Purification of a 175-kDa membrane protein, its localization in smooth and cardiac muscles

Interaction with cytoskeletal protein – vinculin

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A new 175-kDa membrane protein was isolated from chicken gizzard smooth muscle. Antibodies to 175-kDa protein were used for localization of this protein in smooth and cardiac muscles. In both types of muscle 175-kDa protein was localized near plasma membrane. 175-kDa protein was able to interact specifically with vinculin immobilized on polysterene surface. It is suggested that this 175-kDa protein may be involved in physical connection between microfilaments and cell membrane.

175-kDa membrane protein *Vinculin* *Smooth muscle* *Cardiac muscle* *Cytoskeleton-membrane interaction*

1. INTRODUCTION

It is now generally accepted that specific interactions between cytoskeletal filaments and membranes of muscle and non-muscle cells play an important role in cell functioning. Vinculin, metavinculin and the 215-kDa protein were localized at specialized regions of plasma membrane-microfilaments association including the adhesion plaques (focal contacts) of cultured cells, zonula adherens of intestinal epithelial cells, intercalated discs of cardiac muscle and myofibril-sarcolemma attachment regions ('costameres') in striated muscle [1–10]. The intercellular location of vinculin has led to the suggestions that vinculin may be involved in the attachment of microfilaments to the membrane. Since vinculin does not appear to be an integral membrane protein there must be special integral membrane proteins interacting with cytoskeletal proteins and linking actin filaments to the plasma membrane. Here, we describe a new protein which closely associates with cell membrane and is able to form complexes with actin-binding protein - vinculin, in vitro. This protein, with a molecular mass on SDS-polyacrylamide gels of 175 000 has properties of integral membrane protein. The cellular location and biochemical properties of this 175-kDa protein suggest that it may play an important role in microfilament attachment to membranes.

2. MATERIALS AND METHODS

Chicken gizzard smooth muscle vinculin was isolated according to [11]. 200 g of frozen chicken gizzard was consequently homogenized in four different buffers. The homogenates were centrifuged for 20 min at $25\,000 \times g$. The supernatants were discarded (signed as extracts 1–4) and the pellets were resuspended in appropriate extraction buffers. The first extraction was performed at 4°C with deionized water containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) - extract 1, second extraction - at 37°C with buffer, containing 20 mM Tris–HCl, 1 mM EGTA, 0.5 mM PMSF (pH 9.0) - extract 2, third extraction - at 4°C with buffer containing 20 mM Tris–HCl, 0.6 M KCl, 0.5 mM PMSF (pH 7.4) – extract 3,

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fourth extraction – at 4°C with buffer containing 20 mM Tris–HCl, 0.5% Triton X-100, 0.5 mM PMSF (pH 7.4) – extract 4. Proteins (40 mg) in a buffer containing 20 mM Tris–HCl, 0.5% Triton X-100 (pH 7.4) (extract 4) were chromatographed on a 2.6×15 cm column of Whatman DE52 DEAE-cellulose. The column was eluted with 500 ml of linear NaCl gradient (0–250 mM) in a buffer containing 0.5% Triton X-100. Fractions of 3 ml volume each were collected. Their protein content was estimated by measuring absorbance at 291 nm, NaCl concentration was monitored with a conductivity meter.

To raise antibodies to the 175-kDa membrane protein rabbits were immunized by a mixture of 1 ml of antigen solution (0.2–0.4 mg/ml) and an equal volume of Freund's complete adjuvant. After 28 days injections were repeated (0.1–0.2 mg of antigen per rabbit) but without adjuvant. Immunoglobulins were prepared from serum by ammonium sulfate precipitation. Purified antibodies to the 175-kDa protein were obtained by affinity chromatography on a column with 175-kDa protein–Sepharose (1–1.5 mg of 175-kDa protein/ml gel). Purified antibodies (0.25 mg/ml) were stored frozen at −70°C.

For immunofluorescence staining 3–4 μm sections from the frozen tissue were cut on cryostat at −20°C and mounted on glass slides. After incubation with 40 μg/ml affinity purified anti-175-kDa protein for 60 min, sections were washed and then fluorescein conjugated goat anti-rabbit IgG were added to the sections. After incubation for 45 min sections were washed and viewed in a Zeiss epifluorescence photomicroscope III with a 40× objective.

To test the binding of the 175-kDa protein to vinculin an immunochemical method (ELISA) was used [12]. Microtitration plates (Lindbro, Flow Labs, Inglewood, CA) were coated with vinculin, fibronectin and albumin (BSA) (10 μg/ml) overnight at 4°C. Unbound proteins were washed out and 175-kDa protein in 0.14 M NaCl, 0.02 M sodium phosphate buffer (pH 7.4) containing 0.05% Tween 20 and 0.1% Triton X-100, was added. 175-kDa protein was allowed to interact with proteins coating the plate for 90 min at 37°C. Affinity-purified rabbit anti-175-kDa protein antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody were used to detect the 175-kDa protein bound to immubilized proteins. *o*-Phenylenediamine was a substrate for peroxidase.

3. RESULTS AND DISCUSSION

Extraction of chicken gizzard smooth muscle according to the protocol described previously [11] allowed solubilization of the majority of muscle proteins including actin, filamin, tropomyosin, vinculin, α-actinin and some other proteins (fig.1A, a,b,c). The following extraction with buffer containing 0.6 M KCl leads to solubilization of myosin (fig.1A,d). To solubilize integral membrane proteins the nonionic detergent Triton X-100 (0.5–1%) was used. The SDS–gel electrophoretic analysis of Triton X-100 extract demonstrates the presence of the 175-, 120- and 43-kDa proteins (fig.1A,e). The 175-kDa protein represents about 50% of the total protein content of the Triton X-100 extract. Chromatography through DEAE-cellulose of smooth muscle membrane proteins extracted by buffer with 0.5% of Triton X-100 completely separated the 175-kDa protein from other proteins (fig.2; fig.1A,f).

Antibodies against the 175-kDa protein were prepared by the subcutaneous immunization of two rabbits with the proteins purified by preparative SDS–polyacrylamide gel electrophoresis from the 0.5% Triton X-100 soluble fraction. Obtained antisera were characterized by several techniques. In double diffusion analysis the antiserum against the 175-kDa protein formed a single precipitating line with the purified 175-kDa protein as well as with 0.5% Triton X-100 soluble fraction, containing the 175-kDa protein, and did not cross-react with any other smooth muscle proteins tested – vinculin, filamin, myosin, α-actinin (fig.1C). The affinity-purified antibody against 175-kDa protein was found to interact only with 175-kDa protein in immunoblots of SDS-soluble proteins from chicken gizzard smooth muscle (fig.1B,a,e,f). The antibody against 175-kDa protein was used for detection of the 175-kDa protein in different extracts from chicken gizzard smooth muscle (fig.1B). The results revealed that neither low ionic strength buffer (20 mM Tris–HCl, 1 mM EGTA, pH 9.0) which solubilizes most of vinculin, α-actinin and filamin, nor high ionic strength buffer (20 mM Tris–HCl, 0.6 M KCl, pH 7.4) which solubilizes

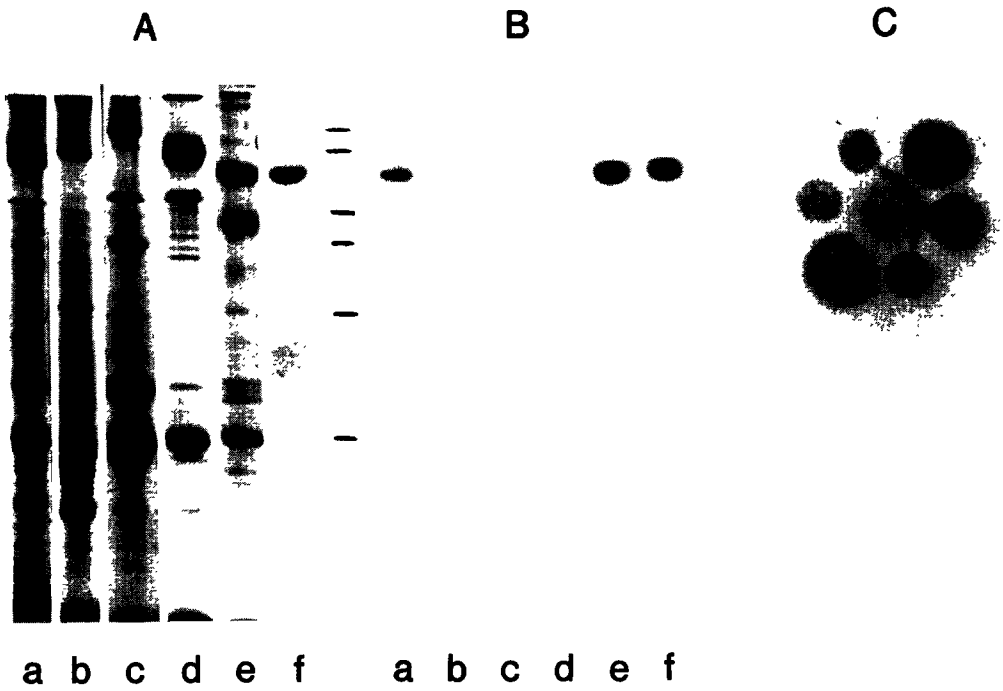

Fig.1. (A) SDS-polyacrylamide gel analysis of chicken gizzard smooth muscle extracts. Aliquots from extracts 1–4 (see section 2) were mixed with 4 × Laemmli SDS sample buffer and then run on a 10% SDS-polyacrylamide gel and visualized by Coomassie blue stain (A). The positions of molecular mass markers (from top to bottom) are indicated by the lines: filamin, 250 kDa; myosin, 200 kDa; vinculin, 130 kDa; α-actinin, 100 kDa; bovine serum albumin, 68 kDa; actin, 43 kDa. Lane a, sample from chicken gizzard smooth muscle homogenized in 10 vols SDS gel electrophoresis buffer; lane b, extract 1; lane c, extract 2; lane d, extract 3; lane e, extract 4; lane f, the purified 175-kDa protein. (B) Detection of 175-kDa protein in chicken gizzard extracts. Lanes a–f show the immunoblots of gels a–f from (A). For immunoblots the proteins were transferred into nitrocellulose paper [13]. The 175-kDa protein was detected by incubating the nitrocellulose paper with affinity-purified anti-175 antibody (50 μg/ml). After intensive washing the paper was incubated with horseradish peroxidase-conjugated goat (anti-rabbit IgG) IgG. 4-Chloro-1-naphthol (Sigma) was used as a substrate for peroxidase. The blue colour was developed during 15 min after adding the substrate. (C) Ouchterlony double-diffusion analysis of anti-175kDa anti-serum. Antiserum from a rabbit immunized with the 175-kDa protein was placed in the center well. Filamin, myosin, vinculin, α-actinin, extract 4 and purified 175-kDa protein were placed in opposite wells and indicated as a–f. After 24 h precipitating lines had formed between the anti-175-kDa and extract 4 (e), purified 175-kDa protein (f).

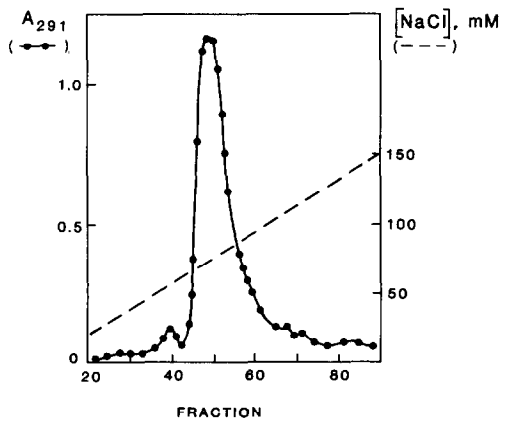

Fig.2. The purification of 175-kDa protein from chicken gizzard smooth muscle on a column with DEAE-cellulose. Selected fractions from the DEAE cellulose column were electrophoresed on 10% SDS-polyacrylamide gels. Fractions containing pure 175-kDa protein (fig.1A,f) were pooled.

most of myosin could extract the 175-kDa protein. These data suggest that to solubilize the 175-kDa protein it is necessary to disrupt mainly hydrophobic interactions.

Unfixed cryostat sections of chicken gizzard smooth and cardiac muscles were used for indirect

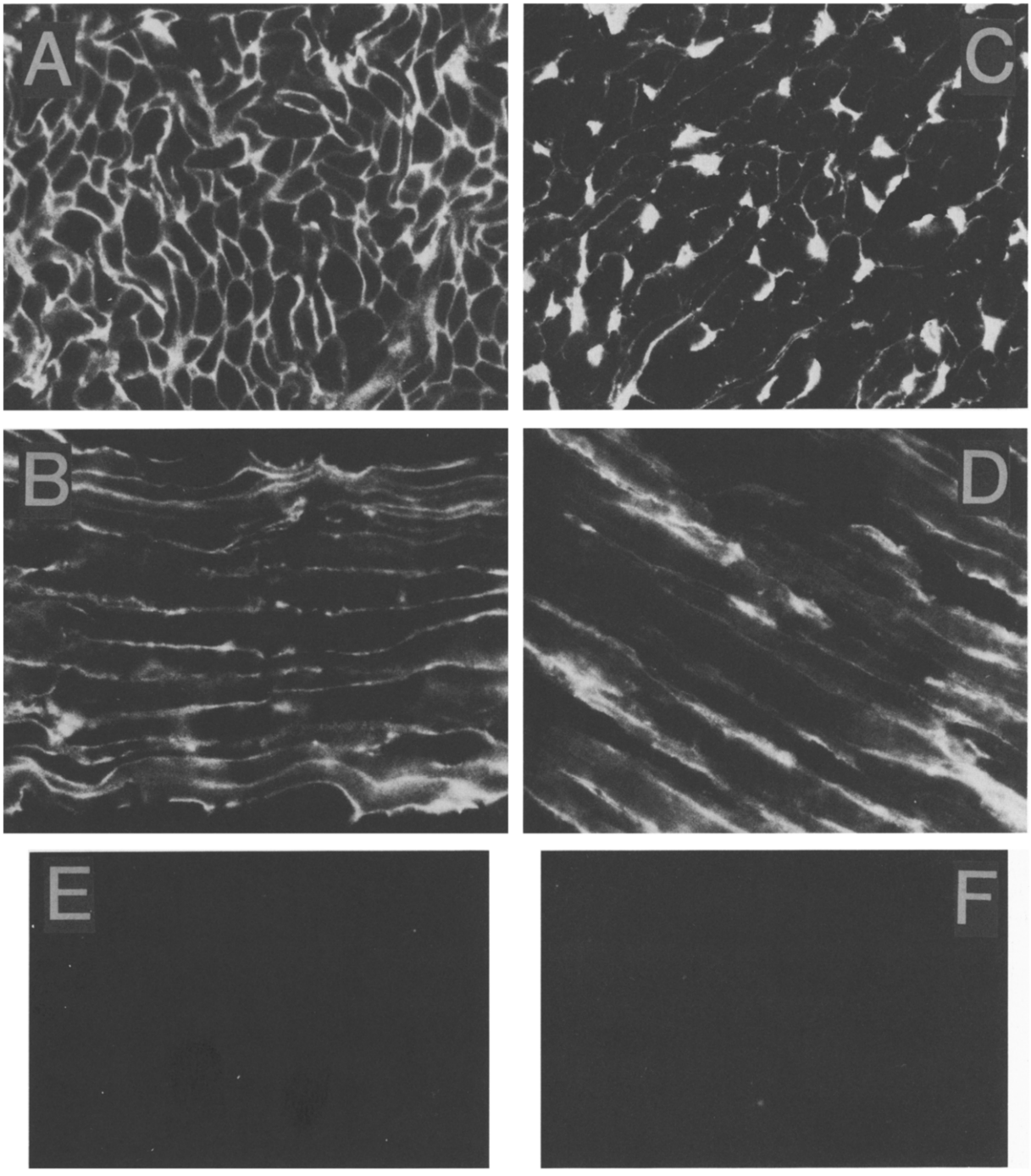

Fig.3. Immunofluorescence localization of the 175-kDa protein in chicken gizzard smooth and cardiac muscles. (A) Transverse section of gizzard smooth muscle. (B) Longitudinal section of gizzard smooth muscle. (C) Transverse section of cardiac muscle. (D) Longitudinal section of cardiac muscle. (E) The same as A, but the antibodies were preadsorbed with 175-kDa protein. (F) The same as A, but section was incubated with non-immune IgG.

immunofluorescence localization of the 175-kDa protein. When transverse sections were examined (fig.3A,C) we observed that the bright fluorescence was confined to the cell margins. No staining is observed intracellulary in fibers that are sectioned transversely. The same results were obtained when longitudinal sections were used for staining (fig. 3B,D). Occasional bright patches of stain (cardiac muscle, fig.3C,D) correspond to vessels. To test whether or not the staining pattern revealed by the antibody against the 175-kDa protein was due to antibodies that recognize the 175-kDa protein, preabsorption experiments were performed (fig. 3E). It was found that preabsorption with purified 175-kDa protein abolished the immunofluorescent staining pattern. The same was found when, instead of antibody against 175-kDa protein, nonimmune rabbit IgG fraction was used (fig.3F). Therefore, the 175-kDa protein is located in smooth and cardiac muscle cells very close to the membrane.

To study the interaction of 175-kDa protein with vinculin and fibronectin the following procedure was used. Vinculin, fibronectin and bovine serum albumin were immobilized on polysterene surface and then purified 175-kDa protein was allowed to bind to adsorbed proteins for 90 min at 37°C. The amount of 175-kDa protein bound to vinculin was determined by enzyme-linked immunoassay. Results of the experiment are shown in fig.4. Under experimental conditions the 175-kDa protein appeared to complex with vinculin, rather than with albumin. The interaction of the 175-kDa protein with such a sticky protein as fibronectin is 10-fold less efficient than with vinculin (to obtain the same binding level a 10-fold higher concentration of 175-kDa protein is necessary). Semiquantitative analysis demonstrated that the K_{diss} for 175-kDa protein complexes with vinculin is approx. 10^6 M^{-1}.

Studies of the solubility properties and localization of 175-kDa protein in cardiac and smooth muscles demonstrated that this protein is closely associated with cell membrane and has properties of an integral membrane protein. Integral membrane-associated proteins have been found to contain a hydrophobic domain which is burried in the lipid bilayer [14]. In vitro 175-kDa protein can form complexes with vinculin – an actin-binding protein which has been identified in microfilaments – membrane attachment regions. Therefore,

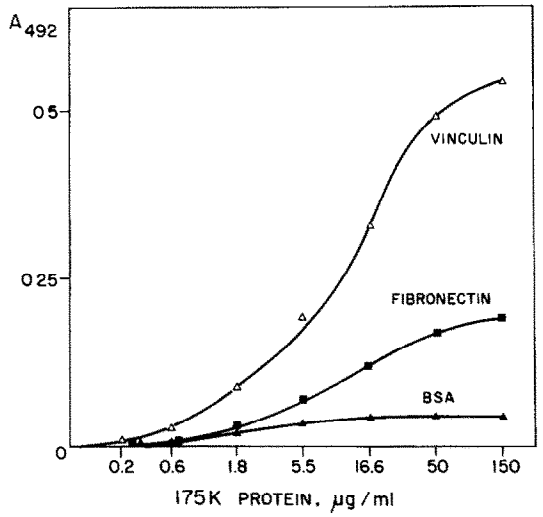

Fig.4. 175-kDa protein binding to proteins immobilized on polysterene surface (ELISA procedure). The wells were coated with vinculin (△), fibronectin (■), BSA (▲).

we suggest that the 175-kDa protein is involved in physical connection between microfilaments and cell membrane. Besides the 175-kDa protein some other proteins were found to have similar functions. Meta-vinculin has solubility properties of an integral membrane protein, is capable of interaction with actin and is located at cytoskeletal-associated membrane specializations [3,7]. Recently, chicken gizzard and nonmuscle cells were shown to contain high M_r vinculin-binding proteins with apparent M_r values of 220000, 190000, 170000 and 215000, 205000, 185000 [15,16]. The 170- and 185-kDa proteins resemble the 175-kDa protein described here. We believe that detection and studies of the integral membrane proteins capable of binding the cytoskeletal component will help to reveal the molecular mechanism of the cytoskeleton-membrane interaction.

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