Localization of Z-Protein in Isolated Z-Disk Sheets of Chicken Leg Muscle

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ABSTRACT Immunoblotting studies with antisera against Z-protein, desmin, and α-actinin showed that Z-protein is clearly distinguishable from desmin and α-actinin. Z-protein is not a proteolytic product of another protein but is an intrinsic component of chicken breast muscle myofibrils. In these experiments, an SDS extract of intact muscle was first electrophoresed in a polyacrylamide gel, and then proteins were transferred to a nitrocellulose paper sheet. Detection of each protein on the sheet was made possible by the application of the indirect immunofluorescence technique with the respective antiserum.

Immunofluorescence microscope studies using these antisera revealed that Z-protein has the same distribution as α-actinin in isolated Z-disk sheets. Anti-Z-protein antiserum and anti-α-actinin antiserum stained the interior of Z-disks. On the other hand, antiserum against desmin stained the periphery of Z-disks in isolated Z-disk sheets.

Since Lazarides and Weber showed the distribution of actin in nonmuscle cells using the immunofluorescence technique with antibody against cell actin (19), immunofluorescence studies of the distribution of proteins involved in cell motility or cytoskeleton structure have been explosively advanced. For example, myofibrillar proteins such as myosin, tropomyosin, and α-actinin have been found distributed widely in nonmuscle cells by use of the immunofluorescence technique (20, 21, 37).

Recently, four kinds of proteins—desmin, vimentin, synemin, and filamin (actin-binding protein)—which were originally thought to be limited to smooth muscle cells or nonmuscle cells, have also been shown to be localized in myofibrils (1, 9, 10, 22). Among them, desmin has been well characterized by several groups of investigators (6, 8, 12, 14, 22, 23, 33).

Granger and Lazarides developed an elegant method to isolate Z-disk sheets from chicken leg muscle (8). This method made it possible to elucidate the roles of Z-band components in myofibrils. Besides actin, the structural proteins constituting the Z-bands of the myofibrils of vertebrate skeletal muscle are classified into two groups by their distribution in isolated Z-disk sheets. One group contains desmin, vimentin, synemin, and filamin (actin-binding protein). They are distributed at the periphery of Z-disks. Only α-actinin has been listed in another group, localized in the interior of Z-disks (8).

Desmin (skeletin), originally discovered as the component of 10-nm filaments of chicken gizzard (33), was found distributed at the periphery of Z-disks in isolated Z-disk sheets by the indirect immunofluorescence technique with antibodies against gizzard desmin. Granger and Lazarides suggested that desmin filaments are connecting adjacent Z-disks of neighboring myofibrils (8). Vimentin filaments have been shown to be present in a variety of cells by the immunofluorescence technique (3). Vimentin, in myofibrils, coexists with desmin and may take the same role as desmin (5, 9). Synemin was regarded as a protein associated with desmin and vimentin filaments. Filamin, a high molecular weight actin binding protein, was isolated from chicken gizzard (35) and has been shown to be identical with actin-binding protein (ABP) isolated from macrophages (11). Antibodies raised against gizzard filamin specifically stained the Z-band regions of chick skeletal muscle using the indirect immunofluorescence technique (1)

There is another branch in the investigation of the components of myofibrillar Z-bands. During a search for regulatory proteins in myofibrils, α-actinin was discovered to be a major component of Z-bands (2, 24). α-Actinin was localized in the interior of Z-disks in isolated Z-disk sheets by the indirect immunofluorescence technique with antibodies against α-actinin (8). Therefore, α-actinin is thought to be the substance constituting Z-disks. However, α-actinin is not regarded as a Z-lattice component. The role of α-actinin in myofibrils has...
not been clearly elucidated. Eu-Actinin was coextracted with α-actinin by a low ionic strength solution from myofibrils (17). A high molecular weight protein (220,000 mol wt) was found in the KI extract of I-Z-I brushes of myofibrils together with actin and α-actinin (26-28). Although the presence of these two proteins at the Z-bands was demonstrated (17, 28), their localization in isolated Z-disk sheets has not yet been determined. We purified Z-protein, whose molecular weight was 55,000, from a KI extract of chicken breast muscle myofibrils (30, 31). Z-protein formed lattice structures in vitro which were similar to Z-band lattice structures in situ. We suggested the possibility that Z-protein is the essential component of the basic lattice structure of myofibrillar Z-disks.

In the present study, we show that Z-protein is localized in the interior of Z-disks in isolated Z-disk sheets and is immunologically different from desmin and α-actinin. This work, we hope, gives a new clue to the study of myofibrillar Z-disks of vertebrate skeletal muscle.

MATERIALS AND METHODS

Preparation of Myofibrils and Z-Disk Sheets

Myofibrils were prepared from chicken breast muscle, *muscus pectoralis*. Muscle strips, ~2 mm in diameter, which were stretched about 1-2 fold beyond the length of resting muscle, were tied to glass rods with cotton threads and immersed for 24 h at 0°C in a solution containing 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2, 10 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 50% glycerine. After the solution was changed once, stretched muscle strips were homogenized three times in a Waring blender at 15,000 rpm, for 10 s each time, in a solution containing 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2, and 10 mM EGTA. Z-Disk sheets were prepared from chicken leg muscle, *muscus iliotibialis*, essentially according to Granger and Lazarides (8). Glycinated muscle strips tied to glass rods, which had been prepared in the same way as above, were immersed in a solution containing 0.6 M KI, 20 mM sodium thiosulfate, 20 mM Tris-HCl, pH 7.5, and 10 mM EGTA for 2-5 d in a cold room. The KI-extracted muscles were homogenized in a Waring blender at 5,000 rpm for 20 s. Z-Disk sheets were released into the solution.

Preparation of Desmin and α-Actinin

Crude desmin was prepared from chicken gizzard according to Hubbard and Lazarides (12) and purified by the method of Huiatt et al. (13) using a hydroxyapatite column and a DEAE-celulose column. Crude α-actinin was prepared from chicken breast muscle according to the method of Masaki and Takaiti (25) and further purified by DEAE-Sephadex column chromatography.

Preparation of Z-Protein

Z-Protein was prepared from chicken breast muscle by the method described elsewhere (30, 31). Five hundred grams of chicken breast muscle was homogenized in a Waring blender with 3 liters of 0.15 M NaCl. The homogenate was centrifuged at 5,000 rpm. The sediment was extracted with the Hasselbach-Schneider's solution, 0.6 M KCl, 10 mM NaH2PO4, 1 mM MgCl2, and 0.1 M potassium phosphate buffer, pH 6.5, for 1 h in a cold room, and the residue of the extracted muscle was collected by centrifugation at 5,000 rpm. The extraction and centrifugation were repeated more than five times. The residue was washed with glass distilled water more than five times. Then, the residue was extracted with 0.6 M KI for 24 h at 4°C. After dialysis against 1 mM NaHCO3, the extract was treated with (NH4)2SO4 between 20 and 40% saturation. The precipitate was dialyzed against 20 mM sodium phosphate buffer, pH 7.5, and then loaded onto a DEAE-Sephalose column (1 x 10 cm). Elution was carried out with a linear concentration gradient of NaCl, from 0 to 0.3 M. Fractions eluted around 0.05 M NaCl contained ~10 mg of pure Z-protein.

Antisera and Indirect Immunofluorescent Microscopy

Antisera against purified chicken myofibrillar Z-protein, chicken gizzard desmin, and chicken myofibrillar α-actinin were produced in rabbits separately according to our routine procedures as described elsewhere (29). Goat FITC-labeled anti-rabbit IgG antibodies were purchased from Miles-Yeda Ltd. (Israel). Myofibrils and isolated Z-disk sheets were mounted on slide glasses and fixed with a solution containing 7% formalin, 50 mM NaCl, and 20 mM sodium phosphate buffer, pH 7.2, for 10 min at room temperature. After being washed thoroughly with a solution consisting of 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2, and 10 mM EGTA, samples were treated with 60-fold or 120-fold diluted antisera for 30 min at room temperature and then were washed with the same NaCl solution. Secondary staining with FITC-labeled anti-rabbit IgG antibodies was carried out in the same way. After being washed again, the specimens were observed under a fluorescence microscope (Fluophot, Nikon) and photographed on Kodak Tri-X film.

Indirect Immunofluorescence on Nitrocellulose Paper Sheets (Immunoblotting Method)

Column-purified Z-protein, desmin, and α-actinin were treated with an equal volume of an SDS solution containing 2% SDS, 0.0625 M Tris-HCl buffer, pH 6.6, 2% 2-mercaptoethanol, and 25% glycerine. Freshly minced chicken breast muscle was put into an appropriate volume of the hot SDS solution mentioned above and boiled for 5 min at 100°C in a water bath. Insoluble residues were removed by centrifugation at 5,000 rpm for 10 min. These samples were electrophoresed essentially according to the method of Laemmli (18), using 10% polyacrylamide gels as separating gels and 3% polyacrylamide gels as stacking gels. The SDS-slab gel electrophoresis was carried out using a solution containing 0.025 M Tris, 0.129 M glycine, pH 8.7, and 0.1% SDS as the electrode buffer. After electrophoresis, peptides were transferred to nitrocellulose paper sheets essentially according to the method of Towbin et al. (34). Polyacrylamide gel sheets were cut into two parts vertically. One part was stained with 0.2% Coomassie Brilliant Blue R-250 in 7% acetic acid and 40% methanol. Another part, put on nitrocellulose paper sheets, was set between two carbon electrodes (10 x 10 cm) covered with chemical sponge sheets. Then peptides were electrophoretically transferred from gels to nitrocellulose sheets. Blotting was performed under the constant current of 200 mA for 2 h at room temperature, using a solution containing 5% ethanol, 0.025 M Tris, and 0.129 M glycine, pH 8.7 as the electrode buffer. The sheets were then soaked in a 1% bovine serum albumin solution containing 0.15 M NaCl and 20 mM sodium phosphate buffer, pH 7.2, for more than 2 h at room temperature. The sheets were treated with 10-fold diluted antisera against Z-protein, desmin, and α-actinin, respectively, for 30 min at room temperature. Secondary reactions of the paper sheets with goat FITC-labeled anti-rabbit IgG antibodies were carried out, after thoroughly washing the sheet surfaces with a solution consisting of 0.15 M NaCl and 20 mM sodium phosphate buffer, pH 7.2, for 30 min at room temperature. After washing the sheet surfaces with the NaCl solution mentioned above, we observed the sheet surfaces under an UV lamp Ultra-Violet Products, Inc., UV-54, wave length = 254 nm) set at the distance of 15 cm, and photographed on Kodak Tri-X film through an UV cut filter, in a dark room. The time of exposure was 5 s when the lens-opening was set at f/3.5.

RESULTS

Immunoblotting of Z-Protein, Desmin, α-Actinin, and Muscle Peptides with Antisera against Z-protein, Desmin, and α-Actinin

Column-purified chicken breast muscle Z-protein, chicken gizzard desmin, and chicken breast muscle α-actinin were blotted onto nitrocellulose paper sheets from SDS-slab electrophoresed polyacrylamide gels. Z-Protein and desmin were electrophoresed on the same gel to compare their mobility. The sample well of this gel was divided into two spaces with a silicon rubber wedge to make the Z-protein lane and desmin lane border on each other. Fig. 1a shows the Coomassie Blue-stained patterns of Z-protein, desmin, and α-actinin. Z-Protein and desmin were not electrophoretically distinguishable from each other. These two proteins also had the same mobility in an SDS polyacrylamide gel electrophoresis by the method of Weber and Osborn (36). Fig. 1 shows the indirect immunofluorescent staining patterns of Z-protein, desmin, and α-actinin with anti-Z-protein antiserum (b), anti-desmin antiserum (c), and anti-α-actinin antiserum (d), respectively. Antiserum
raised against chicken myofibrillar Z-protein stained only the Z-protein band (Fig. 1 b). Antiserum against chicken gizzard desmin and antiserum against chicken myofibrillar α-actinin stained the desmin and α-actinin bands, respectively. Z-Protein and desmin were each applied in an amount of ~1.2 μg per cm gel width and α-actinin was 0.15 μg per cm gel width. There was a faint Coomassie Blue-stained band just under the Z-protein band. This peptide was usually contained in column-purified Z-protein samples. It is unknown whether this peptide is a proteolytic product of Z-protein, a molecular subspecies of Z-protein, or another protein.

Peptides of a whole SDS extract of chicken breast muscle were blotted onto the nitrocellulose paper sheets from the polyacrylamide gels in which fresh chicken breast muscle peptides were electrophoresed. Fig. 2 a shows the Coomassie Blue-stained pattern of chicken breast muscle treated with an SDS solution. Fig. 2 b shows the corresponding indirect immunofluorescence staining pattern with antiserum against chicken gizzard desmin, chicken myofibrillar Z-protein, and chicken myofibrillar α-actinin. Anti-α-actinin antiserum stained very strongly the 100,000 dalton (100 kdalton) peptide band, and stained faintly the 160 kdalton band. The 160 kdalton protein was probably M-protein judging from the molecular weight, since α-actinin samples were often contaminated with M-protein. Anti-desmin antiserum stained the 55 kdalton peptide band. Anti-Z-protein antiserum stained the 55 kdalton peptide band and 110 kdalton peptide band. The SDS solution which dissolved chicken breast muscle was boiled for 20 min at 100°C in a water bath. Fig. 2 c shows the Coomassie Blue-stained pattern of this sample. The corresponding indirect immunofluorescence pattern is shown in Fig. 2 d. A rather strong fluorescent band is observed at the 55 kdalton peptide band and a trace of fluorescence is observed at the 110 kdalton band.

In Fig. 1 a and b, the fluorescent band of desmin stained with anti-desmin antiserum and the fluorescent band of Z-protein stained with anti-Z-protein antiserum are wider than their Coomassie Blue-stained bands. In the case of a whole SDS extract of chicken breast muscle, the fluorescent band stained with anti-α-actinin antiserum is wider than the α-actinin band stained by Coomassie Brilliant Blue (Fig. 2). These results show that the immunoblotting method using FITC-labeled antibodies is a very sensitive method to detect specific antigens. Peptides which had not been stained by Coomassie Brilliant Blue were detected as immunofluorescent bands.

**Immunofluorescence of Myofibrils and Isolated Z-Disk Sheets with Antiserum against Desmin, α-Actinin, and Z-Protein**

As is shown in Fig. 3, Z-bands were strongly fluorescent in every case, when myofibrils were treated with antiserum raised against desmin, α-actinin, and Z-protein. The 60-fold diluted anti-α-actinin antiserum stained Z-bands very strongly, and often stained M-lines very weakly (photographs are not shown). However, the 120-fold diluted anti-α-actinin antiserum seldom stained M-lines. Therefore, in the present study, we used the 120-fold diluted anti-α-actinin antiserum to avoid the effect of contaminant antibodies. M-Line fluorescence was probably due to contaminant anti-M-protein antibodies, judging from the result of immunoblotting experiments (Fig. 2 b).

Anti-desmin antiserum strongly stained the periphery of Z-disks in isolated Z-disk sheets and weakly stained the interior (Fig. 4 a, b). On the other hand, as is shown in Fig. 4 c and d, anti-α-actinin antiserum stained the interior of Z-disks in isolated Z-disk sheets. Immunofluorescence of Z-disks with anti-α-actinin antiserum was very strong. In our preparation, honeycomblike sheets of Z-disks were not often observed, but staining patterns of Z-disk sheets with anti-desmin antiserum and anti-α-actinin antiserum were essentially the same as shown by Granger and Lazarides (8). As is shown in Fig. 4 e and f, the distribution of Z-protein, as revealed by indirect immunofluorescence with antiserum against Z-protein, was in the interior of Z-disks in isolated Z-disk sheets. This is the same as the case with anti-α-actinin.

Z-Disk sheets were obtained from stretched muscle fibers

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**Figure 1** Indirect immunofluorescence on nitrocellulose paper sheets onto which peptides were blotted from electrophoresed polyacrylamide gels. (a) Coomassie Blue-stained polyacrylamide gels before blotting. Z-protein and desmin were electrophoresed on the same gel to compare the mobility. (b, c, d) Indirect immunofluorescent staining patterns on the nitrocellulose sheets with anti-Z-protein antiserum, anti-desmin antiserum, and anti-α-actinin antiserum, respectively. 1, chicken gizzard desmin; 2, chicken myofibrillar Z-protein; 3, chicken myofibrillar α-actinin.
extracted with a KI solution. The KI-extracted fibers seemed to preserve the Z-band components fairly well (7-10). Although both Z-protein and α-actinin can be extracted from myofibrils with 0.6 M KI, the extraction of Z-protein is more difficult than that of α-actinin in our experience. It is very likely that both α-actinin and Z-protein are retained at their original location after extraction with 0.6 M KI.

DISCUSSION

Z-Protein and desmin, both present in Z-bands of vertebrate skeletal muscle, have a similar molecular weight of 55,000 (31).

The two proteins cannot be distinguished by usual SDS gel electrophoresis (Fig. 1 a). As already reported, the amino acid compositions of the two proteins are quite different from each other (31). Desmin has been demonstrated to have two isopro- teins, α- and β-desmins, detectable by isoelectric focusing (14). Z-Protein is focused to several spots by the usual isoelectric focusing technique (Ohashi, K., unpublished observations). The isoelectric point of Z-protein is more basic than those of α- and β-desmins.

We showed the difference between Z-protein and desmin by the double immunodiffusion tests using antisera against chicken myofibrillar Z-protein (31). Antiserum against Z-protein did not cross-react with desmin by double immunodiffusion tests (data are not shown). More directly and clearly, an additional experiment (Fig. 1) indicated the difference among Z-protein, desmin, and α-actinin. Fig. 1 shows that Z-protein is immunologically different from desmin and α-actinin. This result indicates that Z-protein is not a proteolytic product of α-actinin. Both a 55 and a 110 kdalton protein reacted with anti-Z-protein antisera when chicken breast muscle was treated with an SDS solution and boiled for 5 min at 100°C (Fig. 2 b). When chicken breast muscle was treated with an SDS solution for a longer period of time (20 min at 100°C) only a faint band corresponding to the 110 kdalton peptide was observed by the immunoblotting method with anti-Z-protein antisera (Fig. 2 d). The ratio of the 110 kdalton protein to the 55 kdalton protein changed case by case, and one repre- sentative example is shown in Fig. 2. Judging from these results the 110 kdalton protein is probably a disulfide-dimer of Z-protein. The Coomassie Blue-stained pattern of myofibrils treated with an SDS solution for 20 min at 100°C is slightly different from that treated for 5 min (Fig. 2 a, c). This may be accountable by the following reason. When muscle tissue block is reacted with a hot SDS solution for a short period of time, some constituents of myofibrils are insufficiently dissolved into single peptides. Z-Protein may be among the myofibrillar proteins that are less easily extracted with a hot SDS solution.

No other peptide was fluorescent when stained with anti-Z-protein antisera by use of the immunoblotting method. It is very difficult to postulate that the 110 kdalton protein was...
solely digested into the 55 kdalton peptides. Therefore, it is very unlikely that Z-protein is a proteolytic product of any protein of molecular weight higher than that of Z-protein. It may be concluded that Z-protein is an intrinsic component of myofibrillar Z-bands.

The immunofluorescence band of α-actinin (Fig. 2 b, lane 3) is wider than that of Coomassie Blue-stained band of α-actinin (Fig. 2 a). A part of nitrocellulose paper sheet was stained with amido black after myofibrillar proteins were blotted on its surface. The staining pattern was the same as that of the Coomassie Blue-stained gel. Therefore, the broad labeled band is due to the specific reaction of anti-α-actinin antibody with α-actinin not to be detected by Coomassie Blue-staining. Immunoblotting of Z-protein and desmin showed the same cases as is α-actinin in myofibrils (Fig. 1 b, c). However, when a small amount of α-actinin was electrophoresed, the labeled band was sharp (Fig. 1 d). Judging from these results, the content of Z-protein in myofibrils is thought to be very small.

In vertebrate skeletal muscle, each sarcomere in neighboring myofibrils is arranged in register and, therefore, cross striations are observed. Granger and Lazarides obtained a sheet of Z-disks by blending myosin- and actin-extracted myofibrillar bundles (8). Immunofluorescence staining patterns of Z-disk sheets with antisera against desmin and vimentin suggested that each Z-disk is interconnected with adjacent Z-disks by desmin and vimentin filaments (5, 7–9).

**Figure 4** Indirect immunofluorescent staining of isolated Z-disk sheets with anti-desmin antiserum, anti-α-actinin antiserum, and anti-Z-protein antiserum. Upper panels, phase contrast image; Lower panels, indirect immunofluorescent image; (a), (b) Z-disk sheets incubated with anti-desmin antiserum. (c), (d) Anti-α-actinin antiserum. (e), (f) Anti-Z-protein antiserum. Bar, 10 μm.
In the present study, anti-desmin antiserum stained the periphery of Z-disks strongly and the interior of Z-disks weakly. No contaminant antibodies were present in this anti-desmin antiserum, because, as is shown in Fig. 2 a, the anti-desmin antiserum exclusively stained the desmin band among blotted chicken breast muscle peptides. It is probable that the weak fluorescence in the interior of Z-disks is nonspecific immunofluorescence.

The fine structure of Z-disk itself has not yet been revealed on the molecular level. α-Actinin and actin were shown as the components of Z-disks by immunofluorescent staining of Z-disk sheets (8). It is thought that the one end (barbed end) of actin filaments are attached to the lattice structure of Z-disks (16). α-Actinin is not regarded as a basic lattice component of Z-disks, because α-actinin is easily extracted from myofibrils with a low ionic strength solution without destruction of the lattice structure (26). It appears that α-actinin is a cementlike substance affixed to actin filaments at the Z-disk.

We have suggested that Z-protein would be a component of the Z-lattice structure, based on the fact that it forms Z-lattice-like structures in vitro (30). The evidence that Z-protein shows the α-actinin-type distribution (Fig. 4 e, f) substantiates this suggestion. Interesting models of Z-lattice structures have been proposed from the viewpoint of ultrastructural observations under an electron microscope (4, 15, 16, 32). However, they could not avoid some ambiguity because of the lack of biochemical information about the components of Z-lattice structures. We propose that actin filaments attach to the basic lattice structure formed by Z-protein. Questions remaining to be solved in the future include: How do actin filaments attach to the Z-lattice? Is there any connecting component between them?

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