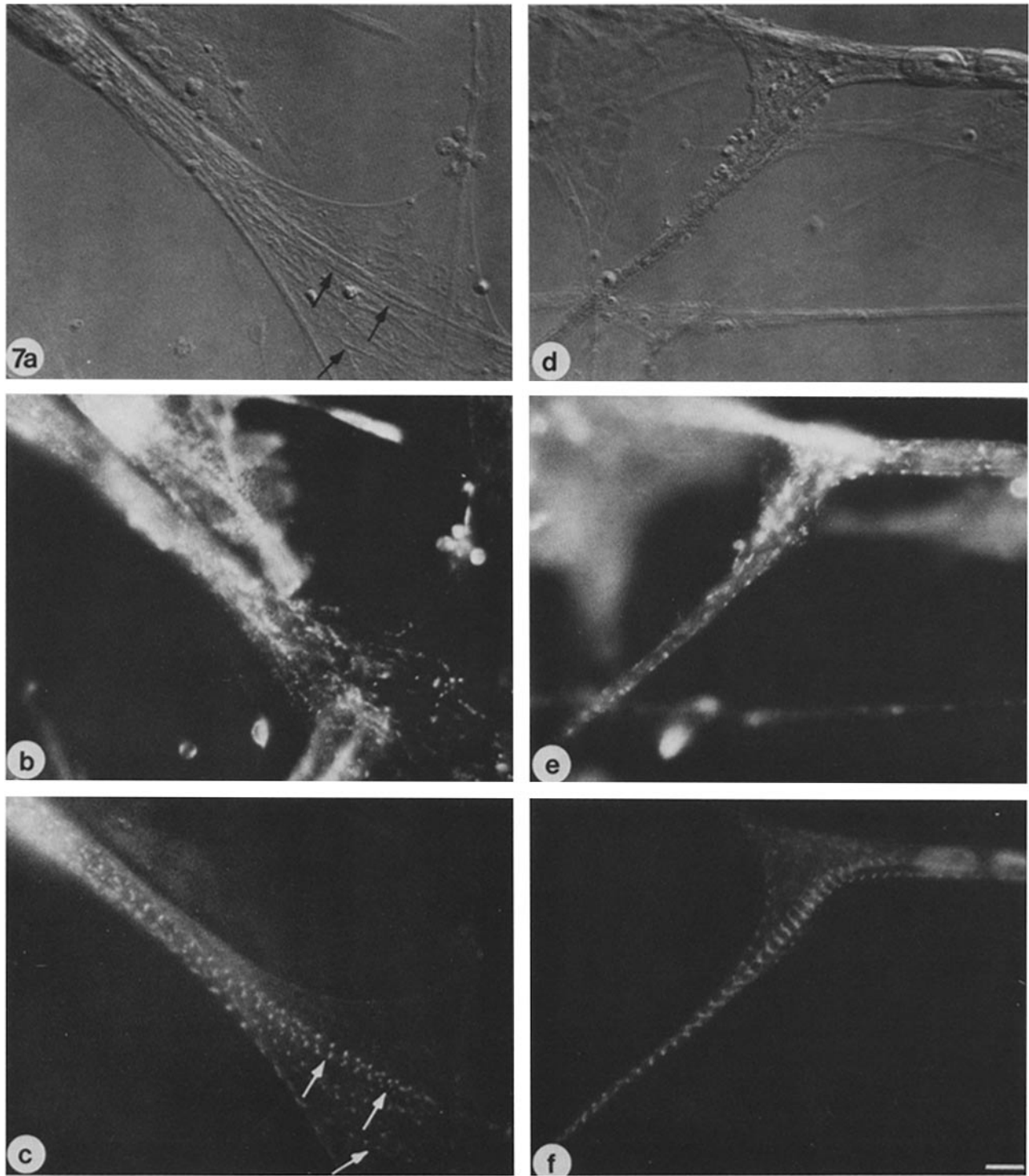


FIGURE 7 Indirect double immunofluorescent labeling of 1-(a-c) and 2-(d-f) cultured chick embryo muscle cells with rabbit anti-chicken gizzard α -actinin and McAb 20. a and d show the Nomarski images, b and e show the staining of the same cells for α -actinin, and c and f show the labeling with McAb 20. Arrows in a and c point to fibrils. Bar, 5 μ m. \times 1,250.

takes several days to complete and does not include EGTA. Indeed, myofibrils prepared by glycerination (35) or with little (1-2 mM) or no EGTA also gave confusing and variable results with immunoblotting. These results suggest that one of the proteases acting on zeugmatin is the Z line-associated Ca^{2+} -activated neutral protease (34). This protein has been reported to cause the degradation of a number of Z line components (39).

It was possible to partially solubilize zeugmatin from KI-extracted myofibrils by overnight treatment with 0.6 M KI which permitted the affinity purification of the protein. This extraction procedure led to some breakdown of zeugmatin. However, all the material eluted from the affinity column did react with McAb 20 on immunoblots, whereas no reaction was seen with material eluted from an unrelated affinity column run with a zeugmatin-enriched extract.

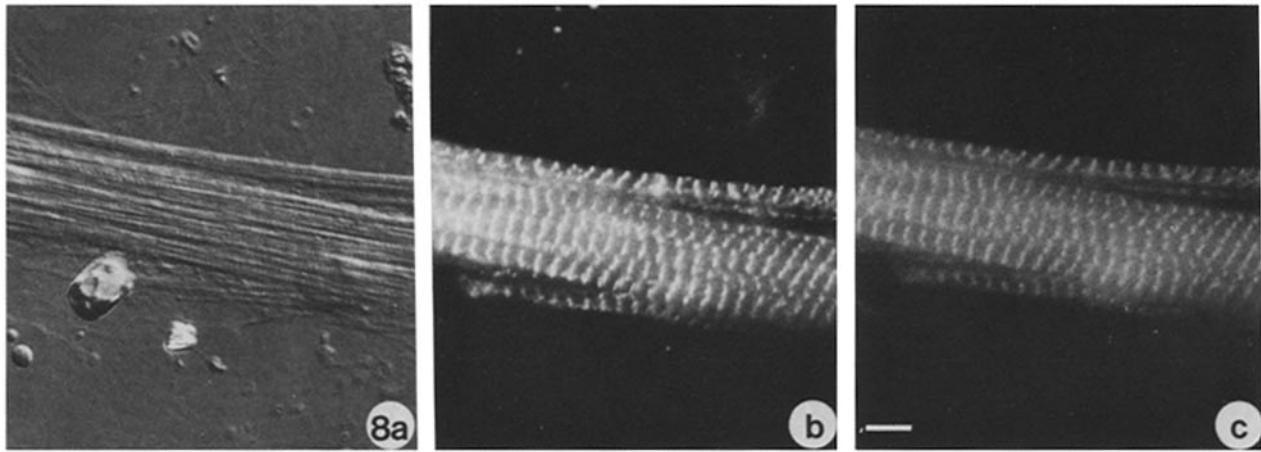


FIGURE 8 Indirect double immunofluorescent labeling of a 6-d cultured chick embryo muscle cell with rabbit anti-chicken gizzard α -actinin (b) and McAb 20 (c). a is the corresponding Nomarski image. Cross-striations are readily apparent throughout the myotube in a. Bar, 5 μ m. \times 1,250.

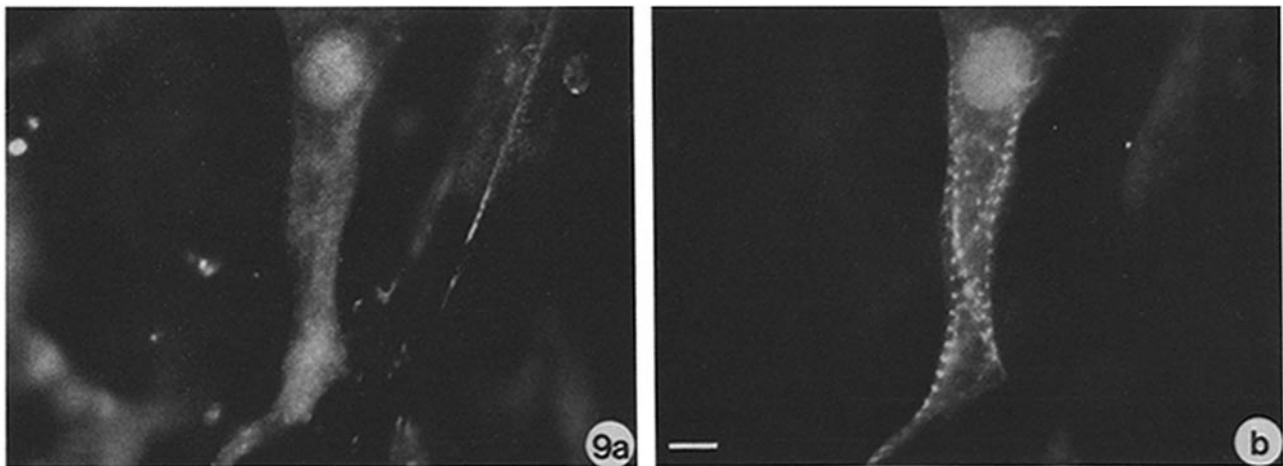


FIGURE 9 Indirect double immunofluorescent labeling of 1-d cultured chick embryo muscle cells with rabbit anti-chicken gizzard α -actinin (a) and McAb 284 (b). Bar, 5 μ m. \times 1,250.

We conclude that the high molecular weight doublet represents the intact form of the polypeptide antigen recognized by McAb 20, as well as by McAb 284. The two bands of the doublet by the intensity of immunoblot staining and Coomassie Blue staining appear consistently to be present in roughly equal amounts in purified preparations. The 125 I-peptide maps of the two components (Fig. 11) show them to be closely similar but not identical polypeptides, with each map exhibiting a few spots not detected in the other. These results suggest that the two components may be independently synthesized polypeptide chains, rather than the smaller one being simply a proteolytic fragment of the larger. These suggestions have to be explored further, however, by translation studies involving the specific mRNA for zeugmatin. If indeed zeugmatin is represented by the high molecular weight doublet of closely similar polypeptides, it differs strikingly from the case of spectrin and its analogues, a family of similarly high molecular weight proteins each of which shows a doublet by SDS PAGE. The two polypeptides of the spectrin doublet have completely different peptide maps (19, 51) and are immunologically distinct as well (19, 51).

Zeugmatin does not appear to be related to any previously reported high molecular weight muscle proteins. Connectin (31), titin (47, 49), and nebulin (48) are all highly sensitive to

proteolysis but are not found in the Z line. Titin and nebulin are also not found in smooth muscle (48). Furthermore, McAb 20 did not react with either titin or nebulin on immunoblots of myofibrils. Plectin (50) has been localized to both Z lines and intercalated discs but it is not muscle specific. In addition it appears to be found in desmosomes and gap junctions as well as fascia adherentes. Zeugmatin also does not seem to be the same as Z-nin (40), a 300–400-kD Z line protein, since their amino acid compositions are quite different. Nor is zeugmatin antigenically related to any one of a large number of other high molecular weight cytoskeletal proteins we have tested, including myosin and filamin. In fact, because of its sensitivity to proteolysis, it is highly unlikely that zeugmatin would have been previously recognized as a single entity and isolated by conventional biochemical techniques. An important feature of monoclonal antibody technology demonstrated by this work is its capacity to detect and to effect the isolation of such an unstable protein.

Localization of Zeugmatin in Muscle Cells

The evidence for the localization of zeugmatin to the Z line comes from immunofluorescent labeling studies with both isolated myofibrils and semithin frozen sections of adult

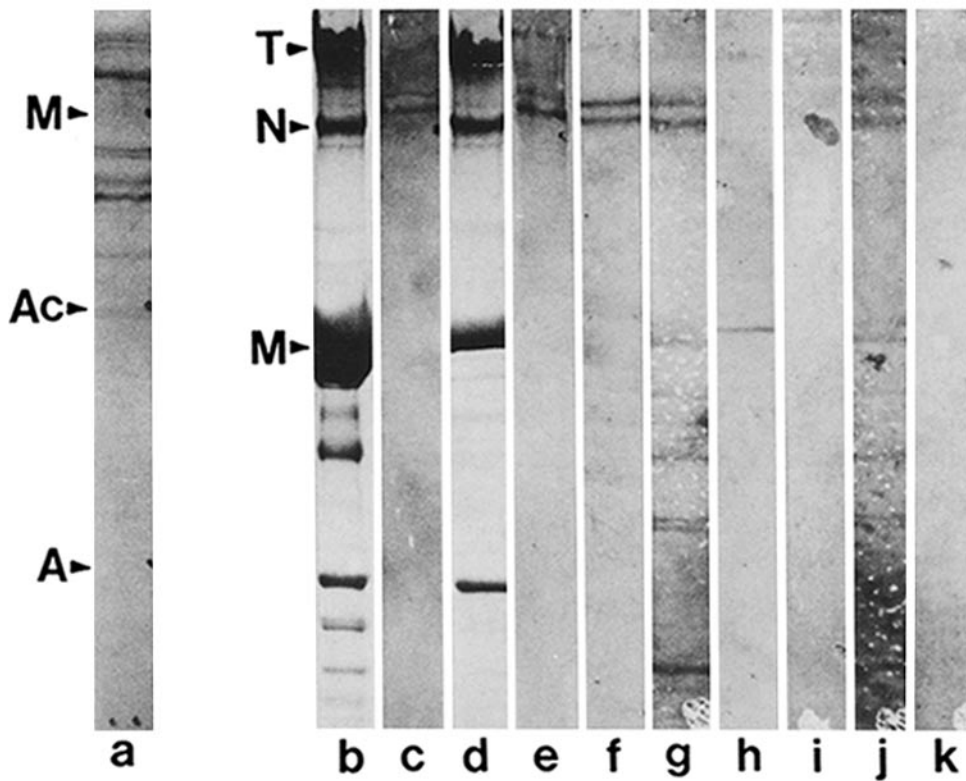


FIGURE 10 SDS PAGE of samples on 7.5% (a) or 5% (b-k) gels stained with Coomassie Blue (b, d, f, and h) or immunoblotted with McAb 20 (a, c, e, g, i, and k) or McAb 284 (j). (a) Purified fascia adherens (50 μ g protein); (b and c) myofibrils (50 μ g protein); (d and e) KCl-extracted myofibrils (50 μ g protein); (f, g, and i) eluant from McAb 20 column; (h and j) eluant from unrelated mouse IgG column; (k) purified sarcoplasmic reticulum (50 μ g protein). A, actin; Ac, α -actinin; M, myosin; N, nebulin; T, titin.

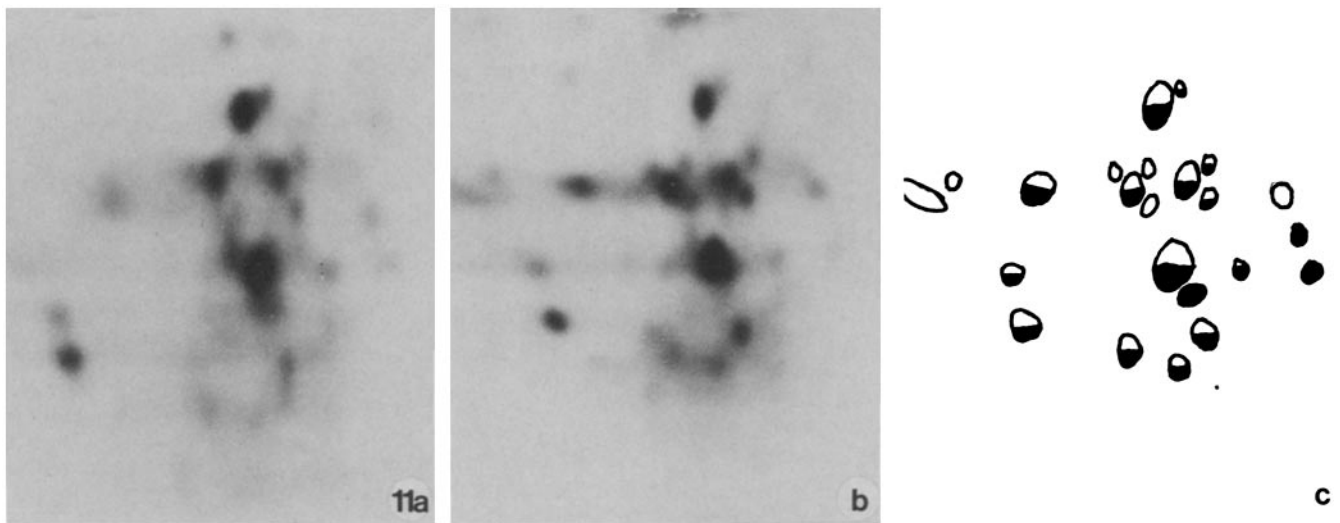


FIGURE 11 Two-dimensional 125 I-peptide maps of trypsin digests of the larger (a) and smaller (b) polypeptide bands of the zeugmatin doublet. c is a tracing showing spots unique to a (●), unique to b (○), and common to both (◐).

skeletal and cardiac muscle (Figs. 2-6). By phase and/or Nomarski optics the Z line can be clearly seen in both these types of samples, and the staining with McAb 20 was always superimposable upon it. From the immunofluorescent labeling pattern, zeugmatin appears to be an integral component of the Z line and not peripherally associated as is desmin (21). This conclusion is supported by the staining patterns seen with both isolated myofibrils and semithin frozen sections of striated muscle (Figs. 2-5). Peripheral Z line proteins such as desmin do not give uniform Z line labeling along the entire length of each isolated myofibril, whereas McAb 20 does. In the frozen sections McAb 20 staining was always present at the Z line throughout all levels of and across the entire widths of the sectioned myofibrils. This would not be expected if it

were labeling a protein peripheral to the Z line.

Although McAb 20 appeared to be staining a Z line protein the possibility existed that it was actually directed against a component of the sarcoplasmic reticulum. Several studies have shown (1, 7, 45, 46) that sarcoplasmic reticulum is specifically associated with the Z line in developing skeletal myotubes. In addition, some association of sarcoplasmic reticulum elements with the Z line has been observed in adult skeletal (46) and cardiac (13) muscle. We tested this possibility by purifying sarcoplasmic reticulum from skeletal muscle and immunoblotting SDS polyacrylamide gels of the sample with McAb 20 (Fig. 10k). No reaction was seen. It could be argued that zeugmatin was extracted from sarcoplasmic reticulum during its preparation. This is very unlikely since we found

TABLE I. Amino Acid Composition of Chicken Breast Zeugmatin

Amino acid	Mol %
Lys	8.9
His	2.9
Arg	5.2
Asx	6.7
Thr	6.2
Ser	8.0
Glx	15.1
Pro	0
Gly	8.4
Ala	8.0
Cys	1.1
Val	8.2
Met	2.2
Ile	5.1
Leu	6.5
Tyr	3.4
Phe	3.0
Trp	0.9

zeugmatin to be extremely resistant to extraction from skeletal muscle with the buffers used for sarcoplasmic reticulum preparation.

Finally, in recent experiments on the immunoelectron microscopic staining of ultrathin frozen sections of cardiac and skeletal muscle with McAb 20 (Cox, G. F., P. A. Maher and S. J. Singer, unpublished results) the presence of zeugmatin at the Z band, across the entire width of a myofibril, was confirmed.

In the studies with the isolated myofibrils the immunofluorescent labeling patterns of skeletal and cardiac muscle Z lines were indistinguishable (Fig. 2). This did not appear to be so, however, in the frozen sections (Figs. 3–5). In these samples, the labeling of skeletal muscle appeared as a pair of narrowly spaced stripes in the Z line region. Several possibilities exist to explain this. First, the pair of stripes could reflect dissociation of the antigen from the Z line during stretching of the muscle. This possibility is rendered unlikely by the fact that relatively unstretched muscle with sarcomere lengths of 2.5–3.0 μm also showed the paired stripe staining pattern. Second, the pair of stripes staining could reflect immunolabeling of an antigen that is located all along the actin filaments, but in the region of overlap of the actin and myosin filaments steric hindrance makes the antigen inaccessible to the antibody. By stretching the muscle to greater lengths, we could decrease the region of overlap of the actin and myosin filaments. The width of the immunofluorescently labeled pair of stripes was, however, constant over a wide range of sarcomere lengths, thus eliminating this possibility. Third, the antigen might be found throughout the Z line but when the muscle is fixed only the antigen at the edges of the Z line is accessible to the antibody. This is unlikely in view of the fact that shorter fixation times do not eliminate the paired stripe staining. However, the possibility remains that the epitope to which McAb 20 is directed is unavailable to react with antibody in the central domain of the Z line. Fourth, the antigen may be located at the edge of the Z line or in the I band very close to the Z line. At the light microscopic level of resolution, one cannot distinguish between these two latter possibilities. Our preliminary immunolabeling results at the level of resolution of the electron microscope, however, provide strong support for the localization of zeugmatin on the outer edges

of the Z band (unpublished results).

The absence of the paired stripe staining at the Z line in cardiac muscle is puzzling in view of the close structural similarities of cardiac and skeletal muscle Z lines. A different distribution of zeugmatin throughout the width of the Z line in the two tissues could explain the different labeling patterns. However, this seems unlikely. It is possible that a greater amount of zeugmatin in cardiac than in skeletal Z lines could lead to an optical fusion of two fluorescent stripes, but this explanation is also unlikely in view of the fact that the McAb 20 Z line immunostaining in skeletal muscle is almost twice as wide (1.0–1.2 μm) as that in cardiac muscle. Another possibility is that the cardiac myofibrils are not as well aligned in all dimensions as in skeletal muscle, and as a consequence the two stripes may always overlap within the thickness of the cardiac muscle section. Overlap of the stripes due to the thickness of the specimen could also explain why a single stripe is seen in the isolated skeletal and cardiac myofibrils. Further analysis of McAb 20 staining in cardiac muscle at the electron microscope level should resolve this confusion.

In cardiac muscle, labeling for zeugmatin was observed at the intercalated discs (Figs. 2 and 3) as well as at the Z lines. This explains why immunization with a purified fascia adherens preparation induced monoclonal antibodies to zeugmatin. These results provide additional evidence for a structural relationship between the fascia adherens of intercalated discs and Z lines (33). Both structures are sites of termination of actin microfilaments. Ultrastructural analysis of cardiac muscle showed the fascia adherens domain of the intercalated disc to be continuous with the Z line (13, 33). In addition, the filamentous mat on the intracellular surface of the fascia adherens was seen to be similar in appearance and staining characteristics to the substance of the Z line in the electron microscope (33). Biochemically, the fascia adherens and Z line have also been found to be similar in early studies that showed both sites were extracted by identical urea concentrations (37). More recently, α -actinin was localized to both sites by immunoelectron microscopy (44). This is not to say, however, that the two structures are identical. The fascia adherens is intimately associated with the cardiac cell membrane, the Z line is not. In addition, the protein vinculin (16, 17) is located at the fascia adherens (and other sites of actin-membrane attachment) but not at the Z line.

In gizzard smooth muscle, immunofluorescent labeling for zeugmatin was concentrated at discrete intracellular sites that by double labeling for α -actinin (17) were identified as the dense bodies (Figs. 2 and 6), and along the plasmalemma of the cells at what were most likely the dense plaques. The dense bodies are structures thought to be homologous to the Z lines of striated muscle (17), and the dense plaques to the fascia adherens membrane domains of cardiac muscle (16, 17, 44), so the presence of zeugmatin at all of these sites is reasonable. To obtain the immunolabeling of the gizzard preparations with McAb 20, however, special treatments were required. The belated recognition of this requirement had led us initially to infer that zeugmatin was not present in gizzard (29) because no immunolabeling was observed with frozen sections of the tissue prepared under the same conditions that allowed the labeling of skeletal and cardiac muscle. However, immunoblotting of extracts of gizzard then showed zeugmatin to be present, and so other specimen preparation procedures were investigated. Only prior prolonged glycerination of the gizzard tissue gave frozen sections that were immunolabeled

with McAb 20, suggesting that the antigen in the dense bodies and dense plaques is not readily accessible to the antibody. The isolated gizzard myofibrils did stain without glycerination, but their preparation included several washes with Triton X-100 and they were not fixed. Fixation of both the isolated myofibrils and the glycerinated gizzard samples for frozen sectioning for more than 20 min in 3% formaldehyde brought about a loss of labeling with McAb 20, also suggesting the possibility that zeugmatin is not readily accessible to the antibody in this tissue. Thus, even mild cross-linking appears to prevent the antibody from interacting with the antigen in this tissue.

Zeugmatin in the Early Myofibrillogenesis of Skeletal Muscle Myotubes

One of the most interesting results obtained from these studies is the observation that zeugmatin is present in a fluorescent cross-striated pattern in myotubes of 1- and 2-d regenerating muscle cell cultures, although it appears to be absent from postmitotic myoblasts (Fig. 1). Since we have shown that zeugmatin is localized to the Z line in adult myotubes, the fluorescent cross-striations observed in early developing myotubes should be due to Z line-localized zeugmatin as well. The fluorescent cross-striations are initially localized to one or more longitudinal fibrils that appear to lie just below the myotube cell surface (Fig. 7). These fibrils often do not appear cross-striated by Nomarski or phase optics. There is a fair amount of cell to cell variation so that ~50% of the cells with two or more nuclei contain fibrils that give fluorescent cross-striations with McAb 20. The remaining multinucleated cells show an overall diffuse fluorescence as well as a punctate staining with McAb 20 along the fibrils. Such variability is common for both developing and regenerating chick embryo muscle cell cultures (8, 11, 22). These observations with McAb 20 are reminiscent of those of Holtzer et al. (22) who studied myosin distribution in developing myotubes of 2-4-d chick embryos using fluorescein-conjugated rabbit anti-myosin. They found their anti-myosin labeling appearing in the myotubes as a banded pattern in the fluorescence microscope at times when cross-striations were not visible by phase optics.

The formation of the first fibrils predominantly just below the cell surface in early cultures of myotubes has been discussed in detail previously (11, 22). The significance of this phenomenon is still not clearly understood, but several groups (25, 33) have suggested the Z line may originate from material that is first associated with or derived from the plasma membrane. Our finding that zeugmatin, a protein that is also associated with the fascia adherens membrane domains of cardiac cells and the membrane-localized dense plaques of gizzard smooth muscle cells, is found in fluorescent cross-striations of membrane-associated immature fibrils in skeletal myotube cultures lends some support to this proposal.

A number of ultrastructural studies have investigated the appearance of Z lines in developing and regenerating embryonic chick muscle (1, 8-11, 22). In all these studies, the Z line, or its progenitor, was identified solely on morphological grounds, by virtue of the appearance of electron dense material associated with the sarcomeric structure of the developing myofibrils. No reports placed the appearance of this electron dense material earlier than 3 d in culture or in ovo. Such early Z lines tend to be quite diffuse and irregularly spaced along

the bundles of actin and myosin filaments. The majority of evidence (11) indicates that the actin and myosin filaments are already organized into interdigitating hexagonal arrays before the appearance of this electron dense Z line material. However, it is not clear whether the actin and myosin filaments self assemble to form these arrays or whether other factors are involved since no confirmed reports of successful reconstruction of myofibrils in vitro have appeared (12).

α -Actinin is a major component of the Z line and as such has been postulated to play a key role in the organization of the myofibril. Thus, the results of our double labeling studies showing that zeugmatin, also a Z line component, is organized into fluorescent cross-striations well before the appearance of α -actinin at Z line locations, are most interesting. In agreement with earlier work we first saw α -actinin in a patchy distribution along the fibrils and the cell periphery (14, 20) with more intense staining around the nucleus (24). The earliest appearance of α -actinin in a cross-striated pattern occurred in some cells in 3-d cultures, again in agreement with earlier studies (14, 20, 24). These striations, however, were wider in the longitudinal direction and more diffuse than those seen with McAb 20 at this time. It was also found in an earlier biosynthetic study (2) that α -actinin did not begin to accumulate in regenerating muscle cells until after 72 h in culture. Our studies demonstrate that at the immunofluorescent level zeugmatin is present at the positions of Z lines in very young myotubes in culture, but α -actinin is not and thus the latter probably does not play a role in the early organization of the myofibrillar apparatus. It may, however, be necessary for myofibrillar growth as a recent study has suggested (38). It has recently been shown (5) that developing chicken skeletal muscle myotubes in vitro contain both a smooth muscle-type and a skeletal muscle-type α -actinin. These two types of α -actinin are differentially organized within the developing myotube such that smooth muscle α -actinin has a diffuse distribution, whereas skeletal muscle α -actinin initially has a punctate, and eventually a cross-striated, distribution. Our antibody to chicken gizzard α -actinin also reacts with skeletal muscle α -actinin since it gives a cross-striated staining pattern in 6-d myotubes (Fig. 8) and adult myofibrils. Furthermore, it is unlikely that the staining of smooth muscle-type α -actinin by our antibody is obscuring a cross-striated pattern of skeletal muscle-type α -actinin in the 1- and 2-d myotubes since cross-striations are not visible in areas where the overall labeling for α -actinin is very low (Fig. 7b).

One difficulty with our findings is that morphological Z bands are not recognized in the electron microscope at times corresponding to those when we find zeugmatin to exhibit a cross-striated pattern by immunofluorescence (1, 8-11, 22). Since the identification of the Z band in the electron microscope requires that it be electron dense, it may be that zeugmatin does not produce an electron dense band when it interacts with the fibrils. Extraction studies (6) that followed the disappearance of the Z band indicated that removal of the electron dense material only removed 2% of the myofibrillar mass, whereas the Z band was estimated to comprise 6% of the myofibrillar mass. A detergent treatment that removed the electron dense Z band eluted only proteins of 95 and 85 kD. Our preliminary experiments indicated zeugmatin is not in this extract. Thus, the absence of electron dense material in the very early myotubes may not mean that no Z band components are present, but simply that Z band components mainly responsible for the electron density are absent.

Resolution of this question will require further studies at the electron microscope level.

In conclusion, we have identified a new Z line component that gives a fluorescent cross-striated pattern during the early stages of myofibrillogenesis. Since this protein appears in the Z line before α -actinin it is a better candidate than α -actinin for a critical role in the organization of the myofibril. The location of this protein at both the Z line and the intercalated disc, particularly in the early-putative Z lines of developing myotubes, suggests that it may directly or indirectly link actin filaments to these structures. This putative linking function and the location of this protein at the Z line are the reasons we have named it zeugmatin ($\xi\epsilon\rho\gamma\mu\alpha \equiv$ yoking).

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