Purification and Characterization of an F-actin-bundling 55-Kilodalton Protein from HeLa Cells*

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An F-actin-bundling protein with $M_0$, 55,000 has been purified from HeLa cells by a simple method using its affinity to F-actin. Briefly, muscle actin was mixed with supernatants of HeLa cell homogenates, and the resultant actin gel was precipitated by low speed centrifugation. The 55-kDa protein in the actin gel was dissociated by depolymerization of F-actin and purified sequentially by chromatography on DEAE-cellulose and hydroxylapatite. The Stokes radius and sedimentation coefficient of the 55-kDa protein were 32 Å and 4.35 ($\bar{s}_{20,w}$), respectively. These results suggest that the 55-kDa protein is a monomeric globular protein with a native molecular weight of 57,000. The globular form of the protein was confirmed by electron microscopy of rotary shadowed specimens. The binding of the protein to actin was saturated at an approximate stoichiometry of 4 actin monomers to one 55-kDa molecule. The protein made F-actin aggregate side-by-side into bundles as has been reported for other F-actin-bundling proteins such as fimbrin ($M_0$, 68,000) and fascin ($M_0$, 58,000). The 55-kDa protein is a new actin-binding protein based on biochemical, morphological, and immunological characterization. Skeletal muscle tropomyosin inhibited the actin-bundling activity of 55-kDa protein by competitive binding to actin, suggesting that the 55-kDa protein binding site on F-actin is in the vicinity of the tropomyosin-binding site.

Actin-containing microfilaments in animal cultured cells are, at least in part, responsible for a variety of cellular activities including cell motility, cell shape changes, cytokinesis, and adhesion to substratum (1-7). Microfilaments have been observed to change their higher ordered organization dynamically during such cellular activities. For example, microfilament bundles (actin cables or stress fibers) form after disassembly, and a contractile ring forms during cytokinesis and adhesion to substratum (8-11). Immunofluorescence microscopy has revealed that microfilaments are composed of several proteins in addition to actin, such as myosin (9, 12, 13), tropomyosin (14, 15), $\alpha$-actinin (16, 17), filamin (18, 19), myosin light chain kinase (20, 21), fimbrin (22), and vinculin (23, 24). Recent biochemical studies have shown that these microfilament-associated, actin-binding proteins are involved in the regulation of the assembly dynamics of microfilaments through cross-linking filaments into networks or bundles, capping an end of the filaments and severing preformed filaments, inhibiting polymerization, and stabilizing the structure of filaments by binding along the length of actin filaments (see, for review, Refs. 25 and 26). However, the molecular mechanisms for regulating microfilament assembly are still unclear. The identification and biochemical characterization of actin-binding proteins responsible for the microfilament assembly are necessary to understand the dynamic regulation.

The studies on temperature-dependent gelation of soluble extracts of cells have allowed the identification and characterization of a variety of actin-binding proteins (see, for review, Refs. 27-33). We have found that soluble extracts of HeLa cells formed gels, even at cold temperature, when an excess of F-actin was added to the extracts. Like temperature-dependent gelation of soluble extracts of other types of cells, the gel from HeLa cells enriched many actin-binding proteins including filamin, $\alpha$-actinin, and an as yet unknown protein with $M_0$, 55,000. This paper describes the purification and characterization of an actin-binding protein with $M_0$, 55,000. The purified 55-kDa protein made F-actin aggregate into bundles like other actin-bundling proteins such as fimbrin (34, 35) and fascin (36). Biochemical and immunological studies suggest that 55-kDa protein is a new actin-binding protein.

**MATERIALS AND METHODS**

*Isolation of 55-kDa Protein from HeLa Cells*—HeLa cells were cultured in 32 liters of spinner bottles containing F13 medium supplemented with 5% calf serum. Cells were harvested by low speed centrifugation at densities of 5-6 x 10^6 cells/ml. About 40 ml of packed cells were obtained in this way. Cells were washed once with PBS 3 at room temperature, extracted for 2 min at room temperature with Triton/glycerol solution (100 mM PIPES, 5 mM MgCl2, 0.2 mM EGTA, 0.05% Triton X-100, 4 mM glycerol, pH 6.9) and then precipitated by brief centrifugation using an SS 34 rotor. (As soon as the rotor speed reached 12,000 rpm, the rotor was decelerated by turning the time knob to zero with the brake on. The procedure took about 4 min in a Sorvall RC-5B centrifuge.) The Triton-extracted HeLa cells were quickly washed 3 times with PBS containing 5 mM MgCl2, 0.2 mM EGTA by the same brief centrifugation, and then resuspended into 60 ml of PBS containing 5 mM MgCl2 and 0.2 mM EGTA. After addition of ATP and PMSF to final concentrations of 5 mM, the cells were homogenized at 0°C by 10 strokes of a motor-driven Potter-Elvehjem (glass-Teflon) homogenizer (Wheaton, overhead stirrer, disl 3-4). The homogenates (lane b of Fig. 1) were centrifuged at

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The abbreviations used are: PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8 mM NaHPO4, pH 7.3); SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N',N"-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; buffer A, 10 mM Tris-Cl, 0.2 mM dithiothreitol, 0.2 mM PMSF, pH 8.0; buffer B, 10 mM sodium phosphate, 0.2 mM PMSF, 0.2 mM dithiothreitol, pH 7.0; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazineethanesulfonic acid.
17,300 × g for 15 min to remove nuclei and cell membranes and the supernatants were ultracentrifuged at 100,000 × g for 1 h. To about 40 ml of the supernatants (lane c of Fig. 1) were added 3 ml of an F-actin solution (10 mg/ml). The mixture was incubated at 0 °C for 90 min to allow gelation and centrifuged at 17,300 × g for 20 min to collect the actin gel. Three ml of the same F-actin solution (10 mg/ml) were again added to the supernatant, and the mixture was incubated at 0 °C for 90 min to induce the gelation of actin again and then centrifuged at 17,300 × g for 20 min to collect the actin gel. This process was repeated one more time. The resulting actin gels (lane d of Fig. 1) were dialyzed against 5 mM NaHCO₃ to depolymerize F-actin and loaded on a DEAE-cellulose column (2.5 × 20 cm). The proteins were eluted with 600 ml (total) of a linear NaCl gradient (0-500 mM). Fractions of 6.3 ml were collected. The elution profile (absorbance at 280 nm) is shown in the upper portion and every other fraction is analyzed by SDS-PAGE (shown in the lower portion). Proteins were stained with Coomassie blue. Arrows in fractions 36, 56, and 76 indicate 55-kDa protein, filamin, and α-actinin (a doublet), respectively. The positions of molecular mass markers (from top to bottom: 200 kDa, 116.5 kDa, 94 kDa, 68 kDa, 45 kDa, 30 kDa, 21 kDa, and 14.3 kDa) are indicated in lane a.
F-actin-bundling 55-kDa Protein

Fig. 2 Hydroxyapatite column chromatography of 55-kDa protein. Fractions eluted from the DEAE-cellulose column (Fig. 1) containing 55-kDa protein were directly applied to a hydroxyapatite column (1.2 x 15 cm). The elution profile (absorbance at 280 nm) of the column developed with a linear gradient of 10-200 mM sodium phosphate in buffer B (200 ml total) is shown in the upper portion. Fractions of 2.5 ml were collected and analyzed by SDS-PAGE (shown in the lower portion). 55-kDa protein was eluted at fraction 36 as a single band. Molecular mass markers (same as in the legend to Fig. 1) are indicated on the left.

**F-actin Binding Assay**—Purified 55-kDa protein at various concentrations (final concentrations, 0-0.5 mg/ml) was mixed with F-actin (final concentration, 0.54 mg/ml) in 70 µl of 100 mM KCl, 20 mM imidazole HCl buffer of pH 7.0. After incubation for 90 min at room temperature, the mixtures were centrifuged in a Beckman Airfuge at 140,000 x g (26 p.s.i.) for 20 min. The 55-kDa protein alone was not precipitated under these conditions. Pellets were suspended in 70 µl of 20 mM imidazole HCl buffer (pH 7.0) containing 100 mM KCl. Both supernatants and pellets were dissolved in the same volume of 2% SDS sample buffer (5% SDS, 15% glycerol, 100 mM dithiothreitol, 80 mM Tris-Cl, 0.001% bromphenol blue (pH 6.8)), run on 12.5% SDS-PAGE, stained quantitatively with fast green (37) and scanned with a Hoefer densitometer (GS 300) as described previously (38). The molar ratios were calculated using values corrected for differential dye uptake between skeletal muscle actin and 55-kDa protein.

**F-actin-bundling Assay by Low Speed Centrifugation**—Low speed centrifugation was used to detect F-actin-bundling activity of 55-kDa protein. F-actin at 0.7 mg/ml was incubated with varying amounts (0-0.2 mg/ml) of 55-kDa protein in 175 µl of 100 mM KCl, 20 mM imidazole HCl, pH 7.0. In some experiments, divalent cations (up to 5 mM concentrations) such as Mg and Ca were added to the above condition, or KCl concentrations were varied from 30 to 500 mM. After incubation for 90 min at room temperature, the mixtures were centrifuged for 15 min at 12,000 x g (Eppendorf centrifuge, model 5412) under conditions where F-actin alone remained in the supernatant. Supernatants were carefully separated from pellet, and both fractions were suspended in an equivalent volume of SDS sample buffer. Samples were run on 12.5% SDS-polyacrylamide gels, stained with fast green, and quantified by densitometry as described above. The pellets were also examined by electron microscopy using the negative staining technique (38).

**Electron Microscopy**—Rotary shadowing was performed essentially
following the method described by Tyler and Brenton (39). One part of the protein solution (70 μg/ml) in 0.3 m KCl, 0.2 mM dithiotheritol, and 20 mM imidazole HCl of pH 7.0 was mixed with 2 parts of glycerol. The solution was sprayed at room temperature using neutralized onto freshly cleaved mica. Samples were allowed to dry in an Eppendorf-Micromax (Denton) by evaporation for 1 h and were negatively stained at an angle of 5° with Pt/Pd vaporized from a heated tungsten filament. The resulting replicas were coated with a supporting film of carbon, floated onto distilled water, and picked up on bare 400-mesh grids. Negative staining electron microscopy was performed as described previously (38). The specimens were observed with a Phillips 201E electron microscope at an accelerating voltage of 80 kV.

**Determination of Sedimentation Coefficients and Stokes Radius—**
Sucrose density gradient centrifugation was done according to the procedure of Martin and Ames (40) to determine sedimentation coefficients. 55-kDa protein in 100 mM KCl and 20 mM imidazole HCl (pH 7.0) was loaded on the 5-20% (w/v) sucrose gradients and centrifuged in a SW 50.1 Ti rotor (Beckman Instruments) at 38,000 rpm for 19 h at 4 °C in a L 5-65 Beckman preparative ultracentrifuge. Protein standards were purchased from Pharmacia Fine Chemicals and included chymotrypsinogen (s20, w = 2.6) and aldolase (s20, w = 7.4). The Stock solutions of the 55-kDa protein was determined using gel filtration with Sephacryl S-300 (Pharmacia Fine Chemicals) according to the method of Siegel and Monty (41). Standards used to prepare a calibration curve were purchased from Pharmacia Fine Chemicals and included blue dextran (Mw = 2,000,000), catalase (Ms = 240,000 and Stokes radius of 52.2 Å), bovine serum albumin (Mw = 68,000 and Stokes radius of 35.0 Å), and ovalbumin (Mw = 43,000 and Stokes radius of 30.5 Å).

**Antibody Production—**
Antibodies against the 55-kDa protein were made by the subcutaneous immunization of rabbits with the protein which was further purified by preparative SDS-PAGE of the hydroxylapatite column fractions. For the first injection, 150 μg (1 ml of PBS) of the 55-kDa protein was emulsified in 1 ml of Freund’s adjuvant. The resulting emulsion was used to immunize rabbits subcutaneously. Seven days after the last injection, serum was collected. The blots corresponding to 55-kDa protein were removed, incubated for 2 h in 3% bovine serum albumin in PBS for blocking, and reacted for 10 h with the antiserum to 55-kDa protein. After extensive washing with PBS, the bound antibody was eluted from the strips with 4 ml of 0.2 M glycine-HCl, pH 2.8, for 2 min. The eluted antibody solution was immediately neutralized with NaOH, diluted with PBS, and centrifuged to 0.2 ml by Amicon ultrafiltration using YM-10 membranes. The solution was sprayed at room temperature using neutrally. The precipitate was found to be bundles of F-actin. The protein doublet appeared to correspond to filamin and α-actinin, respectively, as judged by the elution positions of these proteins (see Fig. 2 in Ref. 52). We found that among these actin-binding proteins, the 55-kDa protein made F-actin pelletable by low speed centrifugation. As described later, this precipitate was found to be bundles of F-actin. The final purification of the 55-kDa protein was achieved by hydroxylapatite column chromatography (Fig. 2). Approximately 2 mg of pure protein were obtained from 32 liters of HeLa cell suspension culture.

**55-kDa Protein Is an Actin-binding Protein—** Stoichiometry of the actin binding of 55-kDa protein was determined by measuring the amounts of actin and 55-kDa protein pelletable by high speed centrifugation (150,000 × g for 20 min). As Fig. 3 shows, saturation was achieved at an approximate molar ratio of one 55-kDa protein to four actin monomers. The binding curve appears S-shaped, suggesting the cooperative binding of 55-kDa protein to actin.

**55-kDa Protein Bundles F-actin Filaments—**
Electron microscopy of F-actin/55-kDa protein mixtures by the negative staining technique showed numerous bundles of F-actin filaments (Fig. 4). At low ratios of 55-kDa protein to actin, only part of F-actin was observed to be aggregated side-by-side. With an increasing ratio of 55-kDa protein to actin, bundles became thicker and free F-actin filaments were hardly seen. However, in any ratio, transverse bands could not be observed in bundles.

We have quantified the F-actin-bundling activity of the 55-kDa protein by measuring the extent to which 55-kDa protein binds to actin filaments in vitro. The binding of 55-kDa protein to actin filaments was measured by performing a titration experiment in which increasing amounts of 55-kDa protein were added to F-actin filaments in the presence of 1 mM ATP. The extent of bundling was determined by measuring the percentage of actin filaments that were bundled with the 55-kDa protein. The results of these experiments showed that the 55-kDa protein has a high affinity for actin filaments and is capable of bundling them. The binding of 55-kDa protein to actin filaments was determined by densitometry of the fast green stained gels (37) as described previously (38).

**RESULTS**

**Purification of the 55-kDa Protein—** We found that the addition of rabbit skeletal muscle F-actin induced gelation of extracts of HeLa cells even at 0 °C. The actin gel was pelletable by low speed centrifugation away from other elements of the extracts. After depolymerization of the actin gel by extensive dialysis against NaHCO3, the polypeptides in the actin gels were separated by DEAE-cellulose column chromatography (Fig. 1). The actin-binding assay revealed that several proteins in the DEAE-cellulose fractions bound to actin. These included a 55-kDa protein in fraction 36, a 95-kDa protein in fraction 50, a 230-kDa protein in fraction 52, a 135-kDa protein in fraction 68, and a 110-kDa protein doublet in fraction 76. Of these, the 230-kDa protein and the 110-kDa protein doublet appeared to correspond to filamin and α-actinin, respectively, as judged by the elution positions of these proteins (see Fig. 2 in Ref. 52). We found that among these actin-binding proteins, the 55-kDa protein made F-actin pelletable by low speed centrifugation. As described later, this precipitate was found to be bundles of F-actin. The final purification of the 55-kDa protein was achieved by hydroxylapatite column chromatography (Fig. 2). Approximately 2 mg of pure protein were obtained from 32 liters of HeLa cell suspension culture.

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FIG. 4. Electron micrographs of actin bundles formed with 55-kDa protein and F-actin at a low (A) and high (B) magnification. F-actin (0.55 mg/ml) was mixed with 0.1 mg/ml of 55-kDa protein and a specimen was prepared by the negative staining technique. Numerous actin filament bundles were observed.

kDa protein by low speed centrifugation as described under "Materials and Methods." Under these conditions, bundles of F-actin were sedimentable whereas free F-actin remained in the supernatant. As Fig. 5A shows, most F-actin became pelletable at a molar ratio of 55-kDa protein to actin higher than 1:7.

In the same conditions, we also measured apparent viscosities of the mixtures of F-actin/55-kDa protein by low shear viscometry to examine F-actin filament-filament interactions (Fig. 5B). Falling ball viscometry with a capillary at angles of 50° and 80° was performed according to the method of MacLean-Fletcher and Pollard (51). Apparent viscosities of 55-kDa protein/F-actin mixtures measured at both angles were increased with an increasing molar ratio of 55-kDa protein to actin up to 1:18. Although viscosities at higher concentrations of 55-kDa protein (except the highest concentration) could not be measured at an angle of 50° (solid line of Fig. 5B), viscometry at an angle of 80° (dashed line) revealed that viscosities were then decreased with further increasing concentrations of 55-kDa protein. As Fig. 5A shows, F-actin became pelletable in these conditions (compare Fig. 5, A and B). This biphasic effect of 55-kDa protein on viscosities could be explained as follows: first, F-actin is cross-linked by 55-kDa protein at low ratios, which results in the increase in the viscosity and then, F-actin is aggregated into bundles by high concentrations of 55-kDa protein, which makes the viscosities lower.

The effects of divalent cations such as Mg²⁺ and Ca²⁺ and KCl concentration on the F-actin-bundling activity were also examined by low speed centrifugation. Neither Ca²⁺ nor Mg²⁺ (each up to 5 mM) had any significant effects on the bundling activity of the 55-kDa protein (data not shown). Fig. 6 shows KCl dependence in that the 55-kDa protein could precipitate F-actin over a broad range of KCl concentrations from 30 to
Fig. 5. Interaction between 55-kDa protein and actin. A. F-actin-bundling activity of 55-kDa protein. F-actin-bundling activity of 55-kDa protein was assayed by low speed centrifugation. 55-kDa protein, at the specified concentration, was mixed with actin (0.7 mg/ml) and adjusted to 20 mM imidazole HCl, 100 mM KCl, pH 7.0. After incubation for 90 min at room temperature, the mixtures were centrifuged 15 min at 12,000 × g (Eppendorf centrifuge). Both supernatants and precipitates were quantitated by densitometry as described in the legend to Fig. 1. B. Low shear viscometry of 55-kDa protein/actin mixtures. Falling ball viscometry was performed according to MacLean-Fletcher and Pollard (51) with the capillary at an angle of 50° (solid line) and 80° (dashed line). The assay conditions are the same as for A.

200 mM. The activity appeared to be optimal around 100 mM KCl, as judged by the density of actin/55-kDa protein bands in the precipitates.

The Interactions of 55-kDa Protein with Tropomyosin—Mixtures of F-actin, skeletal muscle tropomyosin, and 55-kDa protein in various combinations were prepared to examine the effect of skeletal muscle tropomyosin on the bundling activity of 55-kDa protein by low speed centrifugation. Then, the supernatants were further centrifuged for 20 min at 140,000 × g to assess the actin binding of 55-kDa protein and/or tropomyosin. Both supernatants and pellets in the high and low speed centrifugation were examined by SDS-PAGE (Fig. 7), and the amounts of actin, 55-kDa protein, and tropomyosin were quantitated by densitometry.

Neither pure F-actin nor F-actin/tropomyosin complexes were pelletable by low speed centrifugation (series A and B). As series D shows, F-actin became sedimentable by low speed centrifugation when 55-kDa protein was added. However, skeletal muscle tropomyosin inhibited the bundling activity of 55-kDa protein (series C). The amounts of F-actin precipitated by low speed centrifugation were reduced to 26% of the control (compare lane 2 of series C with lane 2 of series D) when 55-kDa protein was added to F-actin-tropomyosin complex. Concomitantly, the actin binding of 55-kDa protein was inhibited to 62% of the control by the presence of tropomyosin (compare the amount of 55-kDa protein bound to F-actin...
actin; first incubated for 1 h with 55-kDa protein and then incubated for 1 h with 55-kDa protein and then incubated for 1 h with 55-kDa protein and then incubated for 1 h with 55-kDa protein and then incubated for 1 h with tropomyosin. Each mixture was first centrifuged for 15 min at 12,000 x g (Eppendorf centrifuge) to see the bundling activity. The supernatants were carefully separated from the pellets and further centrifuged for 20 min at 140,000 x g. Both supernatants (lane 1) and pellets (lane 2) by low speed centrifugation and both supernatants (lane 3) and pellets (lane 4) by high speed centrifugation were dissolved in an equivalent volume of SDS sample buffer and analyzed by SDS-PAGE. Assay conditions were rotary shadowing method. This was confirmed by the direct observation of the electron microscopic specimen prepared by the rotary shadowing technique (Fig. 8). 55-kDa protein migrated as a polypeptide with apparent M, of 55,000 on one-dimensional SDS-polyacrylamide gels. On two-dimensional gels, 55-kDa protein was focused as three isoelectric variants (Fig. 8E). The reason for the existence of three variants is currently under study. Two-dimensional gel analysis also showed that the 55-kDa protein was present in total cell lysates. Fig. 9, A and B, shows the two-dimensional gel patterns of total cell lysates prepared from [35S]methionine-labeled Hut-17 cells. To help the identification of 55-kDa protein on two-dimensional gels, purified 55-kDa protein (unlabeled) was mixed with total cell lysates (35S-labeled) and simultaneously analyzed by two-dimen-

![Fig. 7. Effect of tropomyosin on the bundling activity of 55-kDa protein. Mixtures of actin, tropomyosin, and 55-kDa protein in various combinations were prepared as follows: series A, pure actin; series B, actin mixed with tropomyosin; series C, actin first incubated for 1 h with tropomyosin and then incubated for 1 h with 55-kDa protein; series D, actin mixed with 55-kDa protein; series E, actin first incubated for 1 h with 55-kDa protein and then incubated for 1 h with tropomyosin. Each mixture was first centrifuged for 15 min at 12,000 x g (Eppendorf centrifuge) to see the bundling activity.](image)

![Fig. 8. Electron micrograph of 55-kDa protein prepared by the rotary shadowing method.](image)
FIG. 9. **Two-dimensional gel analysis of 55-kDa protein.** Total cell lysates prepared from [³⁵S]-methionine-labeled Hut-11 cells were analyzed by two-dimensional gel electrophoresis with pH 3–10 ampholytes for the first dimension and 12.5% acrylamide for the second dimension. After electrophoresis, gels were stained with Coomassie blue (left panel) and autoradiographed (right panel). A and B, total cell lysates. C and D, total cell lysates mixed with purified 55-kDa protein. E, purified 55-kDa protein. 55-kDa protein was focused as three variants different in isoelectric points (as indicated by arrows). The 55-kDa protein spots in the autoradiograph of total cell lysates mixed with purified 55-kDa protein (D) were somewhat destroyed by the co-migration of cold protein added. The most acidic spot of 55-kDa protein could not be identified in total cell lysates (B and D) because of its low quantity.
suggestion that 55-kDa protein and fimbrin are not related to each other.

Finally, we examined the localization of the 55-kDa protein in Hut-11 cells by indirect immunofluorescence. As Fig. 11, A and C, shows, staining of microspikes was found prominent. Occasionally, ruffling membranes and stress fibers were also stained. Bretscher and Weber (22) reported that fimbrin is localized in membrane ruffles, microvilli, and microspikes. The localization of fimbrin in Hut-11 cells was examined for comparison. As Fig. 11E shows, the antibody stained ruffling membranes and microspikes as reported. These results suggest that both 55-kDa protein and fimbrin are the structural components of microfilament bundles in microspikes of cultured cells. A study of whether both proteins show the same localization is in progress using monoclonal antibodies to 55-kDa protein.

**DISCUSSION**

In this paper, we described the purification and characterization of an actin-binding protein with $M_r$ of 55,000 from HeLa cells. The in vitro property of the protein is the bundling activity of F-actin filaments. Several proteins have been reported to have actin-bundling activity, including fascin from sea urchin (36), scrub from Limulus sperm (53), and fimbrin from chicken brush border (34, 35). Among them, fascin and fimbrin appear to be most similar to 55-kDa protein in that these proteins are a globular and a monomeric protein, they have a similar molecular weight range (fascin, $M_r = 58,000$; fimbrin, $M_r = 68,000$), and they show similar saturation of actin binding in a molar ratio of 1 molecule to 2-4 actin monomers. The morphologies of actin bundles made with 55-kDa protein are more similar to those of fimbrin/actin bundles than to those of fascin/actin bundles. Like fimbrin/actin bundles, 55-kDa protein/actin bundles showed less ordered structure than fascin/actin bundles and did not show 11-nm transverse bands characteristic of fascin/actin bundles. Furthermore, the antibody against fascin did not cross-react with 55-kDa protein on Western blots, suggesting that 55-kDa protein is immunologically different from fascin.

Despite the similarity in the morphology between 55-kDa protein/actin and fimbrin/actin bundles, 55-kDa protein and fimbrin differ in the actin-bundling activity. The bundling activity of 55-kDa protein was optimal around 100 mM KCl whereas that of fimbrin appears to be weak in this condition (34, 35). Moreover, the binding of fimbrin to actin is inhibited by 5 mM Mg$^{2+}$ or 0.1 mM Ca$^{2+}$ (35) while that of 55-kDa protein was hardly affected by such divalent cations.

The 55-kDa protein is a new actin-bundling protein different from fimbrin as judged by the following pieces of evidence. 1) The 55-kDa protein spots were identified on two-dimensional gels in total cell lysates freshly prepared. 2) The 55-kDa protein was immunologically different from fimbrin or fimbrin core protein. 3) Fimbrin core protein did not co-migrate with 55-kDa protein on two-dimensional gels (55-kDa protein is more basic, data not shown). 4) Fimbrin core protein lost the activity of actin-bundling.

There are two reports on the protein which may be similar to the 55-kDa protein. Carraway and co-workers (54) reported the presence of 58-kDa protein in microvilli with a unique morphology, branched microvilli, which were isolated from a subline of rat mammary ascites tumor cells. This 58-kDa protein is associated both with membrane fractions obtained by fragmenting the microvilli and with the Triton-insoluble microvillus cytoskeletal residues. The localization of this 58-

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\[ P. \text{ Matsudaira, personal communication.} \]

\[ J. \text{ Bryan, personal communication.} \]
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![Fig. 11. Phase-contrast (right panel) and fluorescent (left panel) micrographs of Hut-11 cells indirectly stained with antibodies against 55-kDa protein (A and C) and against fimbrin (E). The 55-kDa protein appears to localize in microspikes and occasionally in ruffling membranes although considerable staining in cell bodies is also observed. Fimbrin antibody stains ruffling membranes and microspikes as reported (22).](image)

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Maekawa et al. (55) purified 53-kDa protein from porcine brain. The 53-kDa protein is a monomer and bundles actin filaments like 55-kDa protein. However, the 53-kDa protein is an acidic protein having a pI of 5.62 whereas the 55-kDa protein is a basic protein showing an approximate pI of 8.5. Moreover, the 53-kDa protein eluted at a phosphate concentration of 15 mM on hydroxylapatite column while the 55-kDa protein eluted at 150 mM. Further biochemical and immunological characterization is needed to elucidate the relation between these 55-, 58-, and 53-kDa proteins.

Like temperature-dependent gelation of cytoplasmic extracts (27–33), we showed that HeLa cell extracts formed actin gels at cold temperature if excess amounts of F-actin were added. Actin in soluble extracts of cells prepared in the cold is believed to be in the nonsedimentable state. In temperature-dependent gelation, actin polymerization is induced on warming. In the gelation at cold temperature, the addition of F-actin in the HeLa cell extracts also induced polymerization of HeLa cell actin at cold temperature, as judged by the incorporation of [35S]methionine-labeled HeLa cell actin in the actin gels. This is probably because the addition of excess
amounts of actin shifts the equilibrium of actin polymerization by blocking the action of some factors such as profilin (56, 57).

It is reported that tropomyosin is not found in microspikes and ruffle membranes by immunofluorescence (58). The staining of the 55-kDa protein antibody in microspikes may be consistent with the competition of actin-binding between 55-kDa protein and muscle tropomyosin. 55-kDa protein aggregated pure F-actin into bundles while it aggregated very poorly the F-actin-tropomyosin complex. Similar inhibition of actin-bundling by tropomyosin was also observed in fascin. 3

Actin binding of nonmuscle tropomyosin is weaker than that of muscle tropomyosin (59). It is necessary, therefore, to investigate whether nonmuscle tropomyosin shows similar inhibition of the bundling activity of 55-kDa protein. Recently, we have shown that rat cultured cells have as many as five forms of tropomyosin (38). These multiple forms of tropomyosin were different in their affinities to actin. 4 Furthermore, these tropomyosin variants are differentially expressed in microfilaments upon cell transformation, suggesting the function of tropomyosin in the changes in the microfilament organization in transformed cells (50, 60). The in vitro interaction between 55-kDa and these nonmuscle tropomyosin variants is currently under progress to help the understanding of the function of 55-kDa protein and tropomyosin in the regulation of microfilament assembly dynamics.

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