

Characterization and Localization of Actinogelin, a Ca^{2+} -sensitive Actin Accessory Protein, in Nonmuscle Cells

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ABSTRACT Actinogelin, which induces gelation of F-actin at Ca^{2+} concentrations below micromolar concentrations but not at higher concentrations, was isolated in the pure state from Ehrlich tumor cells. The protein consists of subunits of 112,000–115,000 daltons and under physiological conditions is present mostly as a dimer. Up to 1 mol of actinogelin (dimer) binds to 10–12 mol of actin monomer. The binding was slightly decreased by the presence of $50 \mu\text{M}$ Ca^{2+} and almost completely inhibited by 300 mM KCl. Antibodies against actinogelin giving a single precipitation line with Ehrlich cell extract and with pure actinogelin were raised in rabbits. Antibody preparations were purified before use in an affinity column containing purified actinogelin.

In mouse embryo fibroblasts and 3T3 cells, staining of actin bundles by the antiactinogelin antibody usually was discontinuous or gave a striated appearance. Most of the crossing points of the actin bundles were intensively stained. In epithelial cells from mouse small intestine, actinogelin was distributed throughout the cell, with the exception of the microvilli, which were devoid of staining. In mouse peritoneal cells, the antibody staining patterns were similar to those of tetramethylrhodamine isothiocyanate-labeled heavy meromyosin, but the former usually were sharper than the latter. Intracellular localization of actinogelin was drastically altered by cytochalasin D treatment at $10 \mu\text{g}/\text{ml}$.

We conclude that actinogelin is present in a wide variety of cell types and discuss the possible participation of actinogelin in the Ca^{2+} -dependent regulation of microfilament distribution.

Recent research indicates that microfilaments in nonmuscle cells not only are responsible for cell locomotion and motility (6, 33, 48) but also provide the mechanical basis for diverse cellular activities, including cell division (27), phagocytosis (40, 56, 57), cell fusion (2, 4, 46, 53), and maintenance of cell shape (5). In contrast to actin filaments in muscle cells, microfilaments in nonmuscle cells seem to change their configuration and aggregation state in response to external conditions and cellular events. It is possible that actin accessory proteins regulate microfilament organization.

Actin-related gelation of cell-free extracts seems to be one of the best methods for the isolation of accessory proteins of microfilaments and, therefore, has been used for the study of a variety of cells, including sea urchin eggs (31), *Dictyostelium* (14), *Acanthamoeba* (35, 36), macrophages (57), HeLa cells (65), *Xenopus* oocytes (13), and Ehrlich tumor cells (28, 41). In all the systems examined, actin has invariably been identified as the major structural component of the gel. However, the

accessory proteins required for gelation seem to differ among cell types, suggesting diverse gelation phenomena. It recently has been shown that, in a number of cell types, micromolar concentrations of Ca^{2+} reversibly inhibit actin-related gelation (21, 26, 30, 41). Inasmuch as intracellular Ca^{2+} at micromolar concentrations is known to affect several cellular activities that might also be related to microfilament function (11), Ca^{2+} seems to be a regulatory factor both for actin-related gelation and for several microfilament-dependent cellular activities. If this is so, then it is possible that Ca^{2+} regulates these Ca^{2+} -sensitive cellular activities by affecting certain types of microfilament organization. Therefore, actin-related gelation should provide an excellent experimental system for studies of some regulatory functions of Ca^{2+} .

Recently, we (42) isolated an actin-binding protein, actinogelin, from Ehrlich tumor cells. This protein produces a Ca^{2+} -sensitive gel when mixed with skeletal muscle F-actin. The purpose of this paper is to report the molecular characterization

of actinogelin and its intracellular localization as determined by immunofluorescence microscopy. From the results reported in this article and previous papers (37, 42), we conclude that actinogelin differs markedly from recently discovered actin accessory proteins of nonmuscle origin that sever F-actin Ca^{2+} dependently (gelsolin [67, 68], fragmin [24], and a serum factor [44]). It is, however, rather similar to an 85,000-dalton protein purified from *Acanthamoeba* (48) and to a 95,000-dalton component of *Dictyostelium* fraction I (26). We also compare actinogelin with a unique Ca^{2+} -dependent actin accessory protein, villin, isolated from the brush border of intestine (7, 9, 15, 39, 43).

MATERIALS AND METHODS

Isolation and Purification of Actinogelin

An 8-azaguanine-resistant strain of Ehrlich tumor cells (39) was propagated in the peritoneal cavities of ddN mice, harvested, and washed as described previously (42). After the cells were washed, the packed cell volume was determined by centrifugation at 650 g for 10 min. Cell pellets were suspended in 1.5 vol of an extraction medium containing 0.34 M sucrose, 1 mM ATP, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 $\mu\text{g}/\text{ml}$ pepstatin, and 20 mM imidazole HCl buffer (pH 7.0 at 20°C). Cell-free extracts were then prepared as described (41).

STEP I: AMMONIUM SULFATE FRACTIONATION: The cell-free extract was adjusted to 0.6 M with respect to KCl concentration by adding 3 M KCl, and stirring was continued for 2–3 h at 4°C. The mixture was fractionated with solid ammonium sulfate, and the precipitate that formed by the addition of 85 g/liter ammonium sulfate was removed by centrifugation and discarded. The pellet that formed by the further addition of 124 g/liter ammonium sulfate (final concentration, 209 g/liter) was collected. The pellet was dispersed with a buffer containing 10 mM imidazole HCl (pH 7.0), 0.1 M KCl, 2 mM MgCl_2 , 0.1 mM PMSF, and 0.1 $\mu\text{g}/\text{ml}$ pepstatin and then dialyzed overnight against 3 liters of the same buffer. By the addition of 3 M KCl, the solution was made 0.6 M with respect to KCl. After stirring for 2 h at 4°C, the solution was spun at 100,000 g for 3 h in a Hitachi RP-40 rotor (Hitachi Koki Co. Ltd., Tokyo, Japan), and the clear supernate was fractionated again by ammonium sulfate in the same way. The resultant pellet was dispersed with a buffer containing 20 mM imidazole HCl (pH 7.2), 0.1 mM PMSF, and 0.1 $\mu\text{g}/\text{ml}$ pepstatin and then dialyzed overnight against 3 liters of the same buffer.

STEP II: DEAE-CELLULOSE ION-EXCHANGE CHROMATOGRAPHY: The insoluble materials were removed by centrifugation at 40,000 g for 10 min in a SS-34 rotor (DuPont Instruments-Sorvall, DuPont Co., Newtown, CT), and the clear supernate was applied to a column of DEAE-cellulose (DE32, Whatman Inc., Clifton, NJ) (3×15 cm) equilibrated with 20 mM imidazole HCl (pH 7.2). The column was washed with 100 ml of 20 mM imidazole HCl (pH 7.2). Elution was performed by a stepped increase in KCl concentration. First, the inactive proteins were eluted with 400 ml of 0.2 M KCl in 20 mM imidazole HCl (pH 7.2). Actinogelin was then eluted with 400 ml of 0.4 M KCl in 20 mM imidazole HCl (pH 7.2).

STEP III: HYDROXYLAPATITE CHROMATOGRAPHY: The actinogelin-containing fractions were combined and dialyzed against 10 mM potassium phosphate (pH 6.8). The dialyzate was applied to a column of hydroxylapatite (2×6.5 cm) prepared according to the method of Tiselius et al. (61). After the column had been washed with 70 ml of 50 mM potassium phosphate (pH 6.8), elution was carried out with a linear gradient prepared from 60 ml of 50 mM potassium phosphate (pH 6.8) and 60 ml of 0.25 M potassium phosphate (pH 6.8) at a flow rate of 10–12 ml/h. Actinogelin was eluted as a major peak between 0.1 and 0.2 M potassium phosphate. Those fractions containing actinogelin detectable by SDS gel electrophoresis were pooled.

STEP IV: SEPHAROSE 6B CHROMATOGRAPHY: The pooled fractions were concentrated from 20 ml to 1 or 1.5 ml with a collodion membrane (Sartorius Co., Ltd., Göttingen, W. Germany) and applied to a column of Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ) (1.2×95 cm) equilibrated with 20 mM Tris-HCl (pH 7.6) containing 50 mM NaCl, 0.1 $\mu\text{g}/\text{ml}$ pepstatin, and 1 mM NaN_3 . The same buffer was used for elution, and 1.2-ml fractions were collected. Fractions containing the 115,000-dalton protein were pooled and identified as pure actinogelin by SDS polyacrylamide gel electrophoresis.

Quantification of Actinogelin-dependent Gelation

Actinogelin-dependent gelation was conducted routinely using 2 mg/ml of skeletal muscle actin at 25°C for 1 h in a standard reaction medium containing

10 mM imidazole-HCl (pH 7.2), 0.5 mM EGTA or 0.5 mM CaCl_2 , 1 mM MgCl_2 , and 50 mM KCl. Quantification of the gelation reaction was performed by measuring the volumes of or the masses (by Lowry's method) of the protein in the pellets after compression of the gels by centrifugation. For quantification by protein measurement, the gels were centrifuged at 49,000 g for 10 min at 20°C, and the resultant supernates were removed with a pasteur pipette. The amount of protein in the pellets was determined either immediately (Fig. 2) or after the gel had been rinsed with a medium containing 0.1 M KCl and 10 mM imidazole HCl (pH 7.2) by recentrifugation at 49,000 g for 10 min.

For the measurement of compressed volume, the gels were centrifuged at 33,000 g for 10 min in a Sorvall SM-24 rotor, and the volume of the resultant supernates was measured with a microsyringe. The volume of compressed gels was calculated by subtraction of the volume of the supernates from the total volume of the reaction mixture.

One unit of actinogelin was defined as follows. F-actin and actinogelin were combined and the complex was centrifuged at 49,000 g for 10 min. An identical amount of F-actin alone was sedimented under the same conditions. Subtracting the mass of the precipitate of F-actin alone from the mass of the precipitate of the complex, one unit was defined as the amount of actinogelin fraction resulting in 1 mg. Specific activity was expressed as units per milligram of protein. The amount of protein precipitated Ca^{2+} -sensitively (EGTA- Ca^{2+} column) was calculated by subtracting the mass of protein precipitated in Ca^{2+} -supplemented (0.05 mM) samples from that precipitated from samples containing 0.5 mM EGTA. Usually, the actinogelin content of the various fractions was also monitored by SDS polyacrylamide gel electrophoresis.

Purification of Rabbit Skeletal Muscle Actin

Rabbit skeletal muscle actin was prepared from acetone powder by the method of Spudich and Watt (55). Purified G-actin was stored at 4°C in 2 mM Tris-HCl buffer (pH 8.0) containing 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl_2 , 1 mM NaN_3 , and 0.2 mM ATP. Stored samples were used within 2 wk.

Electrophoresis

SDS gel electrophoresis was performed according to Fairbanks et al. (20) using 5.6% polyacrylamide gels. The protein samples were heated at 100°C for 3 min in 1% SDS, 10% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1% 2-mercaptoethanol. The protein was stained for ~10 h with 0.04% Coomassie Brilliant Blue in 25% isopropyl alcohol and 10% acetic acid. The gels were destained by diffusion in 10% acetic acid.

Ultracentrifugal Analysis

Sedimentation equilibrium experiments were performed at 6,400 rpm with a model E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 20°C until the protein reached equilibrium (usually for 17–18 h) in the medium containing 50 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.1 $\mu\text{g}/\text{ml}$ pepstatin, and 1 mM NaN_3 . The sedimentation pattern was scanned with an ultraviolet scanner at 280 nm or an interference optical system. The photographic records of the results obtained with the interference optical system were measured on a micro-comparator (Olympus Corp., Tokyo, Japan). The calculations used for molecular weight were the same as those described by Cherminka (12). A partial specific volume of 0.73, which was calculated from the amino acid composition of rat liver actinogelin (Kuo et al., unpublished data), was used for the calculation.

Chemical Cross-Linking

Purified actinogelin at 0.4 mg/ml in 50 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.1 $\mu\text{g}/\text{ml}$ pepstatin, and 1 mM NaN_3 was mixed with an equal volume of 6 mg/ml dimethylsuberimidate dihydrochloride (pH 7.8) in 50 mM Tricine (N-tris[hydroxymethyl]methylglycine)-NaOH. After the mixture was incubated for 3 h at 37°C, 0.5 vol of a medium containing 3% SDS, 30% sucrose, 30 mM Tris-HCl (pH 8.0), 3 mM EDTA and 3% 2-mercaptoethanol was added to stop the cross-linking. The sample was boiled for 3 min and then analyzed by electrophoresis on 4% polyacrylamide gels.

Other Analytical Methods

The level of calmodulin contaminating the actinogelin fractions was monitored by the Ca^{2+} -dependent activation of cyclic nucleotide phosphodiesterase according to the method of Kakiuchi and Yamazaki (29). Protein was determined by the method of Lowry et al. using bovine serum albumin as a standard.

Cells and Tissue Cultures

Primary cultures of mouse embryo fibroblasts were prepared from 10-d mouse embryos. The cells were cultured in Eagle's minimum essential medium (Eagle's

MEM) containing 10% bovine serum in a humid atmosphere of 5% CO₂ and 95% O₂. In the present experiments, cells from passages 1 and 2 were used. 3T3 (Swiss albino) mouse fibroblasts were cultured in Dulbecco's modified Eagle's MEM containing 5% fetal calf serum.

Cells, for the most part macrophages, were obtained from the peritoneal cavities of mice 4 d after the injection of thioglycolate medium as described previously (25). Peritoneal cells suspended in Hanks' medium were placed on cover slips and kept in a moist chamber for 20 min at room temperature. Then the cover slips were rinsed with Hanks' medium and placed in tissue culture dishes 30 mm in diameter. To the dishes were added 1 ml of Eagle's MEM containing 10 mM HEPES-NaOH buffer (pH 7.5) and 0.25 ml of bovine serum; they then were incubated at 37°C for 1 h. After the incubation period, the cover slips were rinsed with phosphate-buffered saline and fixed with 3.6% formaldehyde, as described below.

Antibodies

Antibodies to actinogelin were raised in rabbits. The pure antigen (275 µg) in incomplete Freund's adjuvant was injected subcutaneously at multiple sites. At 2-wk intervals, two booster immunizations (100 µg and 300 µg in incomplete adjuvant, respectively) were given; 3 wk after the second, a final booster (100 µg in complete adjuvant) was injected. Blood was collected 10 d after the last injection. An IgG fraction was prepared by ammonium sulfate fractionation and DEAE-cellulose chromatography. Purification of monospecific IgG was carried out by an affinity column prepared from purified actinogelin (4.0 mg of actinogelin per 4 ml of glutaraldehyde-activated Ultrogel AcA [LKB Wallac Instruments, France] [60]). Fluorescein isothiocyanate (FITC)-conjugated goat IgG against rabbit IgG was purchased from Miles Laboratories Inc. Elkhart, IN and fractionated on DEAE-cellulose. The material with 1 or 2 fluorophores per molecule, determined as described previously (32), was used for immunolabeling.

Heavy meromyosin (HMM) was prepared as described by Yagi and Yasawa (66). Fluorescence labeling of HMM with tetramethylrhodamine isothiocyanate (TRITC) was performed as described by Herman and Pollard (27). HMM preparations containing about 0.5 mol of TRITC/mol of protein, based on *A₆₉₄* and the protein concentration of the conjugate, were used for cell staining.

Fluorescence Labeling of Cells

The cells grown on cover slips were processed for immunofluorescent staining as described by Dedman et al. (18) with slight modifications that are described in the figure legends and in Results. Epithelial cells from mouse intestine were prepared for immunofluorescence microscopy by the method of Bretscher and Weber (7).

Stained cells were observed with a Nikon FL microscope equipped with an EF epiillumination attachment and a 100-power (UV-F 100) objective lens. FITC was analyzed with a Nikon B excitation filter and a 515W barrier filter. TRITC was analyzed with a Nikon G excitation filter and 580W barrier filter. Control experiments were carried out with the IgG fraction prepared from nonimmune sera at 0.10 mg/ml in place of 0.06 mg/ml specific IgG. The photographs were taken on Kodak Tri-X film and developed with Pandol to an ASA of 800.

Electron Microscopy

Rabbit skeletal muscle actin and pure actinogelin were incubated at 25°C for 20 min in the standard reaction medium for gelation measurement as described above, except that actin and actinogelin were added to final concentrations of 82 and 42 µg/ml, respectively, and CaCl₂ was included to a final concentration of 50 µM. In control experiments, actinogelin was omitted from the incubation mixture.

Incubated samples were transferred to carbon-coated copper grids (excess liquid was removed) and stained with 1% uranyl acetate. Micrographs were taken at a magnification of 4,000–20,000 on a JEOL 100C microscope.

Chemicals

RNA polymerase B was purchased from Seikagaku Kogyo Ltd., Tokyo, Japan and bovine serum albumin, RNA polymerase from *Escherichia coli* (core enzyme), and soybean trypsin inhibitor were obtained from Boehringer Mannheim GmbH (Mannheim, W. Germany) as marker proteins for SDS polyacrylamide gel electrophoresis. Dimethylsuberimidate dihydrochloride (DMS) was purchased from Wako Pure Chemical Industries, Ltd. Osaka, Japan. All other reagents are readily obtainable commercially and were of analytical grade.

RESULTS

Purification of Actinogelin

Actinogelin was purified from extracts of Ehrlich tumor cells by a combination of ammonium sulfate fractionation, ion exchange, hydroxyapatite, and gel permeation chromatography, as described in Materials and Methods. Table I summarizes a typical purification of actinogelin, and SDS gel electrophoretograms of samples of each purification step are shown in Fig. 1.

From ~5.7 g of protein from crude cell-free extracts, 2.5 mg of pure actinogelin was recovered in the final fraction. When fractions containing almost pure (>90%) actinogelin were collected together with the pure fractions, 5–6 mg of the protein usually was obtained at the Sepharose 6B step. The fractions containing some contaminants (3.7 mg protein in Table I) either were used as almost pure actinogelin or were further purified by rechromatography on a DE32 column as described in the Step II section of Materials and Methods.

The degree of purification at each step was estimated by measuring the increase in protein precipitation (mostly actin) at a fixed centrifugal force, as described below. Fig. 2 depicts the relationship between centrifugal force applied and amounts of F-actin-actinogelin complex precipitated in a system reconstituted from pure skeletal muscle F-actin and pure actinogelin. If the centrifugal force is as low as 120,000 g × min, compression of the gel is not complete: therefore, an appreciable amount of soluble proteins remains in the gel fraction (i.e., pellet fraction), especially when crude actinogelin preparations are used. Thus, to avoid overestimation of its activity, we selected centrifugation at 49,000 g for 10 min (490,000 g × min) for the assay of actinogelin. Although no Ca²⁺ sensitivity could be observed at the first ammonium sulfate fractionation step, the actinogelin content in each subsequent purification step could be successfully determined and its specific content calculated from the linear portions of the titration curves (Fig.

TABLE I
Purification of Actinogelin

Purification steps	Total protein mg	Total activity* (recovery)				Specific activity‡			(Relative purity)
		+ EGTA	+ Ca ²⁺	EGTA-Ca ²⁺	%	+ EGTA	+ Ca ²⁺	EGTA-Ca ²⁺	
2nd ammonium sulfate	630	252	63	189	(100)	0.4	0.1	0.3	(1.0)
DE32	77	316	100	216	(114)	4.1	1.3	2.8	(9.3)
Hydroxylapatite	16	150	40	110	(58)	9.4	2.5	6.9	(23.0)
Sepharose 6B	6.2	82	25	57	(30)	13.3	4.1	9.2	(30.5)

* One unit is defined as the amount of actinogelin which precipitates 1 mg of protein (mostly actin) by centrifugation at 49,000 g for 10 min. It was calculated from the linear phase of calibration curves (Fig. 3).

‡ Specific activity is expressed as units per milligram of protein.

Purification was started from 5,700 mg of protein from the cell extracts.

3). Low but measurable gelation activity is always observed with pure actinogelin by centrifugal assay, even under high Ca^{2+} conditions, although such activity is much lower as determined by volumetric assay. Thus, the F-actin-actinogelin interaction may not be a simple on/off regulation by Ca^{2+} , and the centrifugal force used in the assay may be sensitive enough to detect small amounts of cross-linking of F-actin, as can be seen from the results shown in Fig. 2.

From the results summarized in Table I (that an approximately 30-fold purification could be attained with 30% recovery and that 6.2 mg of the final preparation was obtained from 5.7 g of protein in the crude extract), the content of actinogelin in the crude extract is estimated to be $>0.36\%$ and $<3.3\%$, although the accuracy of this calculation depends on how much the other actin accessory proteins contaminate the partially purified preparations.

Gel filtration of purified and stored preparations of actinogelin on Sepharose 6B usually exhibits a single peak with a preceding shoulder (data not shown). Since such samples are judged pure by SDS electrophoresis, it is thought that the preceding shoulder is the same actinogelin but with a different oligomeric structure.

Molecular Properties of Purified Actinogelin

The apparent molecular weight of actinogelin was estimated by SDS polyacrylamide gel electrophoresis with 5.6% gels. Actinogelin was found to have a slightly slower mobility than the X subunit of RNA polymerase B of *Thermus thermophilus*, which has a molecular weight of 100,000 (16). In two experiments with different sets of molecular weight markers (one containing RNA polymerase of *E. coli*, bovine serum albumin, and soy bean trypsin inhibitor as the markers and the other using RNA polymerase B of *T. thermophilus*), the apparent molecular weight of actinogelin was determined to be 115,000 and 112,000, respectively (data not shown). A sedimentation equilibrium study conducted at 20°C at an actinogelin concentration of 2.5 mg/ml with an interference optical system showed that native (undenatured) actinogelin had a molecular weight of 242,000 (Fig. 4). In another experiment, in which an ultraviolet scanner was used and the actinogelin concentration was

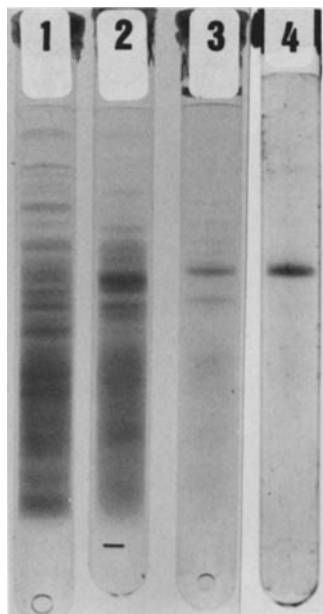


FIGURE 1 SDS gel electrophoretograms of pure and partially purified actinogelin. (1) A sample purified to second ammonium sulfate fraction (Step I). (2) A sample purified to step II (DEAE-cellulose column chromatography). (3) A sample purified by hydroxylapatite chromatography (step III). (4) A final preparation (step IV).

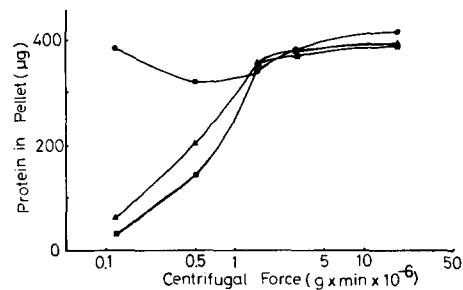


FIGURE 2 Sedimentation profile of F-actin and actin-actinogelin mixture in the presence and absence of Ca^{2+} at various $g \times \text{min}$ values. The reaction mixtures consisted of 21 μg of purified actinogelin, 370 μg of actin, 50 mM KCl, 1 mM MgCl_2 , 10 mM imidazole HCl (pH 7.2), and either 0.5 mM EGTA or 0.05 mM CaCl_2 as indicated, in a total volume of 0.2 ml. After incubation at 25°C mM CaCl_2 as indicated, in a total volume of 0.2 ml. After incubation at 25°C for 1 h, they were sedimented by centrifugation at various $g \times \text{min}$ values as shown in the figure. Total proteins of the resultant pellets and supernates were determined as described in Materials and Methods, except that washing of the pellet with 10 mM imidazole HCl (pH 7.2) containing 0.1 M KCl was omitted, since the gel pellets that formed at low centrifugal force were too loose to be washed without breaking the gel. (▲) Total protein sedimented in the presence of Ca^{2+} , (●) in the presence of EGTA, and (■) without addition of actinogelin, i.e., F-actin alone in the presence of EGTA.

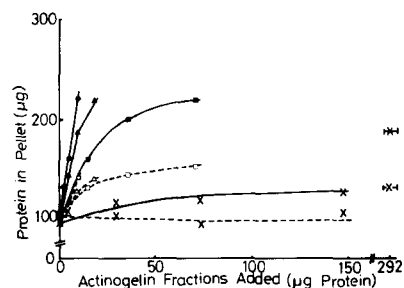


FIGURE 3 Gelation activities of samples from each purification step. Reaction mixtures are similar to that described in Materials and Methods, except that 0.1 ml of actin solution (3.52 mg/ml) and 0–20 μl of the samples dialyzed against 20 mM imidazole HCl (pH 7.2) were added. Total volume of the reaction mixture was made up to 0.2 ml with 20 mM imidazole HCl (pH 7.2). Gelation was performed at 25°C for 1 h in the presence of EGTA (solid line) or 0.05 mM Ca^{2+} (dashed line). (●) sepahrose 6B fraction, (▲) hydroxylapatite fraction, (■) DE32 fraction, and (X) second ammonium sulfate fraction.

0.74 mg/ml, a molecular weight of 252,000 was obtained for native actinogelin. These observations suggest that native actinogelin exists as a dimer. Sedimentation equilibrium experiments indicated that actinogelin contained small amounts of an aggregated population (Fig. 4).

To confirm its dimeric nature, native actinogelin was subjected to the chemical cross-linking treatment described by Davies and Stark (17). As shown in Fig. 5, after this treatment native actinogelin gave a new band having an estimated molecular weight of 230,000 on SDS polyacrylamide gel electrophoresis; the molecular weight was estimated by using cross-linked hemocyanin as an internal standard. However, only about one-third of the total actinogelin was converted to the new band, irrespective of the concentrations of suberimidate employed. The reason for such incomplete cross-linking is unknown, but several proteins have been shown to undergo incomplete cross-linking among their subunits (17). Since the protein concentration (0.4 mg/ml) was kept low to avoid intermolecular cross-linking (17), it was very probable that

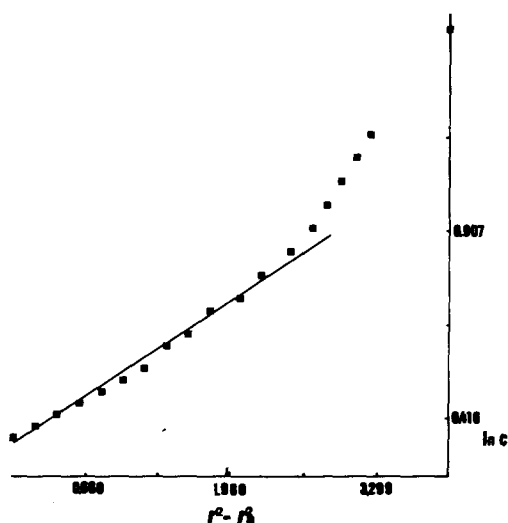


FIGURE 4 Sedimentation equilibrium analysis of actinogelin using interference optics. The abscissa represents $r^2 - r_a^2$, where r corresponds to the distance from the center, and r_a corresponds to the distance from the center to the meniscus of the sample solution. The ordinate represents $\ln [OD_{280}]$, where OD_{280} corresponds to the concentration of actinogelin. The molecular weight of native actinogelin was calculated from the straight line drawn in the figure. The bottom part indicates the presence of a higher molecular weight component(s), suggesting that a small part of the actinogelin may be aggregated.

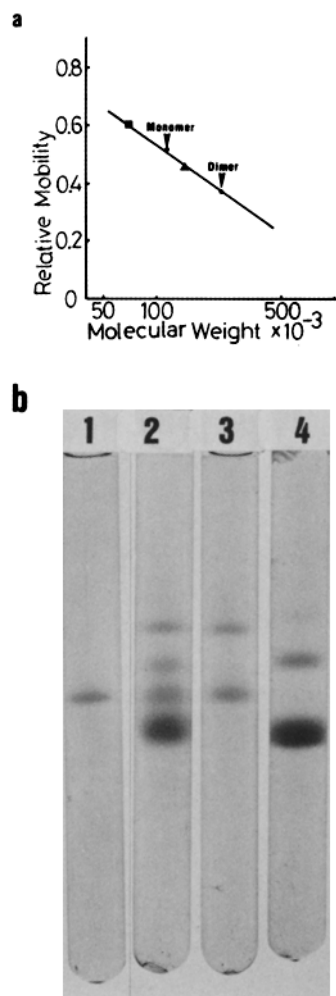


FIGURE 5 Chemical cross-linking of actinogelin. (a) Semilog plot of molecular weight to relative mobility. Cross-linked hemocyanin purchased from Sigma Chemical Co., St. Louis, Mo., was used for the marker protein. The monomer (■) is 70,000 daltons and the dimer (▲) is 140,000 daltons. The arrowhead indicates cross-linked actinogelin (●), corresponding to monomer and presumed dimer. (b) SDS disc gel electrophoresis pattern; column 1, 10 μ g of untreated actinogelin was applied; column 2, 16 μ g of cross-linked actinogelin and 20 μ g of cross-linked hemocyanin were applied together on the column; column 3, 16 μ g of cross-linked actinogelin was applied; column 4, 20 μ g of cross-linked hemocyanin was applied. The results indicate that cross-linked actinogelin has a molecular weight of 230,000 and suggest that it is a dimer of a 115,000-dalton protein. 4% acrylamide gel was used.

cross-linking occurred intramolecularly and that native actinogelin was composed of two identical subunits. When actinogelin was analyzed by SDS polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol, the reducing agent, it exhibited the same electrophoretic mobility as in the presence of reducing agent (data not shown), indicating that actinogelin had no intersubunit disulfide bonding. Actinogelin was colorless and showed no optical adsorption in the visible spectrum.

Ca²⁺-Sensitivity of Actinogelin-dependent Gelation of Muscle Actin

As described above, actinogelin purified to homogeneity induced the gelation of skeletal muscle actin in a Ca²⁺-sensitive fashion. Optimal gelation requires more than 1.0 mg/ml actin (Fig. 6), determined by the volume of the pellet obtained by centrifugation at 33,000 g for 10 min. By contrast, the volume of the pellet of actinogelin-actin mixture in the presence of Ca²⁺ was the same as that of F-actin only.

Fig. 7 shows that optimal gelation occurred at KCl concentrations between 20 and 100 mM. KCl concentrations >300 mM resulted in inhibition of gelation. Formation of F-actin under the conditions employed was confirmed by its sedimentation by centrifugation at 105,000 g for 3 h. Actinogelin-dependent gelation did not require ATP and Mg²⁺: even when both reagents were removed from the reaction medium, gelation occurred normally (data not shown).

Fig. 8 shows the effects of free Ca²⁺ (controlled by Ca²⁺-EGTA buffer [10, 38, 45, 47, 51]), Sr²⁺, and Ba²⁺ concentration on this reconstituted gelation system. Ca²⁺ inhibited this gelation at free-ion concentrations of $\sim 1 \mu$ M. Sr²⁺ also inhibited this gelation, but the ion concentration required for inhibition was 500 times higher than with Ca²⁺. Ba²⁺ at concentrations of 10 mM and higher appeared to decrease the gel volume, but

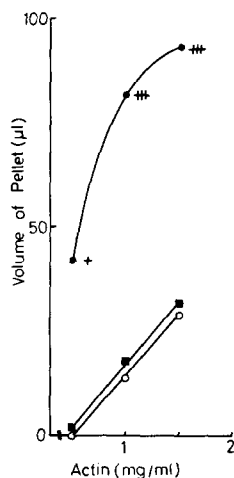


FIGURE 6 Dependence on actin concentrations of the extent of actinogelin-dependent gelation. The reaction mixture consisting of 20 mM imidazole HCl (pH 7.2), 50 mM KCl, 1 mM MgCl₂, either 0.5 mM EGTA (●) or 0.05 mM CaCl₂ (■), 0.07 mg/ml purified actinogelin, and the indicated concentrations of actin. Control experiments (without addition of actinogelin) (○) was performed in the presence of 0.5 mM EGTA. +, +++ shows the degree of gelation assay by tilting tubes containing gels as described previously (41).

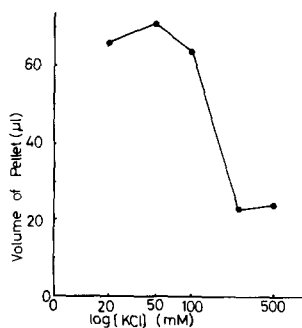


FIGURE 7 Effect of KCl concentrations on actinogelin-dependent gelation of skeletal muscle actin. The reaction mixture contained 4 mM imidazole HCl (pH 7.2), 0.5 mM MgCl₂, 0.5 mM EGTA, 0.07 mg/ml purified actinogelin, 2 mg/ml actin, and the indicated concentrations of KCl in a total volume of 0.2 ml. The reaction was performed at 25°C for 1 h.

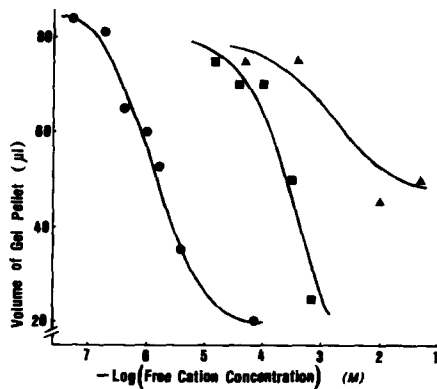


FIGURE 8 Effects of free Ca^{2+} , Sr^{2+} , and Ba^{2+} concentrations on the actinogelin-dependent gelation system. The reaction mixture contained 20 mM imidazole HCl (pH 7.2), 50 mM KCl, 0.5 mM EGTA, 0.25 mM MgCl_2 , 0.07 mg/ml actinogelin, and 2 mg/ml actin. Various concentrations of CaCl_2 were added to the reaction mixture to manipulate free Ca^{2+} concentrations by the EGTA- Ca^{2+} buffer system (●). The total volume of the reaction mixture was 0.2 ml. The extent of gelation (ordinate) was evaluated by the volume of the pellet resulting from centrifugation as described in Materials and Methods. The abscissa shows free concentrations of divalent cations. The effects of Sr^{2+} (■) and Ba^{2+} (▲) were examined by the method used to evaluate the effects of Ca^{2+} , except that the reaction mixture contained 0.1 mM EGTA, 0.05 mg/ml actinogelin, and 1.53 mg/ml actin. For the apparent stability constants of EGTA complex to Ca^{2+} , Sr^{2+} , and Ba^{2+} at pH 7.2, $2.7 \times 10^6 \text{ M}^{-1}$, $3.89 \times 10^4 \text{ M}^{-1}$, and $3.09 \times 10^4 \text{ M}^{-1}$ were used, according to Matsuda and Yagi (34) for Ca^{2+} and Data Book for Biochemistry (edited by Japanese Biochemical Society) for Sr^{2+} and Ba^{2+} , respectively.

this cation did not affect the degree of gelation estimated by eye, as described previously (41), although some increase in turbidity, which may be due to precipitation of F-actin, was noticed at these concentrations. This turbidity may be related to the decrease in gel volume. Thus, we conclude that Ba^{2+} is rather inactive in the induction of solation.

The inhibition by Ca^{2+} was reversible, because gelation could be induced by decreasing the free Ca^{2+} concentration of the inhibited samples by the addition of EGTA. When 10 μl of 10 mM CaCl_2 was placed on the gel formed from 400 μg of actin and 20 μg of actinogelin, the gel began to undergo solation from the upper surface, and complete solation was effected within 1 h (data not shown).

In the experiments described above, gelation was started by mixing G-actin with actinogelin. When actinogelin was added after completion of actin polymerization, normal gelation of preformed F-actin was observed if free Ca^{2+} was depleted by EGTA, whereas in the presence of Ca^{2+} the addition of actinogelin to preformed F-actin did not induce gelation (data not shown; see also reference 37). This suggests that the observed inhibition of actinogelin-dependent gelation by Ca^{2+} was not due to the prevention of the polymerization of G-actin.

Other evidence against the possibility that actinogelin inhibits the polymerization of actin in the presence of Ca^{2+} came from the centrifugal experiments shown in Fig. 2, which compare the sedimentation profile of F-actin with that of an actin-actinogelin mixture at various $g \times \text{min}$ values. As can be seen, in the presence of enough Ca^{2+} to inhibit gel formation, the actin-actinogelin complex tended to be slightly more sedimentable than F-actin alone. Furthermore, by negative-staining electron microscopy, we did not observe any evidence of fragmentation of F-actin by actinogelin at high Ca^{2+} concentrations (Fig. 9). These observations show that free Ca^{2+} plus

actinogelin does not affect gelation by inhibiting polymerization of G-actin to F-actin or by severing preformed F-actin under the conditions employed. Instead, it is likely that free Ca^{2+} somehow inhibits the assembly of F-actin and actinogelin into the three-dimensional gel structure, in contrast to the function of gelsolin, which somehow fragments actin filaments in the presence of Ca^{2+} (68).

Binding of Actinogelin to F-Actin

A titration experiment performed at a low Ca^{2+} concentra-

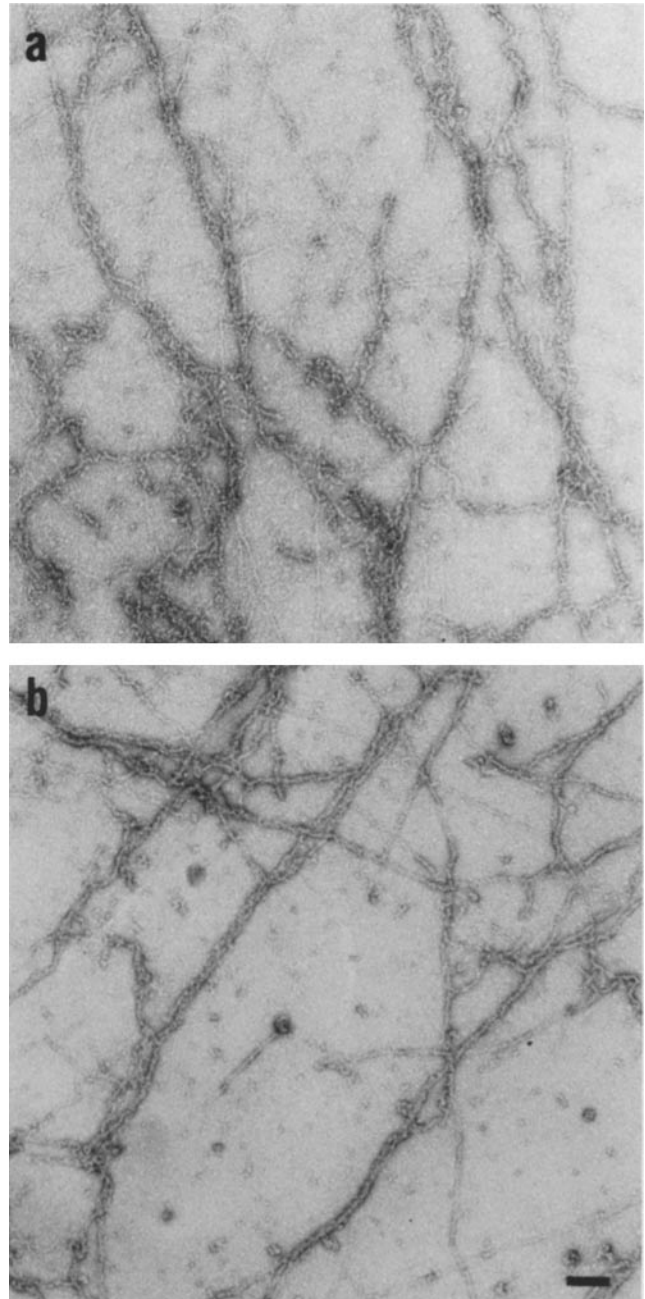


FIGURE 9 Electron micrographs of negatively stained F-actin-actinogelin mixture or F-actin alone incubated at high Ca^{2+} concentration. Electron microscopy was performed by the courtesy of Dr. Reiko Nagai, Faculty of Science, Osaka University, as described in Materials and Methods. No evidence for severing of F-actin with actinogelin plus Ca^{2+} could be observed. (a) Actin-actinogelin mixture. (b) Actin only. Concentrations of actin and actinogelin were 0.082 and 0.024 mg/ml, respectively. Bar, 0.1 μm . $\times 54,000$.

tion indicates that 20 μg of actin could precipitate a maximum of 10 μg of actinogelin: further addition of actinogelin caused no increase in the amount of protein recoverable in the pellet. The remaining actinogelin was recovered in the supernate (data not shown). Assuming molecular weights of 230,000 and 43,000 for actinogelin dimer and G-actin, respectively, it was calculated that, at most, one actinogelin dimeric unit could bind to 10–12 G-actin units. Below the saturation points, almost all the actinogelin added could sediment together with actin. The presence of a stoichiometric relationship in the binding of actinogelin and actin suggests that this binding is specific.

When similar experiments were performed at a higher Ca^{2+} concentration, binding of actinogelin to F-actin decreased measurably at some actin:actinogelin ratios (Fig. 10). This decrease in binding ($\sim 25\%$ at the protein ratios used for gelation) is, however, far less than the inhibition of gelation.

On the other hand, high concentrations of salts (≥ 0.3 M KCl) diminish the binding of actinogelin to F-actin, irrespective of the presence or absence of Ca^{2+} , suggesting that inhibition of actinogelin-dependent gelation at higher ionic strengths (Fig. 7) can be attributed to the release of actinogelin from F-actin.

No Involvement of Calmodulin in Actinogelin Molecule

A specific Ca^{2+} -binding protein, called calmodulin, calcium

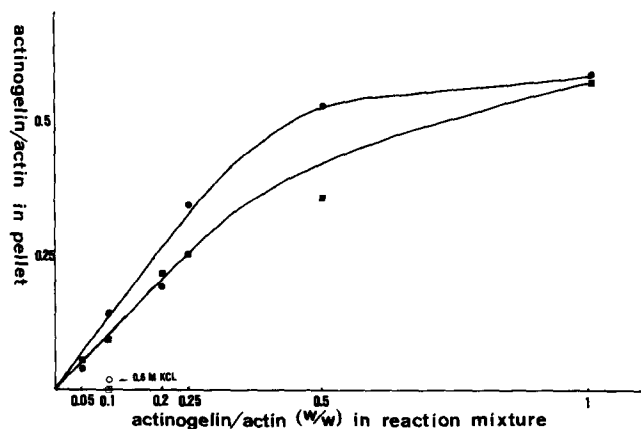


FIGURE 10 Effects of Ca^{2+} on binding capacity of actinogelin to F-actin. An actinogelin solution containing 20 mM Tris HCl (pH 7.6), 50 mM NaCl, 0.1 $\mu\text{g}/\text{ml}$ pepstatin, 50% glycerol, and 1 mM NaN_3 and a G-actin solution containing 2 mM Tris-HCl (pH 8.0), 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl_2 , and 0.2 mM ATP were centrifuged at 100,000 g for 3 h to remove any insoluble material. Resultant supernates were used for this experiment. Pretreated actin (40 μg of protein) was mixed with the indicated amounts of pretreated actinogelin in the medium containing 50 mM KCl, 1 mM MgCl_2 , 0.5 mM EGTA, and 20 mM imidazole HCl (pH 7.2) in a total volume of 80 μl . The final concentration of glycerol contained in the actinogelin sample solution to stabilize actinogelin during storage at -20°C , was made 18.75% (wt/wt) in all experiments, and this concentration of glycerol did not affect gelation activity at the condition employed (data not shown). In the case of $+\text{Ca}^{2+}$, 0.05 mM CaCl_2 was added in place of EGTA to ascertain the effect of Ca^{2+} on the binding capacity. To determine the effect of high ionic concentrations on the binding capacity, the KCl concentration was adjusted to 0.6 M, as shown in the figure. After incubation at 30°C for 1 h, the mixtures were centrifuged at 100,000 g for 3 h and the resultant pellets were analyzed by SDS polyacrylamide gel electrophoresis. The ratio of these two proteins in the pellets was calculated by measuring the area of their peak in the densitometric scans of electrophoretograms stained by Coomassie Brilliant Blue. (●) – Ca^{2+} (■) + Ca^{2+} .

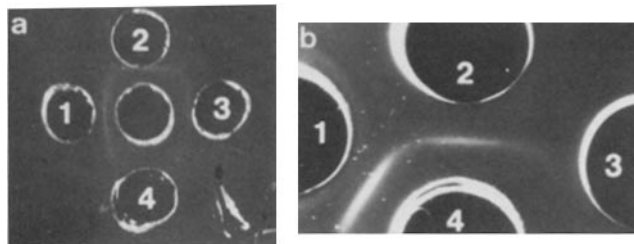


FIGURE 11 Ouchterlony test of anti-actinogelin IgG fraction to the extract of Ehrlich tumor cells and purified actinogelin. (a) The center well contained 25 mg of protein/ml of ETC extracts. The surrounding wells marked 1, 2, 3, and 4 contained anti-actinogelin antibody (IgG fraction) of 43.8, 14.6, 4.87, and 1.62 mg/ml, respectively. (b) The wells numbered 1, 2, and 3 contained antiserum that was diluted with 20 mM imidazole HCl (pH 7.2) onefold, threefold, and ninefold, respectively. Well 4 contained 0.2 mg/ml of purified actinogelin.

modulator protein, or calcium-dependent regulator, has been found in various mammalian tissues and shown to mediate Ca^{2+} -dependent activation of several enzymes. It has further been reported that this protein is a constituent of Ca^{2+} -dependent phosphorylase kinase (62). It was, therefore, of interest to examine whether the Ca^{2+} sensitivity of actinogelin-dependent gelation of F-actin was due to calmodulin-like activity of actinogelin. However, practically no Ca^{2+} -dependent activation of cyclic nucleotide phosphodiesterase was elicited by the addition of pure actinogelin (Dr. R. Yamasaki and Prof. S. Kakiuchi of Osaka University, unpublished results). Furthermore, gel filtration of actinogelin through a Sephacryl S-200 Pharmacia Fine Chemicals AB, Uppsala, Sweden column in the presence of high salt concentration and EGTA did not abolish the capacity of actinogelin to induce Ca^{2+} -sensitive gelation of actin, though this treatment would separate actinogelin from low molecular weight calmodulin, if present. These findings led to the conclusion that calmodulin-like activity was not involved in the Ca^{2+} -sensitive gelation of actin by actinogelin.

Localization of Actinogelin in Nonmuscle Cells

Antibodies to actinogelin were elicited in rabbits. These anti-actinogelin IgGs are specific to actinogelin, as judged by an immunodiffusion (Ouchterlony) test, which showed a single precipitation line between anti-actinogelin IgG and crude Ehrlich cell extracts (Fig. 11a), and also with pure actinogelin (Fig. 11b). Because this IgG fraction contained only a small amount of IgG specific for actinogelin, monospecific IgG was purified from the IgG fraction by affinity chromatography with actinogelin-Ultrogel AcA prepared as described in Materials and Methods.

Immunofluorescence microscopy with this monospecific IgG in a wide variety of nonmuscle cells revealed that actinogelin is localized within these cells. Fig. 12 shows the fluorescence patterns of (a) mouse embryo fibroblasts and (c and d) 3T3 Swiss albino mouse fibroblasts stained with monospecific anti-actinogelin IgG. In mouse embryo fibroblasts (Fig. 12a), actinogelin was present along the stress fibers of an elongated cell. The fluorescent actinogelin-containing filaments spanned the interior of cells and were parallel to one another along the long axis of the cells, but this type of localization was rather rare. Well-spread mouse embryo fibroblasts showed a pattern of staining similar to that of 3T3 cells (Fig. 12c and d). In these cases, the antibody stained stress-fibers discontinuously, whereas TRITC-HMM showed continuous fluorescence along

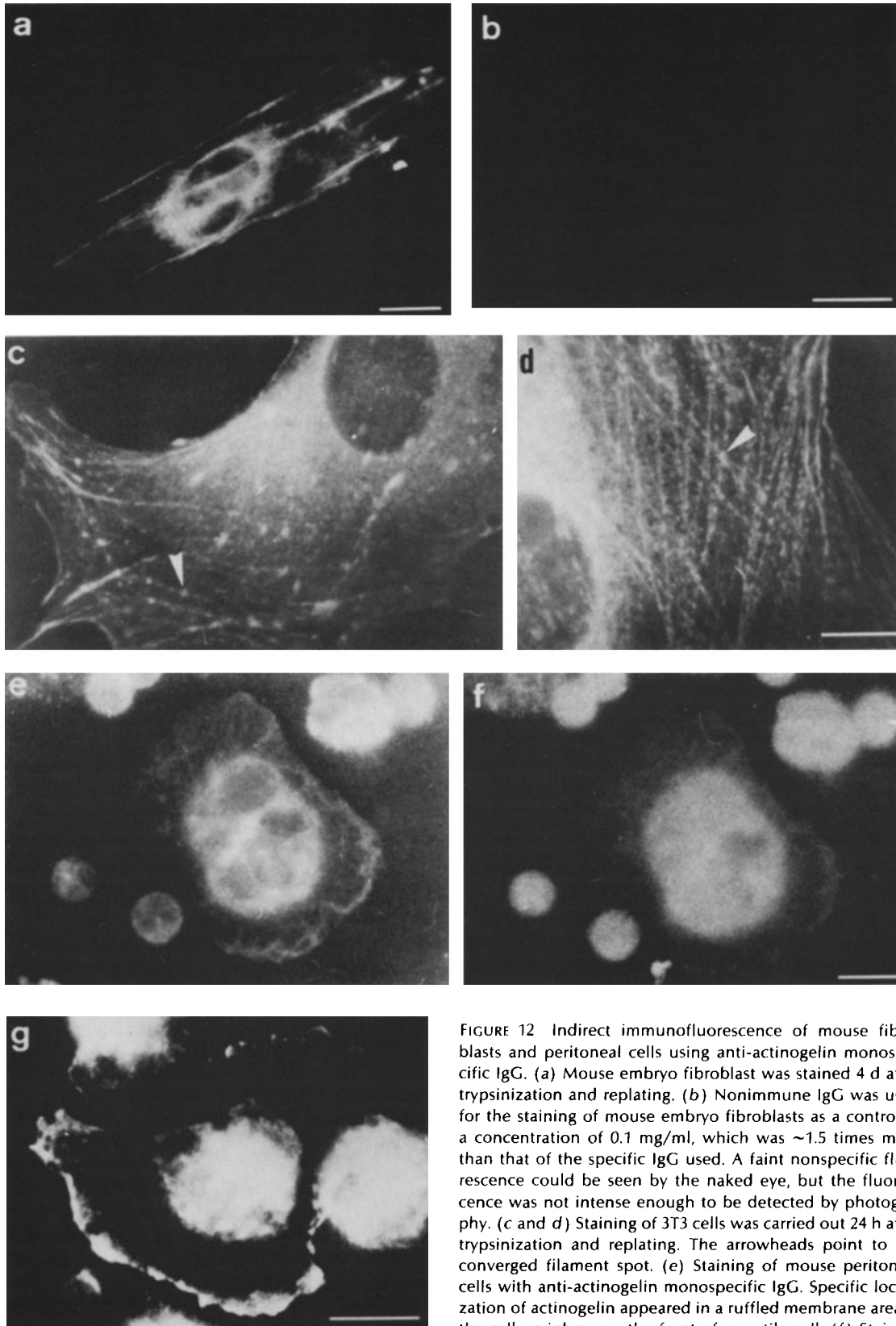


FIGURE 12 Indirect immunofluorescence of mouse fibroblasts and peritoneal cells using anti-actinogelin monospecific IgG. (a) Mouse embryo fibroblast was stained 4 d after trypsinization and replating. (b) Nonimmune IgG was used for the staining of mouse embryo fibroblasts as a control at a concentration of 0.1 mg/ml, which was ~1.5 times more than that of the specific IgG used. A faint nonspecific fluorescence could be seen by the naked eye, but the fluorescence was not intense enough to be detected by photography. (c and d) Staining of 3T3 cells was carried out 24 h after trypsinization and replating. The arrowheads point to the converged filament spot. (e) Staining of mouse peritoneal cells with anti-actinogelin monospecific IgG. Specific localization of actinogelin appeared in a ruffled membrane area at the cell periphery on the front of a motile cell. (f) Staining

with TRITC-labeled heavy meromyosin; localization of actin revealed by this method was very similar to that of actinogelin but more diffuse. (g) Mouse peritoneal cells which were incubated with cytochalasin D (10 μ g/ml) at 37°C for 20 min were stained for actinogelin with FITC-labeled antibody. Bars, 10 μ m. (a) \times 900; (b–d) \times 1,300; (e and f) \times 1,000; and (g) \times 1,500.

microfilament bundles (data not shown). The length of the fluorescent segments and the space between these spots are in general variable but occasionally are almost continuous at the

cell periphery. In some places where the fibers cross each other or converge, fluorescent staining was particularly strong, exhibiting a starlike pattern. These observations suggest that

actinogelin is present at the crossing points of stress fibers and on stress fibers themselves. Perinuclear regions sometimes showed strong fluorescence, but the nucleoplasm was never stained.

When well-spread mouse peritoneal cells consisting mostly of macrophages were stained, the cell peripheries were heavily stained (Fig. 12*e*). The staining patterns were similar to those obtained with TRITC-labeled HMM (Fig. 12*f*), although most of the HMM-stained places were stained by the anti-actinogelin antibody.

When mouse intestinal epithelial cells were examined by indirect immunofluorescence, actinogelin was found also to be present in the terminal web region of the brush border (Fig. 13*b*). The intensely fluorescent polygonal shapes may correspond to actin bundles that interconnect junctional complexes. However, no evidence for the presence of actinogelin in the microvilli region was obtained, whereas fluorescence-labeled HMM treatment resulted in some staining both in the terminal web and in microvilli regions (Fig. 13*c*).

Mouse embryo fibroblasts treated with nonimmune IgGs showed very little fluorescence (Fig. 12*b*). Treatment of cells with a microfilament inhibitor, cytochalasin D, drastically changed the pattern of staining by the anti-actinogelin antibody. As shown in Fig. 12*g*, cytochalasin D treatment at 10 $\mu\text{g}/\text{ml}$ resulted in heavy staining by the antibody around the nuclear and cell periphery regions.

DISCUSSION

Among the several proteins of mammalian origin that induce gelation of F-actin, actinogelin seems to be unique (41), inasmuch as the other proteins, including actin-binding protein isolated from macrophages (23) and filamin isolated from chicken gizzard (63) and Ehrlich tumor cells (42), induce Ca^{2+} -insensitive gelation, whereas actinogelin-induced gelation is reversibly blocked by micromolar concentrations of Ca^{2+} . It has been reported that a protein fraction prepared from *Dictyostelium* exhibited a Ca^{2+} sensitivity similar to that of actinogelin (26), and purification from *Acanthamoeba* of a protein that is very similar to actinogelin recently been reported (48). Villin is the only other mammalian protein that induces cross-linking of F-actin only in the absence of Ca^{2+} (9, 15, 39, 43), but it seems to occur only in the microvilli of intestinal epithelial cells (8). Furthermore, its effects on F-actin in the presence

of Ca^{2+} are entirely different from those of actinogelin as described below.

Actinogelin is also different from the other proteins that induce Ca^{2+} -dependent solation of gels composed of F-actin and actin-binding protein(s). Gelsolin, which is isolated from alveolar macrophages (67), induces solation of filamin-F-actin gel in the presence of micromolar concentrations of Ca^{2+} . In this case, solation is due to Ca^{2+} -dependent splitting of F-actin by gelsolin (68). No such mechanism seems to be operating in the case of actinogelin-dependent gel-sol transformation of F-actin: no other protein is required for the solation reaction, and no evidence for splitting of F-actin after solation was obtained by viscosity measurements with a rotary viscometer, by flow birefringence measurements (37); or by electron microscope observation. Furthermore, the amount of F-actin sedimented by ultracentrifugation was not decreased by the addition of actinogelin, even in the presence of Ca^{2+} . Instead, slightly more protein was sedimented in the latter case than by F-actin alone. Thus, solation of actinogelin-induced gel seems to occur without the severing of F-actin. Villin, which cross-links F-actin in the absence of Ca^{2+} , as does actinogelin, is, on the other hand, known to split it when Ca^{2+} is present at micromolar concentrations (9, 15, 39, 43). Therefore, the mechanism of solation of the actinogelin/F-actin system is different from that of these F-actin-severing proteins.

Although solation at high ionic strength ($\geq 0.3 \text{ M KCl}$) is due to the release of actinogelin from F-actin, actinogelin-dependent solation by Ca^{2+} seems to be more complex, because no significant release of actinogelin accompanies solation. The molecular mechanism of solation is not clear at present, but there are several possibilities that would explain the gel-sol transformation, including the following two. The first possibility is that Ca^{2+} shifts the tetramer-dimer equilibrium to a less aggregated state. Thus, the cross-linking ability of the molecule decreases in the presence of Ca^{2+} without appreciably affecting F-actin binding. Although there is some evidence for the presence of tetramers (or higher oligomers) in purified preparations, the occurrence of such oligomers in the native state has not been proved, since this protein tends to aggregate during storage, as described below. The second possibility is that the actinogelin molecule has two different binding sites for F-actin, one insensitive to Ca^{2+} and the second inhibited by Ca^{2+} . Thus, no appreciable effect of Ca^{2+} on actinogelin

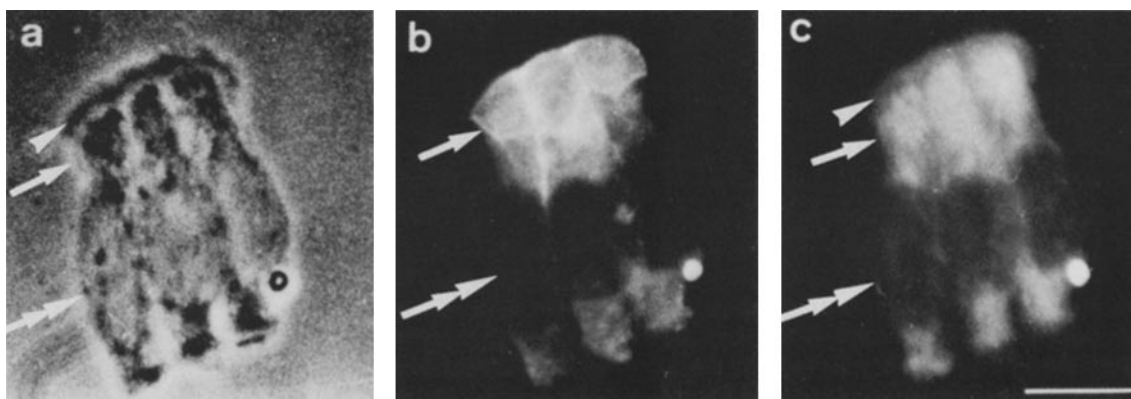


FIGURE 13 Fluorescent staining of intestinal brush border cells. (a) Intestinal epithelial cells observed by phase-contrast microscopy. (b) Indirect staining with anti-actinogelin monospecific IgG and FITC-labeled anti-rabbit IgG antibody (IgG fraction). Polygonal structures were seen in the apical part of the cells. These cells may have been slightly deformed during preparation, and therefore polygonal microfilament bundles are somewhat distorted to be in an oblique position. (c) Staining with TRITC-labeled heavy meromyosin. An arrowhead points to microvilli that are present on the luminal surface of the cells. An arrow shows the terminal web region, and the double arrow points to nuclei of the cell. Bar, 10 μm . $\times 1,400$.

binding to F-actin would be expected, but, for cross-linking of F-actin, Ca^{2+} would be a strong inhibitor.

The maximum binding of actinogelin to F-actin (1 dimer to 10–12 actin monomers) is similar to that of other actin-accessory proteins. For example, reported molar ratios, at saturation, of the other proteins to actin are as follows: filamin, 1:8–12 (3, 64); tropomyosin, 1:7 (19); and α -actinin, 1:9–11 (22). Among these actin-binding proteins, α -actinin is similar to actinogelin in that both are dimeric proteins of similar size (the subunit molecular weight of α -actinin is $\sim 100,000$ [58]) and bind to F-actin with similar stoichiometry. However, gelation of F-actin induced by α -actinin is not sensitive to Ca^{2+} (37), and no antigenic reactivity between these proteins was detected by Ouchterlony tests (unpublished observation).

From sedimentation equilibrium experiments and SDS polyacrylamide gel electrophoresis, native actinogelin seems to be a dimeric molecule consisting of two subunits of identical size. The molecular weight of the subunits has been measured to be 112,000 or 115,000 daltons and that of native actinogelin to be 242,000–252,000 daltons. Chemical cross-linking studies have also supported the dimeric nature of actinogelin: a molecular weight for the dimer of 230,000 has been obtained by this method. However, actinogelin has a tendency toward aggregate formation. In fact, aggregate formation can be readily observed upon freeze-thawing of actinogelin and concentration by means of a Sartorius collodion membrane. The molecular weight of native actinogelin determined by hydrodynamic methods is slightly larger than that calculated from the molecular weight of the subunits, possibly because the presence of aggregated forms affects the sedimentation of the molecules of native actinogelin.

To calculate free Ca^{2+} concentrations, we used an apparent binding constant that is measured with a Ca^{2+} electrode and is the most accurate available (38). Since this value is lower than that calculated from Schwarzenbach's binding constant (50), the calculated values of free Ca^{2+} tend to be higher than those reported by others (1, 20, 50, 52, 53, 54, 59). When we use a binding constant for Ca^{2+} and EGTA as high as $10^{11.0}$ (50), the free Ca^{2+} concentration giving half-maximal inhibition of gelation becomes 0.4 instead of 1.6 μM .

An immunofluorescence microscopy study of intracellular localization of actinogelin clearly showed that actinogelin is a constituent of the microfilament system. Stress fibers of fibroblasts and actin bundles of intestinal epithelial cells were stained. Interestingly, however, not all actin bundles were stained. For example, staining of stress fibers was not continuous in fibroblasts in most cases, and the core filaments of microvilli were not stained. Since the stain on stress fibers was not regularly spaced, and since in many cases intense staining seems to correspond to crossing points of actin bundles, we speculate that actinogelin has a regulatory role in the intracellular organization of actin filaments. Similar staining patterns with antibodies to smooth-muscle α -actinin have been reported by Lazarides (34), but biochemical identification of α -actinin in non-muscle cells is lacking. Although no immunological cross-reactivity between the two proteins has been detected, a detailed comparison of actinogelin with α -actinin in both biochemical and immunological terms is now underway in our laboratory.

Actinogelin seems to be quite ubiquitous, inasmuch as it can be isolated from cancer cells of mouse mammary gland origin (Ehrlich tumor cells) and from normal rat liver cells (Kuo, Mimura, and Asano, unpublished observation) and can also

be immunologically detected in several mouse cell types, such as fibroblasts, epithelial cells, macrophages, and lymphocytes. It may be possible that actinogelin not bound to actin filaments is also present in cytoplasm, since the supernatant obtained after compression of a gel prepared from crude cell-free extracts still showed appreciable actinogelin activity. However, the percentages of unbound and bound forms in the cell extracts have not been determined.

Although the same ubiquitous distribution of gelsolin has not been reported, it is possible that gelsolin or a gelsolin-type Ca^{2+} -dependent regulator is present not only in alveolar macrophages but also in the other cell types. A protein similar but with a different molecular weight has been isolated from the plasmodium of *Physarum* (fragmin) (24). Thus, the presence of gelsolin-type protein (i.e., protein inhibitor of filamin-dependent gelation of F-actin) was surveyed in Ehrlich tumor cell extracts. Although protein fractions that inhibit gelation independent of Ca^{2+} were found, efforts to detect a gelsolin-type Ca^{2+} -sensitive inhibitor in the extracts have been unsuccessful.

Note Added in Proof: After submission of this report, a paper describing ubiquitous distribution of gelsolin appeared (69).

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