A high molecular mass protein isolated from chicken gizzard: its localization at the dense plaques and dense bodies of smooth muscle and the Z-disks of skeletal muscle

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SUMMARY

We purified a 450 kDa protein from a low-salt alkaline extract of chicken gizzard smooth muscle. This high molecular mass protein could be extracted with the low-salt alkaline solution at 37 °C but not at 4 °C. The 450 kDa protein was isolated from the extract by ammonium sulfate fractionation and following sequential column chromatography using hydroxyapatite, DEAE-Cellulofine A-800m and phenyl-Sepharose CL-4B resins. The partially purified protein molecule resembled a flexible rod with a globular head and an irregular-shaped tail. Its length was approximately 300 nm. The nucleotide sequence of the partial cDNA encoding this protein was determined and analyzed with a data base. The analysis showed that the protein revealed significant homology with the rod region of chicken filamin (57% homology in amino acid sequence). Immunoblot analysis showed that an affinity-purified antibody reacted exclusively with the 450 kDa protein band of smooth, skeletal and cardiac muscle tissues. By indirect immunofluorescence microscopy, we examined the localization of the 450 kDa protein in smooth and skeletal muscle cells. The affinity-purified antibody against the 450 kDa protein stained the dense plaques and dense bodies of smooth muscle, the peripheral region of Z-disks and the subsarcolemmal region of skeletal muscle. Immunoelectron microscopy confirmed the localization of the 450 kDa protein at the peripheral regions of the actin anchoring structures mentioned above. Judging from its amino acid sequence, molecular size, molecular shape, immunological reactivity and localization in muscle cells, the 450 kDa protein seemed to be a new component associated with the actin-anchoring structures of muscle tissues. We tentatively call this protein ‘fulcin’. Key words: dense body, dense plaque, Z-disk, fulcin, 450 kDa protein, smooth muscle

INTRODUCTION

In muscle cells, actin filaments of myofibrils are bound to the plasma membrane at dense plaques of smooth muscle, intercalated disks of cardiac muscle and myotendinous junctions of skeletal muscle. Further, muscle cells have specialized intracellular actin-anchoring structures, dense bodies of smooth muscle and Z-disks of striated muscle where actin filaments from opposite sides are bundled together (Huxley, 1963; Bond and Somlyo, 1982; Small et al., 1992). In each case, actin filaments attach themselves to the plasma membrane or intracellular structures by their barbed ends (Huxley, 1963; Bond and Somlyo, 1982; Geiger, 1983; Tsukita et al., 1983). These actin-anchoring structures are important for transmitting the force generated by the system including actin and myosin. α-Actinin is a common component of dense bodies, dense plaques, Z-lines and intercalated disks (Masaki et al., 1967; Lazarides and Granger, 1978; Geiger et al., 1981; Tokuyasu et al., 1981; Fay et al., 1983), while vinculin (Geiger et al., 1981; Shear and Bloch, 1985; Volberg et al., 1986; Draeger et al., 1989), talin (Tidball et al; 1986; Volberg et al., 1986; Draeger et al., 1989), Paxillin (Turner et al., 1990, 1991) and zyxin (Crawford and Beckerle, 1991) are not found in cytoplasmic structures such as Z-disks and dense bodies. Filamin, a component of the cytoskeletal domain of smooth muscle cells, is not found in the core of the dense bodies but localizes at the cytoplasmic surface of the dense plaques of smooth muscle (Small et al., 1986) and the periphery of Z-disks of striated muscle (Gomer and Lazarides, 1981). Various other Z-disk components have been also reported: a 220 kDa peptide (Muguruma et al., 1981), β-actinin (Maruyama et al., 1990) which is identical to Cap Z (Casella et al., 1987), eu-actinin (Kuroda et al., 1981), Z-protein (Ohashi et al., 1982), zeugmatin (Maher et al., 1985) and so forth. Zeugmatin also localizes at the dense bodies of smooth muscle. Dense bodies and Z-disks are known not only as actin-anchoring structures but also as intermediate filament-binding structures (Granger and Lazarides, 1979; Tsukita et al., 1983). Intermediate
filament-associated proteins such as synemin (Granger and Lazariades, 1980), plectin (Wiche et al., 1983) and paranemin (Breckler and Lazariades, 1982) are also localized at the periphery of the Z-disks.

Several cytoskeleton-associated proteins such as α-actinin, filamin, vinculin, talin and zyxin have been isolated from the 37°C low-salt alkaline extract of avian gizzard smooth muscle (Burridge and Connell, 1983; Feramisco and Burridge, 1980; Crawford and Beckerle, 1991). In the present study, we purified a high molecular mass protein from the 37°C low-salt alkaline extract of the chicken gizzard. This protein localized not only at the membrane-associated structures such as intercalated disks and dense plaques but also at such cytoplasmic structures as Z-lines and dense bodies. We tentatively call this protein ‘fulcin’ (from the Latin word ‘fulcio’, meaning to ‘prop up’) in this paper.

MATERIALS AND METHODS

Extraction and purification of fulcin from gizzard smooth muscle

All procedures were performed at 4°C unless otherwise noted. 200 g of chicken gizzard smooth muscle were homogenized in 2 l of cold PBS (0.15 M NaCl and 20 mM sodium phosphate buffer, pH 7.2) containing 10 mM EDTA (ethylenediaminetetraacetic acid) and 0.1 mM PMSF (phenylmethylsulfonyl fluoride) with a domestic mixer for 2 minutes. The homogenate was then centrifuged at 7,000 g for 10 minutes. The pellet was resuspended in the same buffer and washed 8 times in the same manner. Fulcin was distinguished on an SDS-PAGE gel as a distinct band from the other peptides of the myofibril.

The solution containing partially purified fulcin (Fig. 1o) was supplemented with slight modifications. Nonspecific binding sites on the filters and antibody screening, the method of Huynh et al. (1985) was used with slight modifications. For preparation of antigen-bound nitrocellulose membrane filters and antibody screening, the method of Huynh et al. (1985) was used with slight modifications. Nonspecific binding sites on the filters

solution for 2 hours and centrifuged in the same manner. This procedure was repeated 6 times. Some fulcin, actin, filamin, α-actinin and the other minor proteins were dissolved in this solution. The soluble protein fractions were collected together (crude fulcin, Fig. 1j) and dialyzed overnight against buffer A containing 20 mM NaCl and 0.1 M sodium phosphate buffer (pH 8.0). These amounted in total to approximately 350 mg.

The crude fulcin was applied to a hydroxylapatite column (1.5 × 7.5 cm) equilibrated with buffer A containing 20 mM NaCl and 0.1 M sodium phosphate buffer (pH 8.0). Most of the actin and α-actinin flowed through the column. Fulcin, filamin and a small part of the actin were adsorbed on the column (Fig. 1k). Proteins retained by the column matrix were eluted with buffer A containing 20 mM NaCl and 0.2 M sodium phosphate buffer (pH 8.0). Approximately 3.6 mg of protein was eluted from the column. The eluent containing fulcin, which was dialyzed against buffer A containing 0.17 M NaCl and 0.1 mM EDTA, was applied to a phenyl-Sepharose CL-4B (Pharmacia) minicolumn (0.2 ml) to remove part of the actin and other minor contaminants which were trapped in the resin bed. This minicolumn was eluted with 0.6 M NaCl (Fig. 1m). The fraction not retained in the minicolumn was reapplied to a usual DEAE-Cellulose A-800m column (1.5 × 5.5 cm). Proteins adsorbed on the resin beads were eluted with buffer A containing 0.3 M NaCl and 0.1 mM EDTA. The amount of the adsorbed protein was 1.6 mg. The eluent containing fulcin and actin (Fig. 1n) was diluted twice with buffer A and applied once more to the DEAE-Cellulose column (1.5 × 5.5 cm), then eluted with a 100 ml linear gradient of 0.15 M-0.3 M NaCl in buffer A. Fulcin was eluted as a broad peak around 0.22 M NaCl. Then 8 M urea and 0.5 M Tris-acetate buffer (pH 8.0) were added to the fractions containing fulcin and actin (Fig. 1o) to make the solution, finally, 4 M urea, 10 M Tris-acetate buffer, 60 mM NaCl, 15 mM 2-ME and 0.1 mM PMSF. The sample solution was immediately applied to a small DEAE-Cellulose A-800m column (0.5 ml) which had been equilibrated with a solution of the same composition. The column was washed with the same buffer and eluted with a solution consisting of 4 M urea, 0.1 M NaCl, 20 mM Tris-acetate buffer, pH 7.2, 15 mM 2-ME and 0.1 mM PMSF. The eluent containing pure fulcin (Fig. 1p) was immediately dialyzed against 20 mM KCl, 20 mM sodium phosphate buffer, pH 7.2, 15 mM 2-ME and 0.1 mM PMSF for 8 hours. The yield was rather low, approximately 0.1 mg from 200 g of chicken gizzard. Its relative molecular mass was estimated as 450 kDa on SDS-PAGE using chemically crosslinked myosin heavy chain oligomers as molecular mass markers according to the method of Hu et al. (1989).

Rotary-shadowing

The solution containing partially purified fulcin (Fig. 1o) was supplemented with an equal volume of glyceraldehyde and sprayed onto freshly cleaved mica. The samples were rotary-shadowed at 7.5° with platinum and carbon and then at 90° with carbon on the uncooled specimen stage of a JEOL JFD-9000 freeze-fracture apparatus (Sato et al., 1992). Replicas were float off on a surface of distilled water and mounted on copper grids. The grids were observed under an electron microscope (JEOL 100S).

Screening of the cDNA library

A chicken gizzard cDNA library (CLONTECH Laboratories, USA) prepared in Agt11 was screened with affinity-purified antibody against fulcin. For preparation of antigen-bound nitrocellulose membrane filters and antibody screening, the method of Huynh et al. (1985) was used with slight modifications. Nonspecific binding sites on the filters
were saturated by preincubation for 30 minutes with TTBS (20 mM Tris-HCl, pH 8.0, 500 mM NaCl and 0.05% Tween-20) containing 1% gelatin. Filters were then incubated with the affinity-purified antibody in TTBS overnight, washed with TTBS to remove unbound antibody, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 1 hour. After 3 washes with TTBS followed by a wash with TBS (20 mM Tris-HCl, pH 8.0, and 500 mM NaCl), color reaction of the filters was started using a Konica Immunostaining HRP kit.

**Cloning and sequencing**

The EcoRI inserts were excised from the immuno-positive λgt1 recombinant and subcloned into plasmid vector pHC118. Serial deletion derivatives were prepared using exonuclease and mung bean nuclease (Henikoff, 1987). Each deletion derivative was sequenced by the chain termination method (Sanger et al., 1977). Analysis of nucleotide sequences and homologies were carried out using the program GENETYX version 9.0 (Software Development Co. Ltd). It was confirmed that determined sequences of both strands of the cDNA were consistent.

**Production of λgt11 fusion proteins**

To obtain fulcin and β-galactosidase fusion protein, crude lysates of λgt1 recombinant lysogeny in *Escherichia coli* coli Y1089 were prepared as described by Huynh et al. (1985). Recombinant lysogeny were cultured in 50 ml of L-broth medium containing ampicillin at 32°C to 0 Abso=0.2. After a temperature shift up to 42°C for 20 minutes, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 10 mM. After incubation at 37°C for 2 hours, cells were pelleted and resuspended in sample buffer (50 mM Tris-HCl, 1.5% SDS, 50 mM dithiothreitol, 4 M urea) at 3% of the original volume and mixed well by passing several times through a 21-gauge needle. The β-galactosidase fusion protein was subjected to immunoblot analysis.

**Preparation and polymerization of actin**

Actin was prepared from the acetone powder of rabbit back muscle, according to the method of Spudich and Watt (1971). G-actin (0.1 mg/ml) was polymerized for 1 hour at room temperature in the presence or absence of 0.015 mg/ml of purified fulcin, in a solution consisting of 0.1 M KCl, 20 mM sodium phosphate buffer, pH 7.2, 1 mM MgCl2, 0.5 mM ATP and 10 mM 2-ME (polymerizing buffer). Next, fulcin was added to F-actin. G-actin (0.2 mg/ml) was prepolymerized in the polymerizing buffer for 1 hour at room temperature. Fulcin dissolved in a polymerizing buffer was mixed with the F-actin solution and incubated for 1 hour at room temperature. The final concentration of actin and fulcin were, respectively, 0.1 and 0.015 mg/ml. A drop of each sample solution was applied to a collodion-coated copper grid, fixed with 3% formaldehyde in the polymerizing buffer and negatively stained with 1% uranyl acetate. The grid was then examined under an electron microscope (JEOL 100CX).

**Antibodies**

Antibody against fulcin was raised in a rabbit according to our usual method (Ohashi et al., 1982). Partially purified fulcin (0.5 mg, Fig. 1o) was electrophoresed on an SDS-polyacrylamide gel and the protein band was cut out after being stained with Coomassie Brilliant Blue R-250. The gel strip was homogenized in PBS and emulsified with an equal volume of Freund’s incomplete adjuvant. 0.1 mg of emulsified protein was subcutaneously injected 3 times at 2-week intervals. The rabbit was bled on the tenth day, following the last injection.

The antibody against fulcin was purified from the antiserum by membrane affinity adsorption according to the modified method described by Smith and Fisher (1984). Partially purified fulcin (Fig. 1k) was subjected to SDS-PAGE and transferred to a piece of polyvinylidenedifluoride membrane (PVDF) membrane (Immobilon-P, Millipore). A band of fulcin stained with Ponceau-S was excised, de-stained in 0.01 M Tris-HCl, pH 8.8, and cut into small pieces. The membrane pieces were blocked with 1% bovine serum albumin (BSA) in PBS overnight 4°C. The antisera against fulcin, diluted ten-fold with PBS and the membrane pieces were washed thoroughly with TTBS. Antibody molecules bound specifically to fulcin were recovered in 50 mM glycine-HCl buffer, pH 2.5. The antibody fraction was immediately neutralized by adding 0.5 M Tris-HCl, pH 8.8, diazylized against PBS and stored with 1% BSA at 4°C. The affinity-purified antibody was used for immunoblot analysis, indirect immunofluorescence microscopy and immunoelectron microscopy. The anti-t-actinin antibody (Ohashi et al., 1982) was affinity-purified and used for immunoelectron microscopy. A goat antibody against chicken gizzard filamin was purchased from BioMakor Co. Ltd and used for immunoblotting.

**Frozen sections of muscle tissues**

Adult chicken gizzard and breast muscle were dissected into small cubes (1 x 1 x 1 mm) and fixed on ice for 10 minutes with 3.5% formaldehyde in PBS. The cubic pieces of fixed tissue were frozen in Tissue Mount (Chiba Medical, Japan) by immersion in liquid nitrogen and sectioned at −30°C.

**Isolation of smooth muscle cells**

Single gizzard smooth muscle cells were isolated without the use of any protease. Fresh gizzard smooth muscle was cut into small pieces with a razor blade, suspended in PBS containing 20 mM EGTA and 0.1 mM PMSF and left on ice for 1 hour. The EGTA-PBS solution was exchanged and the suspension was homogenized gently with a Waring blender for a few seconds. Then the smooth muscle cells were isolated from the tissue in the suspension. Isolated smooth muscle cells were then suspended in 50% glycerol, 0.15 M NaCl, 20 mM sodium phosphate buffer, pH 7.2, 10 mM EGTA and 0.1 mM PMSF and stored at −20°C for more than 2 weeks.

**Immunofluorescence microscopy**

Isolated smooth muscle cells were mounted on a glass slide, and covered with a coverslip. They were washed with PBS containing 10 mM EGTA and 0.1 mM PMSF, fixed with 3.5% formaldehyde in PBS, washed thoroughly with PBS and blocked with 1% BSA in PBS for 30 minutes under coverslips. A drop of each solution was added from one side of the coverslip and drawn with a strip of filter paper from the opposite side. Reaction with antibodies was performed in the same way. The sample was incubated with the affinity-purified anti-fulcin antibody for 2 hours at room temperature, washed well with PBS and incubated with the FITC-labeled goat anti-rabbit IgG antibody (Cappel, USA) for 1 hour. The specimen was then washed with PBS, fixed with 3.5% formaldehyde in PBS, washed again with PBS and embedded in the glycerol/PBS solution of Citifluor (Agar, USA). Cryosections of muscle tissues mounted on glass slides were fixed in PBS containing 3.5% formaldehyde for 10 minutes at room temperature. The sections were washed with PBS and blocked with 1% BSA in PBS for 30 minutes. Antibody treatment and embedding of sections were performed on glass slides according to the procedure described above.

**Immunoelectron microscopy**

Chicken gizzard smooth muscle was dissected and cut into small blocks (1 mm). The block was fixed in a solution containing 3.5% formaldehyde, 0.15 M NaCl, 10 mM EDTA, 0.1 mM PMSF and 20 mM sodium phosphate buffer, pH 7.2, for 30 minutes, washed 3 times with PBS for 20 minutes, dehydrated and embedded in LR White (London Resin). The sample was ultrathin sectioned, blocked with 1% BSA in PBS, reacted with primary antibodies for 2 hours and washed 5 times with PBS for 5 minutes. The sections were labeled with the secondary antibodies coupled to colloidal gold particles (15 nm, E-Y
LABS, USA) in the same manner. The immunostained sections were postfixed with 2.5% glutaraldehyde in PBS, stained with 4% uranyl acetate and 0.1% lead citrate and observed under an electron microscope (JEOL 100CX). In the case of the breast muscle, the pre-embedding method was used. The tissue block was fixed in PBS containing 3.5% formaldehyde, washed with PBS, blocked with 1% BSA in PBS, reacted overnight with the primary antibody and washed with PBS. Reaction with the secondary antibody coupled to colloidal gold particles (5 nm, E-Y LABS) was carried out in the same manner and the tissue block was embedded in Epon.

SDS-PAGE and immunoblotting

SDS-PAGE was essentially carried out according to the method of Laemmli (1970), using 3-15% gradient separating gels. Immunoblotting analysis was performed by modification of the procedure developed by Towbin et al. (1979). The SDS-electrophoresed peptides were transferred electrophoretically to PVDF sheets. The PVDF sheets were blocked with 1% BSA in PBS, washed with TTBS, reacted with the first antibody for 1 hour at room temperature, washed with TTBS and incubated with the alkaline phosphatase-labeled second antibody for 1 hour at room temperature. Development of enzyme activity on the PVDF membrane was carried out according to the manual of Bio-Rad Co. Ltd.

RESULTS

Molecular shape of fulcin

The partially purified fulcin sample just before the urea treatment (Fig. 1o) was used for rotary-shadowing electron microscopy. The molecule looked like a flexible rod with a globular head and an irregular-shaped tail (Fig. 2). The length of the molecule was approximately 300 nm.

cDNA clones encoding fulcin

To isolate a cDNA clone encoding fulcin, λgt11 gizzard cell cDNA library was screened with the affinity-purified antibody against fulcin. An immunopositive clone, designated λgt11-Fu1, contained a cDNA insert of 1,398 bp (Fig. 3). The nucleotide sequence encoding fulcin was compared against the GenBank/EMBL Data Bank. This comparison revealed significant homology with chicken retina filamin (Barry et al., 1993). Based on the alignment in Fig. 4, the calculated percentage identities of the amino acid sequence between fulcin and filamin (1189-1644) was 57%.

Immunoblot analysis of cDNA-encoded protein

A clone, λgt11-Fu1, was used to generate a λ lysogen after infection of E. coli Y1089. Immunoblot analysis shown in Fig. 5 demonstrated that the fulcin and β-galactosidase fusion protein reacted with the affinity-purified anti-fulcin antibody. However, the fusion protein did not react with an anti-filamin antibody. These results confirmed that the isolated clone is a partial cDNA of fulcin.

Actin filaments were entangled in the presence of fulcin

G-actin was polymerized in the presence of fulcin at the molar ratio of 1:70. Aggregates of short actin filaments were formed (Fig. 6C), while control actin filaments were only sparsely distributed (Fig. 6A). When fulcin was mixed with F-actin, large aggregates of entangled actin filaments were formed (Fig. 6D). Several actin filaments were associated laterally in the aggregates. By itself, purified fulcin mainly formed amorphous aggregates within the polymerizing buffer (Fig. 6B), and filamentous structures were occasionally observed (arrow).

Immunoblot analysis of muscular tissues with the anti-fulcin antibody

The specificity of the membrane-affinity-purified anti-fulcin antibody was examined using the immunoblotting method. The myofibril-rich fraction of chicken gizzard smooth muscle was electrophoresed on a polyacrylamide gel and transferred to a sheet of PVDF membrane. The anti-fulcin antibody reacted noticeably with the bands corresponding to fulcin (Fig. 7a,b). The preimmuneserum did not react with the fulcin band (Fig. 7c). When the antiserum against fulcin was absorbed with pure fulcin (Fig. 1p), no immunostained band was developed (Fig. 7d) on the blotted membrane. Muscular tissues were SDS-electrophoresed on a 3-15% gradient gel. In the whole peptides of

![Fig. 1. SDS-PAGE of each step in the preparation of fulcin from chicken gizzard. (a) myofibril-rich fraction; (b) soluble fraction in distilled water; (c) soluble fraction in the cold low-salt alkaline (LSA) solution; (d) extract with Hasselbach-Schneider’s solution; (e) residue of the extraction with Hasselbach-Schneiders’ solution; (f) soluble fraction of the residue (e) in distilled water; (g) extract with the LSA solution at 4°C; (h) extract with LSA at 37°C; (i) resultant residue extracted with the warm LSA solution; (j) crude fulcin; (k) fulcin fraction eluted from a hydroxylapatite column; (l) fraction adsorbed on a phenyl-Sepharose minicolumn; (m) fraction adsorbed on a DEAE-Cellulofine minicolumn; (n) eluent from a DEAE-Cellulofine column with 0.3 M NaCl; (o) fulcin fraction rechromatographed on a DEAE-Cellulofine column; (p) pure fulcin. The arrow shows fulcin. P, filamin; M, myosin; α, α-actinin; D, desmin; A, actin.](image-url)
the chicken gizzard, breast muscle and heart, a peptide of the same molecular mass as fulcin was reacted with the anti-fulcin antibody in each lane (Fig. 8). In the skeletal muscle lane, the immunologically-stained band had a slightly higher degree of mobility than did the nebulin band (Fig. 8, lane 2 and 2′). The bands of fulcin were not distinct in the whole peptides of muscular tissues stained with Coomassie Brilliant Blue R-250. Only in the smooth muscle lane (Fig. 8, lane 1), was the fulcin band (arrow) less distinctly identifiable on the gel than the myofibril-rich fraction (Fig. 1a, lane (Fig. 8, lane 1′ and 2′). The nucleotide sequence and predicted amino acid sequence

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Localization of fulcin in smooth muscle

By indirect immunofluorescence microscopy using the anti-fulcin antibody, we examined the isolated smooth muscle cells (Fig. 9) and frozen sections of chicken gizzard smooth muscle (Fig. 10). Intensely fluorescent dots aligned at the periphery of the single cell (Fig. 9A,B, long arrows) and rather strong fluorescence seemed to dot the inner region of the cell. Two fluorescent patches on the plasma membrane appeared to correspond to the dense bodies (short arrows) and the highly fluorescent patches on the plasma membrane appeared to correspond to the dense plaques (arrowheads). The preimunoserum (Fig. 9C,D) and immunoabsorbed anti-fulcin antiserum (Fig. 9E,F) did not specifically stain isolated smooth muscle cells.

On the longitudinal frozen section of the gizzard (Fig. 10A,B), intensely fluorescent dots were seen along the plasma membrane and weak fluorescent dots were scattered throughout the cytoplasm except in the nuclear region (arrowheads). The fusiform or ovoid fluorescent structures corresponding to the dense plaques seemed to straddle the boundary between

Fig. 2. Molecular shape of fulcin. The fulcin molecule looks like a flexible rod with a globular head and an irregular-shaped tail. Its length is approximately 300 nm. Bar, 100 nm.

Fig. 3. The nucleotide sequence and predicted amino acid sequence of partial DNA encoding fulcin. This sequence has been deposited in the DDBJ/EMBL/NCBI DNA data bases (accession no. D38755).

ACCATCGCCGCGCGG

T I Q R G

G G A Q A E V C V E D N G D G T Y N I G

70     80     90     100     110     120
neighboring cells. At these sites, cells seemed to adhere tightly to their neighbors with plasma membrane between. On the cross section of the gizzard smooth muscle (Fig. 10C,D), immunofluorescence specific to fulcin was highly concentrated at the plasma membrane. The cytoplasmic fluorescence was rather weak (Fig. 10B,D) compared with that observed in the isolated cells.

On the ultrathin section of gizzard smooth muscle, most of the colloidal gold particles (15 nm) representing fulcin were observed at the peripheral region of dense bodies (Fig. 11B, large arrows) and the region around the cytoplasmic surface of dense plaques (Fig. 11A, small arrows). On the same section, they were more frequently found around the dense plaques than the dense bodies (Fig. 11A). The colloidal gold particles were rarely found in the core region of dense bodies (Fig. 11A). On the other hand, α-actinin localized in the core region of dense bodies and dense plaques (Fig. 11C). The colloidal gold particles (15 nm) were more scarce on dense plaques (small arrow) than on dense bodies (large arrows).

**FULCIN**

1. GQAEEVCEVDENGCTYNTALSPGHSITLGYQVGPVPFPRVKVE
2. FILAMIN
3. TVSKAEI1DQKHCTTYVTYPPLAGHNTIRKMYGGQVPRFPAPRKYVE
4. FULCIN
5. PADGAARFVRQGQEGKVGFSGCGALTVEHTFVDARALAGAAATIPSP
6. FILAMIN
7. PAVOTSRVKGQVRGKVFVREATTDFTDAPLFTARGGHDHTQITSP
8. FULCIN
9. SGGVIAAVEDGCTYGVTYQYEQYVSHVYSGQSP1FAPRNPVT
10. FILAMIN
11. SGSTDCIQQNACDCTAAYETPFEKHTVNYTVDQVPNPSPVPVVT
12. FULCIN
13. ECDPSRVRVGPPICAGTATQPMFVFVGTGGLGMGQPAEQL
14. FILAMIN
15. ECDPSRVRVAGFVPLAQTGQPMFVFVGTGGLGMGQPAEQL
16. FULCIN
17. SC2DAKGGSCVYRPAPCTGTSLNTYGRGRPGPSFRKPVVQETGGS
18. FILAMIN
19. SC2DAKGGSCVYRPAPCTGTSLNTYGRGRPGPSFRKPVVQETGGS
20. FULCIN
21. AADGMCAGPLKGTKEVQVFPVDVCDRPGTAEALKVFVGKVV
22. FILAMIN
23. AADGMCAGPLKGTKEVQVFPVDVCDRPGTAEALKVFVGKVV
24. FULCIN
25. --DEGKCIAPCGL-FTAVKRPVGFQTFSPKAVAGPQPVQIV
26. FILAMIN
27. --DEGKCIAPCGL-FTAVKRPVGFQTFSPKAVAGPQPVQIV
28. FULCIN
29. EPVEVDNGCTSVSVPSRGPSYSISVRYQQEQRFSFVRKPALPSHD
30. FILAMIN
31. EPVEVDNGCTSVSVPSRGPSYSISVRYQQEQRFSFVRKPALPSHD
32. FULCIN
33. ARVRASGPGLNTGVPASLVEFVTDIAODVAGFLGLHAGQPQD
34. FILAMIN
35. ARVRASGPGLNTGVPASLVEFVTDIAODVAGFLGLHAGQPQD
36. FULCIN
37. LIRONGCTYTYSYPTDGRYTTLLIKYGDIEIFPFSRIRAVPVGDAS
38. FILAMIN
39. LIRONGCTYTYSYPTDGRYTTLLIKYGDIEIFPFSRIRAVPVGDAS
40. FULCIN
41. DIHMGDCQYTTVYPTDKRXQYICYDDGDIPIIPQ-ASFPAGDAQA
42. FILAMIN
43. CVTQGPLVPTIQM
44. FULCIN
45. CLATGLQAFVPRTG

**Fig. 4.** Comparison of amino acid sequences of fulcin and filamin (Barry et al., 1993) over their homologous region. Asterisks indicate the positions of the identical residues.

**Fig. 5.** Western blot analysis of the fulcin and β-galactosidase fusion protein. Control Y1089 bacteria (lane 1) and Agt11-Fu1 lysogenic Y1089 bacteria (lane 2) were subjected to 10% SDS-PAGE and blotted onto nitrocellulose membrane. (A) Coomassie Brilliant Blue staining pattern; (B, C) immunostaining patterns with anti-β-galactosidase, anti-fulcin and anti-filamin antibodies, respectively.
Localization of fulcin in skeletal muscle

On the longitudinal frozen section of the chicken breast muscle, the anti-fulcin antibody most strongly stained the subsarcolemmal region and periodically stained the Z-disks (large arrows) of myofibrils (Fig. 12A, B). Very faint fluorescence was observed at the M-lines. On the cross section of the breast muscle, specific fluorescence was condensed at the subsarcolemma (Fig. 12C). It is notable that subsarcolemmal fluorescence was not uniformly distributed. A chain of fluorescent dots (Fig. 12C, small arrow) decorated the sarcolemma. Fluorescent nets were observed in the inner region of a muscle fiber. Gaps between muscle fibers were not distinct in the fluorescence micrograph. Therefore, fulcin should mainly localize in the vicinity of the sarcolemmal inner surface.

We also examined the localization of fulcin in muscle fibers by pre-embedding immunoelectron microscopy. On the longitudinal ultrathin section, colloidal gold particles (5 nm), which recognize anti-fulcin antibody molecules, bound abundantly to the subsarcolemmal structures which connected Z-disks to cell membranes (Fig. 13A, large arrows) and bound to the Z-disks at the cytoplasmic periphery (Fig. 13A, small arrow). Several gold particles were also found in the I-bands. On the cross section, clusters of colloidal gold particles (Fig. 13B, large arrows) surrounded the Z-disks. The gold particles densely decorated the subsarcolemmal structures associated with the Z-disks. A cluster of gold particles labeled the intermyofibrillar structure (Fig. 13B, small arrow) which seemed to connect neighboring Z-disks.

DISCUSSION

In this paper, we describe the purification, partial characterization, and localization of a high molecular mass protein, fulcin, from gizzard smooth muscle. The partial nucleotide sequence of fulcin revealed the significant homology with the central repeated β-sheet motifs of filamin molecules (Barry et al., 1993; Figs 3 and 4). However, the affinity-purified antibody against fulcin did not react with filamin (Fig. 7b) and an anti-filamin antibody did not react with the fulcin and β-galactosidase fusion protein (Fig. 5), or the mother molecules of fulcin (data not shown). The results showed that fulcin was similar but not identical to filamin. Several high molecular mass proteins of muscle tissues have been reported, but fulcin differs from these. Connectin (Maruyama et al., 1981), which is identical to titin (for a review see Trinick, 1992), a well-known high molecular mass protein (~3,000 kDa) of striated muscle, has not been found in vertebrate smooth muscle. Nebulin (Trinick, 1992; Wang and Williamson, 1980) is a unique protein of skeletal muscle and does not exist in either cardiac or smooth muscles. The molecular mass of nebulin (~800 kDa) is larger than that of fulcin. Also, the anti-fulcin antibody did not react with the nebulin band (Fig. 8).

Zeugmatin, which is larger than nebulin, is thought to be an integral component of the Z-disk, and a monoclonal antibody against zeugmatin stained the inner region of the gizzard smooth muscle cell much more strongly than the membrane region (Maher et al., 1985). Still, its molecular mass and localization are different from fulcin’s. Kettin, a large protein of over 500 kDa of insect flight muscle (Lakey et al., 1993), localizes on the lateral faces of the Z-disc where actin filaments are attached, and it has repeats which are homologous to class II (immunoglobulin-C2-like) domains. Although dystrophin’s molecular mass is similar to that of fulcin’s, its distribution in muscle cells is restricted to the membrane region (Byers et al., 1991; Straub et al., 1992; North et al., 1993). Additionally, its central rod domain is composed of spectrin-like α-helical motifs (Koenig et al., 1988), and anti-dystrophin antibody reacted with a protein of lower molecular mass than fulcin (data not shown). Tenuin, which has been isolated from rat liver adherens junctions (Tsukita and Tsukita, 1989; Tsukita et al., 1991; Straub et al., 1992; North et al., 1993). Additionally, its central rod domain is composed of spectrin-like α-helical motifs (Koenig et al., 1988), and anti-dystrophin antibody reacted with a protein of lower molecular mass than fulcin (data not shown). Tenuin, which has been isolated from rat liver adherens junctions (Tsukita and Tsukita, 1989; Tsukita et al., 1991; Straub et al., 1992; North et al., 1993). Additionally, its central rod domain is composed of spectrin-like α-helical motifs (Koenig et al., 1988), and anti-dystrophin antibody reacted with a protein of lower molecular mass than fulcin (data not shown).
Fig. 9. Localization of fulcin in isolated gizzard smooth muscle cells by immunofluorescence microscopy. Both phase-contrast (A) and immunofluorescence (B) images of a single smooth muscle cell were obtained at the same focal plane. The stringlike arrays of intensely fluorescent dots were seen at the surface of the cell (long arrows show the array). Less intense fluorescence dotted the inner part of the cell. The preimmuniserum (C,D) and immunoabsorbed anti-fulcin antiserum (E,F) did not specifically stain the isolated cells. (G-J) Higher-powered views of smooth muscle cells. Immunofluorescence dots with the anti-fulcin antibody in the inner region of the cell coincided with the dense bodies (G,H, short arrows). The dense plaques were more intensely stained with the anti-fulcin antibody (I,J, arrowheads). (A,C,E,G,I) Phase images; (B,D,F,H,J), immunofluorescence images. Bars, 10 µm.
A high molecular mass protein associated with actin-anchoring structures in muscle

al., 1989), reportedly has a molecular mass similar to that of fulcin. Its specific antibodies, however, do not stain the Z-lines of the cardiac muscle (Tsukita et al., 1989). On the basis of these findings, we consider fulcin to be a new fimatin-related protein.

Fulcin seemed to promote the aggregation of actin filaments in vitro, even when the molar ratio to actin was 1:70 (Fig. 6C,D). When fulcin contaminated with a small amount of actin (Fig. 1o) was mixed with G-actin before urea treatment, similar actin aggregates were also formed (data not shown). These results suggest that fulcin has an actin-binding ability. It would be natural to assume that fulcin, a fimatin-related protein, has actin-binding domain(s). In a physiological salt solution, purified fulcin formed aggregates large enough to be sedimented at 15,000 g for 10 minutes (Fig. 6B). For this reason, the co-sedimentation of fulcin with actin was not successfully performed.

In order to prevent errors caused by contaminated antibodies, we used the affinity-purified antibody against fulcin for all immunological experiments. This specific antibody exclusively reacted with the 450 kDa peptide of muscle tissues (Fig. 8). The preimmuniserum and the antiserum against fulcin absorbed with purified fulcin (Fig. 1p) did not react with the peptides of gizzard smooth muscle (Fig. 7c,d). Therefore, the antibody that we used in the present paper is concluded to be a highly specific antibody against fulcin.

By immunofluorescence microscopy, we found that the structures of muscle cells stained with the anti-fulcin antibody were of two classes: intensely stained membrane-associated structures such as dense plaques and less intensely stained cytoplasmic structures such as dense bodies and Z-disks. It has now been confirmed that these two are not biochemically equivalent. Since the immunofluorescence staining pattern of isolated smooth muscle cells with the anti-fulcin antibody (Fig. 9) was similar to that with the anti-α-actinin antibody (Fay et al., 1983), the ultrastructural localization of fulcin in smooth muscle was compared with that of α-actinin. Post-embedding immunoelectron microscopic studies showed that fulcin was closely associated with actin-anchoring structures in muscle.

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**Fig. 10.** Frozen sections of the chicken gizzard immunostained with anti-fulcin. On the longitudinal section (A,B), intensely fluorescent fusiform dots were arranged along the plasma membrane of the smooth muscle cells. The arrowhead shows a nucleus that is not stained. On the cross section (C,D), immunofluorescence was condensed at the cell membrane. In both sections, relatively weak fluorescence dots were scattered inside the cells. (A,C) Phase-contrast images; (B,D) immunofluorescence images. Bar, 10 µm.

**Fig. 11.** Ultrastructural localization of fulcin in chicken gizzard smooth muscle by immunoelectron microscopy. (A,B) Labelled with anti-fulcin antibody; (C) labelled with anti-α-actinin antibody. Small arrows, dense plaques; large arrows, dense bodies. Bar, 0.5 µm.
tissues such as dense bodies and dense plaques. However, it seemed not to be the component of their substance (Fig. 11).

The breast muscle fiber was examined by the pre-embedding method. The antigen-antibody reaction on longitudinal ultrathin sections of breast muscle fiber was difficult to detect by the post-embedding method due to the low content of fulcin in breast muscle. In breast muscle, colloidal gold particles representing fulcin labeled the subsarcolemmal structures (Fig. 13) containing costameres, where vinculin (Shear and Bloch, 1985) and talin (Tidball et al., 1986) are localized. In the cross section of skeletal muscle, by immunofluorescence microscopy, intensely fluorescent dots observed along the plasma membrane (Fig. 12C, arrow) probably represented Z-disk-sarcolemma connecting sites. Colloidal gold particles also labeled the structures between the Z-disks of neighboring myofibrils (Fig. 13A,B, small arrows). The fluorescent nets observed in the cross section of muscle fibers (Fig. 12C) were probably caused by antibody molecules bound to the periphery of Z-disks. Besides these structures, several colloidal gold particles were found at the I-band region and in the inner part of the Z-disks. The possibility that a small amount of fulcin is present there cannot be excluded. Fulcin is localized at the intercalated disks and Z-disks in cardiac muscle (Data not shown). Judging from its localization in muscle cells, fulcin probably serves to prop up actin filament bundles, especially at actin-anchoring structures. The localization of filamin in striated muscle cells (Gomer and Lazarides, 1981; Koteliantsky et al., 1986) is very similar to that of fulcin, while filamin localizes in the cytoskeletal domain in smooth muscle (Small et al., 1986). Of all the actin-associated proteins, only filamin has been known to locate at the periphery of Z-disks (Gomer and Lazarides, 1981). There could possibly be interaction between fulcin and filamin at the periphery of the Z-disks. The staining pattern of myofibrils by the anti-fulcin antibody also resembled that of the desmin antibody. Desmin and vimentin filaments are known to surround Z-disks and connect them to neighboring Z-disks (Granger and Lazarides, 1979). Such intermediate filament-associated proteins as synemin (Granger and Lazarides, 1980), plectin (Wiche et al., 1983) and paranemin (Breckler and Lazarides, 1982) usually accumulate at the peripheral regions of Z-disks. The question of whether fulcin binds to them or not remains an interesting problem for further study.

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REFERENCES


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