

THE ROLE OF ACTIN IN TEMPERATURE-DEPENDENT GEL-SOL TRANSFORMATION OF EXTRACTS OF EHRlich ASCITES TUMOR CELLS

MASAHIRO ISHIURA and YOSHIO OKADA

From the Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka
565, Japan

ABSTRACT

Ehrlich ascites tumor cell extracts form a gel when warmed to 25°C at pH 7.0 in sucrose solution, and the gel rapidly becomes a sol when cooled to 0°C. This gel-sol transformation was studied quantitatively by determining the volume or the total protein of pellets of gel obtained by low-speed centrifugation. The gelation depended on nucleotide triphosphates, Mg²⁺, KCl, and a reducing agent. Gelation was inhibited reversibly by 0.5 μM free Ca²⁺, and 25–50 ng/ml of either cytochalasin B or D, but it was not affected by 10 mM colchicine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that the gel was composed of six major proteins with mol wt >300,000, 270,000, 89,000, 51,000, 48,000, and 42,000 daltons. The last component was identified as cell actin because it had the same molecular weight as muscle actin and bound with muscle myosin and tropomyosin.

The role of actin in gelation was studied by use of actin-inhibitors. Gelation was inhibited by a chemically modified subfragment-1 of myosin, which binds with F-actin even in the presence of ATP, and by bovine pancreatic DNase I, which tightly binds with G-actin. Muscle G-actin neutralized the inhibitory effect of DNase I when added at an equimolar ratio to the latter, and it also restored gelation after its inhibition by DNase I. These findings suggest that gelation depends on actin. However, the extracts showed temperature-dependent, cytochalasin-sensitive, and Ca²⁺-regulated gelation as did the original extracts when the cell actin in the extracts was replaced by muscle actin, suggesting that components other than cell actin might be responsible for these characteristics of the gelation.

KEY WORDS Ehrlich ascites tumor cells ·
actin · gelation · Ca²⁺ regulation ·
cytochalasins

Recently, it has become possible to study the assembly and disassembly of actin-containing filaments (microfilaments) in vitro. Kane first found

that actin filaments form a gel when extracts of sea urchin eggs are warmed in the presence of ATP, ethyleneglycol-bis (β-aminoethylether)-N,N'-tetraacetate (EGTA), and KCl (26, 27). Similar temperature- and ATP-dependent gelation of cell extracts and subsequent contraction of the gel have also been reported for various kinds

of nonmuscle cells including rabbit pulmonary macrophages (18, 56), HeLa cells (62, 63), human leukocytes from patients with chronic myelogenous leukemia (5), platelets (35), *Acanthamoeba* (46, 47), *Amoeba proteus* (60), *Dictyostelium discoideum* (7, 59), and Ehrlich ascites tumor cells (ETC) (39). It is interesting, in connection with cytochalasin effects on cells, that cytochalasin B reversibly inhibits the gelation of cell extracts (7, 18, 47, 62). Hartwig and Stossel demonstrated that cytochalasin B reversibly inhibits the gelation of actin caused by the interaction of actin and actin-binding protein (18). Despite these previous studies, however, much remains to be determined about the gelation, such as its temperature-dependence, Ca^{2+} -regulation, and ATP-requirement, and the protein components involved. The difficulty in studying gelation is partly because of the lack of a reliable quantitative method for measuring the gelation.

This paper reports a quantitative method to measure the extent of gelation or solation of cell extracts. By this method, we studied the temperature-dependent reversible gel-sol transformation of ETC extracts, and also examined the role of actin in the transformation quantitatively by use of two actin inhibitors, a chemically modified subfragment-1 of myosin (CMB-S-1) and bovine pancreatic DNase I.

MATERIALS AND METHODS

ETC

A mutant of ETC, resistant to 8-aza guanine (43), was propagated in the abdomen of ddO mice. After 10–13 d of *in vivo* culture, the cells were harvested from the abdomen. All subsequent treatments of the cells were carried out in ice-cold conditions. The cells were washed three–four times with 0.15 M NaCl containing 15 mM sodium citrate by centrifugation at 120 g for 3 min to remove ascites fluid and blood cells. Then the cells were suspended in 15 vol of distilled, deionized water to lyse residual erythrocytes. The preparation was mixed by pipetting, and then rapidly centrifuged at 1,150 g for 10 min. When there was no apparent contamination with blood, the water treatment was omitted. The packed cells were immediately used for preparation of cell extracts.

ETC Extracts

Cell extracts were prepared by a procedure based on the methods of Stossel and Hartwig (56), and Weithing (62). Freshly prepared packed ETC were suspended in an equal volume of an extraction solution containing 0.45 M sucrose, 5 mM ATP, 1 mM EGTA, 10 mM

dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.6% Triton X-100, and 20 mM Tris-maleate (pH 7.0 at 25°C), and gently stirred for 10 min under ice-cold conditions. This procedure effectively ruptures all the cells, as monitored by phase microscopy. In some experiments, the extraction solution was slightly modified. The ruptured cells were centrifuged at 160,000 g for 90 min at 3°C, and the resulting clear supernatant fraction (ETC extract) was collected. Unless otherwise mentioned, the supernatant fraction was dialyzed against 25 vol of buffered sucrose solution (0.34 M sucrose and 20 mM Tris-maleate [pH 7.0 at 25°C]) containing 0.25 mM ATP, 0.5 mM EGTA, 0.5 mM DTT, and 0.2 mM PMSF, for 12 h in ice-cold conditions, with one change of the buffer. After dialysis, the fraction was clarified by centrifugation at 1,150 g for 30 min at 4°C, and used for experiments. In some experiments, ETC extracts were desalted by chromatography on Sephadex G-25.

Rabbit Skeletal Muscle Proteins

Myosin was prepared from rabbit skeletal white muscle by the method of Perry (45). Heavy meromyosin (HMM) was prepared by tryptic digestion of myosin by the method of Szent-Györgyi (57) with slight modification (51). Subfragment-1 of myosin (S-1) (mol wt = 1.2×10^5) was prepared by tryptic digestion of HMM, followed by chromatography on Sephadex G-200 (20, 52).

Myosin was chemically modified with *p*-chloromercuribenzoate (CMB) as described previously (52). S-1 modified with CMB (CMB-S-1) was prepared by two-step tryptic digestion of the modified myosin by the procedure used for preparation of S-1.

Purified G-actin (mol wt = 4.2×10^4) was prepared from an acetone powder of rabbit skeletal white muscle by the method of Spudich and Watt (55). F-actin was prepared by polymerizing G-actin by adding 1 mM MgCl_2 and 50 mM KCl.

Tropomyosin (heterodimer, mol wt = 6.8×10^4) was extracted with 1 M KCl from the residue of rabbit skeletal white muscle which remained after extraction of actin, and was purified in the presence of 10 mM β -mercaptoethanol by a procedure based on the method of Bailey (1). Briefly, the 1-M KCl extracts were acidified to pH 4.5 with 1 N HCl. The resulting precipitate was collected by centrifugation and dispersed in 10 vol of water, and the pH was readjusted to 7.0 with 1 N NaOH. The solution was clarified by centrifugation and protein was salted out with 41–70% saturation of $(\text{NH}_4)_2\text{SO}_4$. Then, isoelectric fractionation at pH 4.5 in the presence of 1 M KCl followed by fractionation at pH 7.0 with 53–60% saturation of $(\text{NH}_4)_2\text{SO}_4$ was repeated three times.

Reagents

Bovine pancreatic DNase I (DL-CL) (mol wt = 3.1×10^4) was purchased from Sigma Chemical Co. (St.

Louis, Mo.). It was >90% pure as judged by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gel. Cytochalasin B was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.), and cytochalasin D was a gift from Shionogi Co. (Osaka, Japan). CMB, 5,5'-dithio-bis (2-nitrobenzoate) (DTNB), and iodoacetamide (IAM) were obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan), and *p*-chloromercuriphenylsulfonate (CMBS) from Sigma Chemical Co. *N*-Ethylmaleimide (NEM) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PMSF was purchased from Sigma Chemical Co. All other chemicals were reagent grade.

Temperature-Dependent Gel-Sol Transformation of ETC Extracts

The temperature-dependent gel-sol transformation of ETC extracts was measured by determining the gel volume or total protein in the gel. Gelation was defined as the increase of the gel volume or total protein in the gel, and solation as their decrease. Gelation was induced by warming the preparation to 25°C in a small test tube graduated by 25 μ l (6 \times 80 mm), and solation by cooling the preparation to 0°C. After incubation for gelation or solation, extracts were reproducibly separated into gel and liquid layers by centrifugation at 1,150 *g* for 30 min or at 2,060 *g* for 10 min. After removing the liquid layer carefully with a Pasteur pipette, the gel volume and (or) total protein in the gel was determined. To obtain reproducible results, gelled extracts were not stirred: this is very important because the gels are fragile, easily broken, and give white precipitates when stirred.

SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was performed on gels containing 5 or 7.5% acrylamide, 0.1% SDS, and 50 mM sodium phosphate buffer at pH 7.2, by the method of Weber and Osborn (61), with slight modification (20). After electrophoresis, the gels were stained at 45°C for 1 h with 0.1% Coomassie Brilliant Blue R 250. Electropherograms were scanned with a Gilford model 2000 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio.) at a wavelength of 550 nm.

The molecular weights of polypeptides were calculated by the method of Weber and Osborn (61) by using the following proteins as standards: human erythrocyte spectrin (mol wt = 2.4×10^5 and 2.2×10^5), rabbit skeletal muscle myosin (heavy chain, mol wt = 2.0×10^5), *Escherichia coli* β -galactosidase (mol wt = 1.3×10^5), rabbit skeletal muscle α -actinin (mol wt = 9.0×10^4), bovine serum albumin (mol wt = 6.8×10^4), rabbit muscle pyruvate kinase (mol wt = 5.7×10^4), bovine brain tubulin (mol wt = 5.5×10^5), pig cardiac muscle fumarase (mol wt = 4.9×10^4), rabbit skeletal muscle actin (mol wt = 4.2×10^4), and rabbit muscle

glyceraldehyde-3-phosphate dehydrogenase (mol wt = 3.6×10^4).

Protein composition was estimated roughly by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis. The peak areas of polypeptides in the densitometric scans were cut out and weighed on an analytical balance by using the procedure of Gorovsky et al. (15).

Protein Determination

Protein concentration was estimated by the biuret reaction (14) or by the copper-Folin method (34) with bovine serum albumin as a standard. Proteins were precipitated from crude extracts with 5% trichloroacetic acid (TCA), and contaminating nucleic acids were hydrolysed by incubating the precipitates at 90°C for 10 min in 5% TCA. The precipitates were washed three times with 5% TCA, dissolved in 0.1 N NaOH, and used for protein determination. The concentration of tropomyosin was estimated from the ultraviolet absorbance using a value of $A_{277}^{1\%} = 3.3$ (64).

RESULTS

General Features

QUANTITATIVE MEASUREMENT OF THE GELATION OF ETC EXTRACTS: To study the gel-sol transformation of ETC extracts in detail, we first established a quantitative method to measure the extent of gelation of the extracts. Recently, the gelation of cell extracts of rabbit pulmonary macrophages (18), *Acanthamoeba* (46), human leukocytes (5), and *A. proteus* (60) was measured turbidimetrically. This method, however, was not applicable to ETC extracts because the turbidity of the extracts easily changed independently of the extent of gelation. Therefore, in this study we measured the gelation of ETC extracts quantitatively by the following procedure. After incubation for gelation or solation, the extracts were separated into gel and liquid layers by low-speed centrifugation without stirring. The liquid layer was removed, and the gel volume or the total protein in the gel was measured.

Fig. 1 shows the linear relationship between the protein concentrations of ETC extracts and the extent of gelation, measured as the volume or total protein of the gel. Above a critical protein concentration of the extracts, the gel volume and the percentage of total protein in the gel increased linearly until saturation, with increase in the protein concentration of the extracts. This shows that we can measure the extent of gelation of ETC extracts quantitatively, as the volume or total protein of the gel.

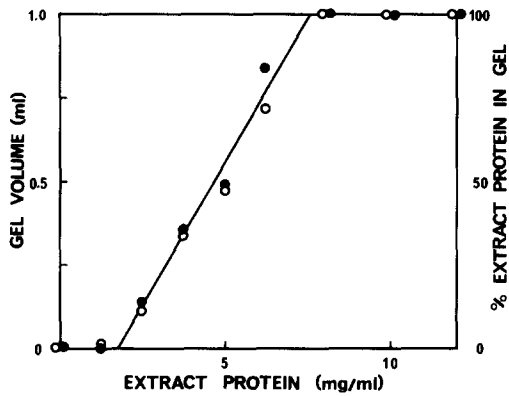


FIGURE 1 Linear relations between the protein concentration of ETC extracts, the gel volume, and the percentage of the total protein in the gel. ETC extracts were dialyzed against 25 vol of buffered sucrose solution (0.34 M sucrose, 20 mM Tris-maleate, pH 7.0 at 25°C), which contained 0.25 mM ATP, 0.5 mM EGTA, 0.5 mM DTT, and 0.2 mM PMSF, for 12 h under ice-cold conditions with one change of buffer. Reaction mixtures containing various amounts of the extracts, 57 mM KCl, 1.4 mM ATP, 3.9 mM MgCl₂, 2.5 mM EGTA, 2.5 mM DTT, and 0.2 mM PMSF were incubated at 25°C for gelation, and the resulting gels were separated by centrifugation at 1,150 g for 30 min at 25°C. ○, Percentage of total protein in the gel; ●, gel volume.

TEMPERATURE-DEPENDENT GEL-SOL TRANSFORMATION OF ETC EXTRACTS: We studied the temperature-dependent gel-sol transformation of ETC extracts quantitatively by the centrifugation method described above. Fig. 2a shows the time-course of gelation induced by warming, and subsequent solation induced by cooling. ETC extracts formed a gel when warmed to 25°C, and the gel liquified when cooled to 0°C. The rate and extent of gelation increased with an increase in the protein concentration of the extracts. At a protein concentration of ~5–6 mg/ml, the extracts gelled within 30 min when warmed to 25°C, and the gelled extracts liquified rapidly within 15 min when cooled to 0°C. When re-warmed to 25°C, the liquified extracts gelled again within 5 min; that is, more rapidly than the original extracts. Even if F-actin prepared from rabbit skeletal muscle was present in the reaction mixtures for gelation, the extracts never gelled without warming. This indicates that the presence of F-actin only is not sufficient for inducing gelation.

Fig. 2b shows the reversibility of the temperature-dependent gel-sol transformation. When

ETC extracts in reaction mixtures were alternately warmed to 25°C for gelation and cooled to 0°C for solation, the extracts showed reversible gel-sol transformation. This transformation cycle could

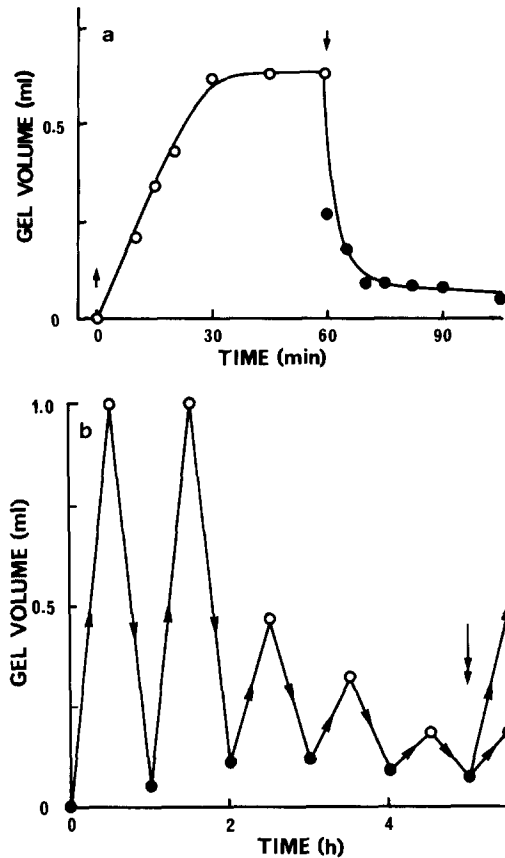


FIGURE 2 Temperature-dependent reversible gel-sol transformation of ETC extracts. Gelation was induced by warming the mixture to 25°C, and solation by cooling it to 0°C. After various incubation times, the gelled extracts were separated into gel and liquid layers by centrifugation at 2,060 g for 10 min at 25°C, whereas solled extracts were centrifuged at 4°C to prevent their regelation. Other conditions were the same as for Fig. 1. ↑, Warming to 25°C; ↓, cooling to 0°C; ↓, addition of 1 mM Mg-ATP; △, extracts to which 1 mM Mg-ATP was added at 5 h; ○△, extracts centrifuged at 25°C; ●, extracts centrifuged at 4°C. (a) Time-course of gelation on warming and subsequent solation on cooling. ETC extracts (5.4 mg/ml extract protein), in 1 ml of reaction mixture, were incubated at 25°C, and then at 0°C. (b) Reversibility of the gel-sol transformation. ETC extracts (8.0 mg/ml extract protein), in 1 ml of reaction mixture, were incubated for 30 min at 25°C for gelation, and then for 30 min at 0°C for solation. This warming-cooling cycle was repeated.

be repeated at least six times. On repeating the cycle, the extent of gelation decreased and unliquified material increased. Addition of 1 mM Mg-ATP partially restored the extent of gelation of extracts after several transformation cycles (Fig. 2*b*), suggesting the ATP-requirement for gelation described below in detail.

ION AND NUCLEOTIDE REQUIREMENTS FOR THE GELATION: To show ion and nucleotide requirements for the gelation, we rapidly desalted the extracts by chromatography on Sephadex G-25, and then added various amounts of KCl, MgCl₂, or various nucleotides and phosphoric compounds to the reaction mixtures for gelation. Fig. 3 shows the dependence of gelation on the concentrations of KCl and MgCl₂. When warmed in the absence of either KCl or MgCl₂, the extracts formed only a slight precipitate and showed no gelation. The extent of gelation increased linearly with an increase in the KCl concentration and became maximal at 75 mM KCl; high concentrations of KCl were rather inhibitory as observed with *D. discoideum* extracts (7). The extent of gelation also increased with an increase in the MgCl₂ concentration, and in the presence of 1 mM ATP it reached a saturation level at 2 mM MgCl₂.

Table I shows the nucleotide-requirements for the gelation. When added to reaction mixtures at concentrations of 1 mM, nucleotide triphosphates, such as ATP, CTP, GTP, ITP, and UTP, were all effective for sustaining the gelation, whereas inorganic phosphate, pyrophosphate, AMP, and ADP had no effect. Fig. 4 shows the dependence of gelation on the concentration of ATP. The gelation was detectable upon addition of 10 μM ATP. The addition of 250 μM–1 mM ATP resulted in maximal gelation, but high concentrations of ATP were rather inhibitory. In addition to sustaining gelation, ATP apparently stabilized the gelation activity of ETC extracts, because the extracts irreversibly lost activity in the absence of ATP but not in its presence.

EFFECTS OF REDUCING OR SH-BLOCKING REAGENTS ON THE GELATION: When ETC extracts were dialyzed for 12 h against buffered sucrose solution without a reducing agent, they formed insoluble material which contained a 42,000-dalton polypeptide, presumably cell actin, as a main protein component, and lost gelation activity. Addition of DTT did not restore the lost activity. Inclusion of DTT in the dialysis solution

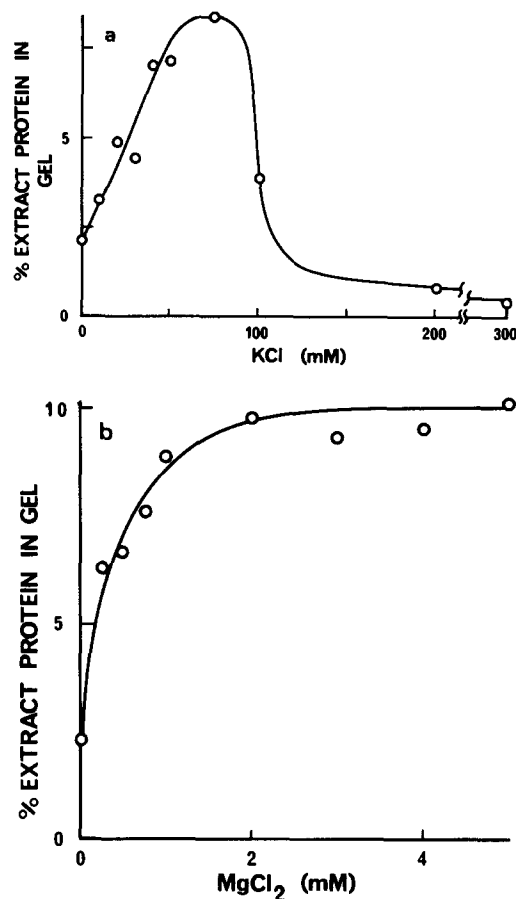


FIGURE 3 Effects of KCl and MgCl₂ on gelation. 8 ml of ETC extract was desalted by chromatography on Sephadex G-25 (3 × 25 cm), equilibrated with buffered sucrose solution containing 0.5 mM EGTA and 1 mM DTT, and the void fractions were collected. The void fractions were incubated in 1 ml of reaction mixture for 1 h at 25°C for gelation. The gels were separated by centrifugation at 1,150 g for 30 min at 25°C, and the total protein in the gels was determined. (a) Effect of KCl. Reaction mixtures contained various amounts of KCl, 5.0 mg/ml extract protein, 0.5 mM ATP, 1 mM MgCl₂, 0.5 mM EGTA, and 1 mM DTT in buffered sucrose solution. (b) Effect of MgCl₂. Reaction mixtures contained various amounts of MgCl₂, 5.0 mg/ml extract protein, 50 mM KCl, 1 mM ATP, 0.5 mM EGTA, and 1 mM DTT in buffered sucrose solution.

stabilized the activity, presumably by reducing the SH-groups of protein components.

Fig. 5 shows the inhibitory effects of SH-blocking reagents on the gelation. When added to reaction mixtures containing 0.5 mM DTT just

TABLE I
Nucleotide Requirements for Gelation

Nucleotide and phosphoric compound	Total protein* in gel	Gel volume*
	μg	μl
None	114 \pm 49	<10
Inorganic phosphate	174 \pm 2	<10
Pyrophosphate	185 \pm 9	<10
AMP	164 \pm 5	<10
ADP	142 \pm 12	<10
ATP	1,591 \pm 563	210 \pm 90
CTP	730 \pm 112	80 \pm 10
GTP	1,418 \pm 225	200 \pm 30
ITP	1,851 \pm 132	270 \pm 10
UTP	1,854 \pm 239	260 \pm 50

Reaction mixtures contained the nucleotide or phosphoric compound indicated at 1 mM concentration, 6.8 mg/ml extract protein, 50 mM KCl, 2.5 mM MgCl_2 , and 1 mM DTT in 1 ml of buffered sucrose solution. Other conditions were the same as for Fig. 3.

* Means \pm SD for triplicate experiments.

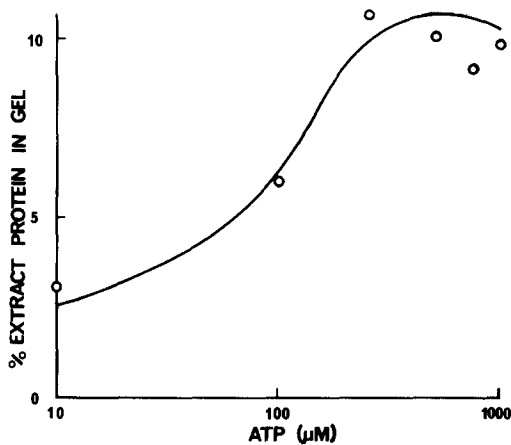


FIGURE 4 Dependence of gelation on ATP concentration. Reaction mixtures contained various amounts of Mg-ATP, 5.0 mg/ml extract protein, 50 mM KCl, 1 mM MgCl_2 , 0.5 mM EGTA, and 1 mM DTT in 1 ml of buffered sucrose solution. Other conditions were the same as for Fig. 3.

before warming to 25°C, 1 mM DTNB, 2 mM NEM, 2 mM CMB, or 2 mM CMBS caused complete inhibition, and 7 mM IAM caused 50% inhibition. The gelation inhibited by 1 mM DTNB or 2 mM CMBS was restored to 72 and 61%, respectively, of the control value without the reagents by adding 39 mM DTT. These findings indicate that SH-groups of protein components involved in the gelation should be kept reduced

for their functional interactions resulting in gelation.

REGULATION OF THE GELATION BY FREE Ca^{2+} : When EGTA was not included in the solutions for extraction, dialysis, and incubation of ETC extracts for gelation, the gelation was partially or almost completely inhibited. This inhibition seemed to be caused by contaminating calcium or heavy metals, because the inhibited gelation was restored when EGTA was added to the reaction mixture for gelation. This fact also indicates the reversibility of the inhibition. To examine the possible role of Ca^{2+} in regulating the gelation of ETC extracts, we first measured the extent of gelation at various concentrations of free Ca^{2+} obtained with Ca-EGTA buffer. Fig. 6 shows that low concentrations of free Ca^{2+} inhibited the gelation: 50% inhibition was observed with 0.06 μM Ca^{2+} , and complete inhibition with 0.5 μM Ca^{2+} . In addition, Table II shows that the gelation inhibited by low concentrations of free Ca^{2+} was partially restored by adding excess EGTA.

We also examined the solution of gelled extracts by Ca^{2+} . Table III shows the results. When 1 ml of CaCl_2 in 100 mM Tris-HCl buffer (pH 7.6) was overlaid on 1 ml of gelled extracts containing 1

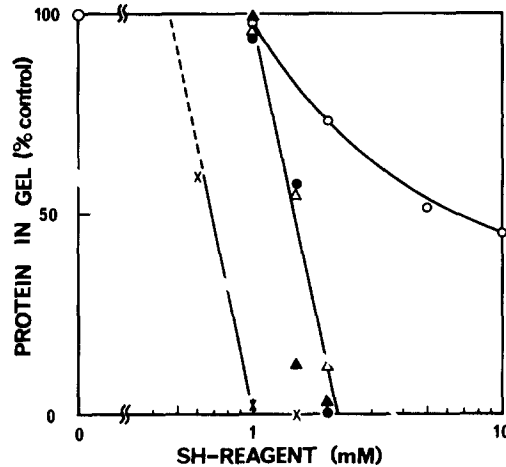


FIGURE 5 Inhibition of gelation by SH-reagents. ETC extracts were dialyzed against 25 vol of buffered sucrose solution containing 0.25 mM ATP, 0.5 mM EGTA, and 0.5 mM DTT, with one change of buffer. The extracts (10.5 mg/ml extract protein), in 1 ml of reaction mixture, were incubated for 30 min at 25°C for gelation in the presence of 0.5 mM DTT and various amounts of SH-reagents. Other conditions were the same as for Fig. 1. \circ , IAM; \bullet , NEM; Δ , CMB; \blacktriangle , CMBS; \times , DTNB.

mM EGTA, for 1.5 h at 25°C, a 1 mM CaCl₂ solution (final concentration, ~10⁻⁶ M free Ca²⁺) caused a 50% reduction of the gel volume. Be-

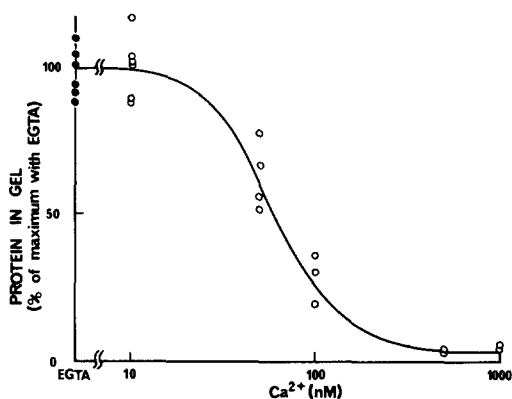


FIGURE 6 Inhibition of gelation by free Ca²⁺. ETC extracts were dialyzed against 25 vol of buffered sucrose solution containing 0.25 mM ATP, 0.5 mM EGTA, and 1 mM DTT, with one change of buffer, and then against 25 vol of buffered sucrose solution containing 0.25 mM ATP and 1 mM DTT, with one change of buffer. The extracts (6.0 mg/ml extract protein), in 1 ml of buffered sucrose solution containing 50 mM KCl, 1 mM ATP, 2 mM MgCl₂, 1 mM EGTA, and 1 mM DTT, were incubated for 1 h at 25°C for gelation at various free Ca²⁺ concentrations obtained with Ca-EGTA buffer. Free Ca²⁺ concentrations of reaction mixtures were calculated by adopting the value of 5.13 × 10⁶ M⁻¹ as the apparent stability constant of the Ca-EGTA complex at pH 7.0 (44). Other conditions were the same as for Fig. 1.

cause ETC extracts do not show contraction phenomena such as those reported for other cell extracts, this suggests that low concentrations of free Ca²⁺ caused weak solution of gelled extracts.

EFFECTS OF CYTOCHALASINS AND COLCHICINE ON THE GELATION: Fig. 7 shows the inhibition of gelation by cytochalasins, which are metabolites of mold that affect microfilaments. Cytochalasins B and D were equally effective in inhibiting the gelation, and both inhibited the gelation of ETC extracts (5.8 mg/ml extract pro-

TABLE II
Reversibility of the Inhibition of Gelation by Free Ca²⁺

Condition of free Ca ²⁺	Total protein in gel μg
Control with 1 mM EGTA	3,431 ± 199 (100)*
Ca ²⁺ → +0 mM EGTA	321 ± 111 (9)
Ca ²⁺ → +5 mM EGTA	1,388 ± 128 (40)
Ca ²⁺ → +10 mM EGTA	1,713 ± 155 (50)

ETC extracts were incubated at 25°C for 1 h in the presence of 0.35 mM CaCl₂ and 0.5 mM EGTA (~5 × 10⁻⁷ M free Ca²⁺) in 1 ml of reaction mixture for gelation. Then, 0.1 ml of EGTA (pH 7) was added to the extracts at the final concentrations indicated and the extracts (6.1 mg/ml extract protein) were further incubated at 25°C for 1 h. Other conditions were the same as for Fig. 1. The initial free Ca²⁺ concentration of ~5 × 10⁻⁷ M was calculated as described for Fig. 6.

* Percentage of the value for the 1 mM EGTA control.

TABLE III
Solution of Gelled Extracts by Addition of CaCl₂ or Cytochalasin B

Addition	Total protein in gel	Gel volume
	μg	μ
A. CaCl ₂ (1 ml)		
0 mM	8,280 ± 732 (100)*	840 ± 70 (100)*
1 mM	4,475 ± 510 (54)	410 ± 90 (49)
2 mM	4,921 ± 160 (59)	430 ± 40 (51)
B. Cytochalasin B (0.1 ml)		
0 μg/ml	NM‡	310 ± 10 (100)
10 μg/ml	NM	200 ± 40 (65)

(A) The gelled extracts (10.3 mg/ml extract protein) in 1 ml of reaction mixture were prepared by warming ETC extracts at 25°C for 1.5 h in the presence of 1 mM EGTA, as described for Fig. 1. Then 1 ml of various concentrations of CaCl₂ in 100 mM Tris-HCl (pH 7.6) was overlaid on 1 ml of gelled extracts and the extracts were further incubated at 25°C for 1.5 h. Other conditions were the same as for Fig. 1.

(B) The gelled extracts (4.2 mg/ml extract protein) in 0.9 ml of reaction mixture for gelation were prepared, and then 0.1 ml of 0 or 10 μg/ml cytochalasin B in 10 mM Tris-maleate (pH 7.0) was overlaid on 0.9 ml of gelled extracts. Other conditions were the same as for (A).

* Percentage of the value for the buffer control.

‡ NM, not measured.

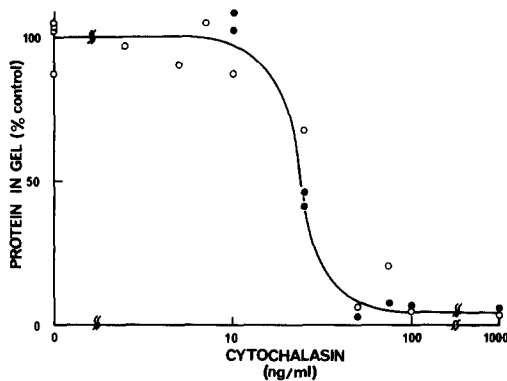


FIGURE 7 Inhibition of gelation by cytochalasins B and D. Cytochalasins B and D dissolved in dimethylsulfoxide at a concentration of 1 mg/ml were diluted with water and added to ETC extracts. Dimethylsulfoxide did not affect the gelation at concentrations of 0.1% or higher. ETC extracts (5.8 mg/ml extract protein), in 1 ml of buffered sucrose solution containing 50 mM KCl, 1.3 mM ATP, 2.3 mM MgCl₂, 0.5 mM EGTA, and 1 mM DTT, were incubated for 1 h at 25°C for gelation with various concentrations of cytochalasins B and D. Other conditions were the same as for Fig. 1. ○, Cytochalasin B; ●, cytochalasin D.

tein) by 50% at 25 ng/ml and completely at 50 ng/ml (~0.1 μM). The amounts of cytochalasins required for inhibition increased with an increase in the protein concentration of the extracts. Table IV shows the reversibility of the inhibition of gelation by cytochalasin B. When extracts treated with 0.1 μg/ml cytochalasin B were extensively dialyzed against buffered sucrose solution, the gelation activity was partially recovered. In addition, Table III shows that when cytochalasin B was overlaid on gelled extracts it caused weak solation of the extracts as observed with *D. discoideum* extracts (7).

In contrast to cytochalasins, colchicine, which prevents the assembly of tubulins into microtubules, did not affect the gelation even at a concentration of 10 mM, thus suggesting that assembly of tubulins was not involved in the gelation.

Involvement of Actin

PROTEIN COMPOSITION OF THE GEL: After completion of gelation, the gelled extracts were broken by vigorous agitation in a Voltex mixer (Scientific Industries, Inc., Bohemia, N.Y.), and then separated into a small volume of broken gels and supernates by low-speed centrifugation. Then, the protein compositions of the

TABLE IV
Reversibility of the Inhibition of Gelation by
Cytochalasin B

Condition of cytochalasin B	Total protein in gel	Gel volume
	μg	μl
(-) → (-)	6,190 ± 287 (100)*	1,000 (100)*
(-) → (+)	94 ± 12 (2)	<10 (<1)
(+) → (-)	3,925 ± 198 (63)	640 (64)
(+) → (+)	232 ± 23 (4)	10 (1)

ETC extracts treated with 0.1 μg/ml cytochalasin B for 30 min at 25°C in reaction mixture for gelation were dialyzed against 42 vol of buffered sucrose solution containing 1 mM EGTA, 0.25 mM ATP, and 0.5 mM DTT, under ice-cold conditions. The buffer was changed twice at 6-h intervals. ETC extracts without cytochalasin treatment were also dialyzed similarly. Then, extracts (6.4 mg/ml extract protein) in 1 ml of reaction mixture were incubated at 25°C for 30 min in the presence or absence of 0.1 μg/ml cytochalasin B, for gelation. Other conditions were the same as for Fig. 7.

* Percentage of the value for the control without cytochalasin B.

broken gels, the supernates, and the original extracts were analyzed by SDS-polyacrylamide gel electrophoresis. The relative amounts of the major gel components in the gels and the original extracts were also roughly estimated by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis.

When ETC extracts immediately after preparation were gelled by warming to 25°C for 1 h, their pellets contained polypeptides of 42,000, 48,000, 51,000, 89,000, and 270,000 daltons, and a very large polypeptide (VLP, the largest component easily detected on SDS-polyacrylamide gels of ETC extracts stained with Coomassie Blue after electrophoresis) with a mol wt >300,000 daltons, with numerous minor unidentified polypeptides. Washing the gels several times with gelation solution at 25°C removed minor polypeptide bands, but did not significantly alter the relative amounts of the major polypeptide bands (Fig. 8d).¹ The amount of the polypeptides of 42,000, 51,000, 89,000, and 270,000 daltons in the supernates²

¹ Washing the gels also removed some of the 270,000-dalton polypeptide, but a significant amount of the polypeptide remained in the gels even after extensive washing.

² The supernates prepared without the agitation also showed the same SDS-polyacrylamide gel pattern.

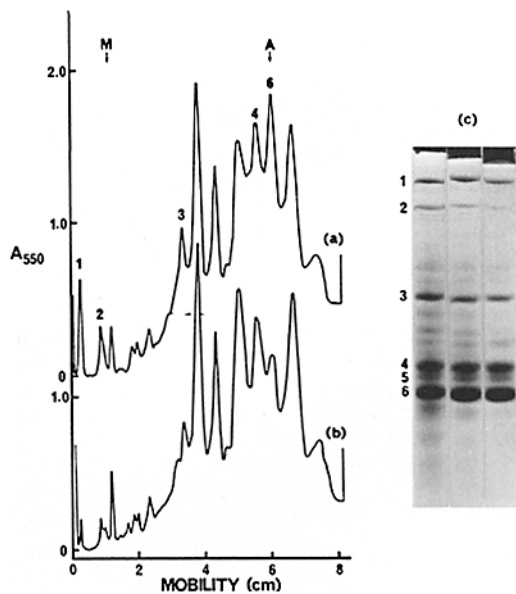


FIGURE 8 Protein composition of the gels analyzed by SDS-polyacrylamide gel electrophoresis. ETC extracts immediately after preparation, in 1 ml of reaction mixture for gelation, were gelled by warming them to 25°C for 1 h. The gelled extracts were broken by vigorous agitation in a Voltex mixer, and the broken gels were collected by low-speed centrifugation (1,150 g for 30 min at 25°C). The precipitated gels were resuspended in 2 ml of reaction mixture for gelation at 25°C, and collected again by low-speed centrifugation. The gels were washed again. The protein compositions of the broken gels, the supernates after gelation, and the original extracts were analyzed by SDS-polyacrylamide gel electrophoresis on 5% polyacrylamide gels. Other conditions were the same as for Fig. 1. The numbers indicate the polypeptide peaks (or bands) corresponding to mol wt >300,000(1), 270,000(2), 89,000(3), 51,000(4), 48,000(5), and 42,000 daltons (6), respectively. The arrows indicate the position of heavy chain of rabbit muscle myosin (M) and actin (A), respectively. Densitometric scans of electrophoretic gels stained with Coomassie Blue: (a), original extracts; (b), supernates after gelation. (c), SDS-polyacrylamide gels, from left to right, of the unwashed, once washed, and twice washed gels.

was less than in the original extracts, and most of the VLP in the original extracts was concentrated in the gels (Fig. 8a and b). Fig. 9 and Table V show the relative amounts of the major polypeptides of the washed gels.

When the gels were prepared from extracts dialyzed against buffered sucrose solution or stored under ice-cold conditions in the absence of PMSF for 12 h, the gels contained only two major

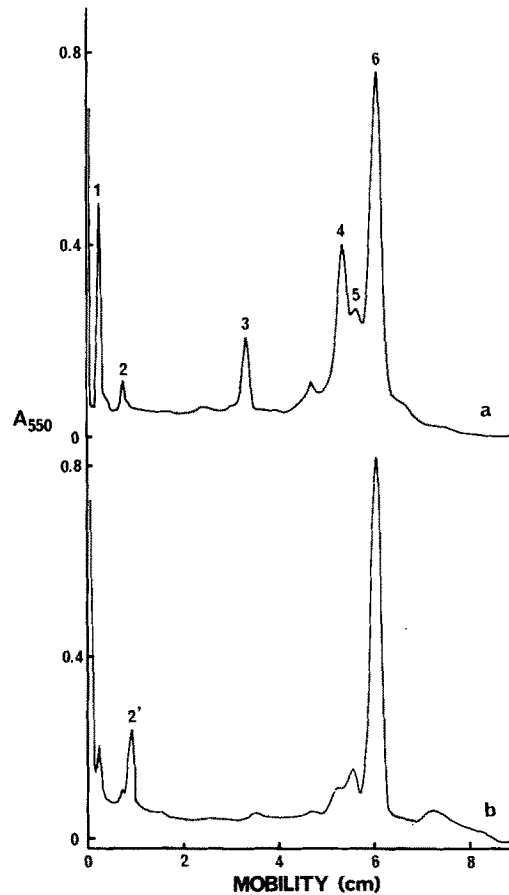


FIGURE 9 Densitometric scans of SDS-polyacrylamide gels of ETC-extract gels stained with Coomassie Blue. The extracts immediately after preparation or dialysis against buffered sucrose solution without PMSF for 12 h were warmed for 1 h at 25°C for gelation. The gelled extracts were broken by vigorous agitation in a Voltex mixer, and the broken gels were collected by low-speed centrifugation and washed three times with reaction mixture for gelation. Other conditions were the same as for Fig. 8. The numbers indicate the polypeptide peaks corresponding to mol wt >300,000(1), 270,000(2), 230,000(2'), 89,000(3), 51,000(4), 48,000(5), and 42,000 daltons (6), respectively. (a), Gels prepared immediately after preparation of ETC extracts; (b), gels prepared after dialysis of the extracts against buffered sucrose solution without PMSF.

polypeptides, of 42,000 and 230,000 daltons, with a trace of the VLP (Fig. 9 and Table V). The extracts before gelation contained only a trace of the VLP but there was much more of the 230,000-dalton polypeptide than in the ETC extracts just after preparation. This suggests that the VLP was degraded into a 230,000-dalton polypeptide by

TABLE V
Protein Composition of the Gel

Polypeptide daltons	% of total stainable polypeptide		
	Extract*	Gel I‡	Gel II§
>300,000	0.33 ± 0.09	6.4 ± 0.7	<0.1
270,000	NM	1.6 ± 0.5	<0.1
230,000	NM	<0.1	14.5 ± 0.2
89,000	NM	5.2 ± 0.8	ND¶
51,000	NM	19.5 ± 0.1	9.4 ± 0.8**
48,000	NM	6.3 ± 0.7	
42,000	13.9 ± 1.7	37.2 ± 6.4	73.0 ± 0.8
Others	NM	23.8 ± 5.8	3.1 ± 0.3

The extracts immediately after preparation or dialysis against buffered sucrose solution without PMSF for 12 h were warmed at 25°C for 1 h for gelation. The gels were washed three times with reaction mixture for gelation, as described for Fig. 8. The relative amounts of the major components in the gels and in the extracts just after preparation were roughly estimated by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis.

* Fresh extracts just after preparation.

‡ Gels prepared immediately after preparation of ETC extracts.

§ Gels prepared after dialysis of the extracts against buffered sucrose solution without PMSF for 12 h.

|| NM, not measured.

¶ ND, not detected.

** 51,000-dalton polypeptide plus 48,000-dalton polypeptide.

endogenous protease.³ If ETC extracts were further dialyzed or stored, they lost their gelation activity and these extracts were found to have little VLP or 230,000-dalton polypeptides.

The polypeptide of 42,000 daltons comigrated with rabbit muscle actin on SDS-polyacrylamide gels. To determine whether it was in fact cell actin, first we examined its selective binding with muscle myosin. ETC extracts and rabbit muscle myosin were extensively dialyzed together against a low ionic strength solution without ATP, and then centrifuged at low speed in the absence or presence of ATP. Then the supernates and washed pellets were analyzed by SDS-polyacrylamide gel electrophoresis. In the absence of ATP, ~40% of the 42,000-dalton polypeptide selectively coprecipitated with myosin. On the other hand in the presence of ATP, neither this polypeptide nor muscle myosin precipitated. This indicates that at least 40% of the 42,000-dalton polypeptide was functional cell actin.

Second, we studied the binding of muscle tropomyosin with presumptive cell actin. Rabbit muscle tropomyosin, up to 0.6 mg/ml, did not

³ It is also possible that some of the 230,000-dalton polypeptide was derived from 270,000-dalton polypeptide.

affect the gelation. So, the gelled extracts were made in the presence of various amounts of muscle tropomyosin. Then, the protein composition of the washed gels was analyzed by SDS-polyacrylamide gel electrophoresis. Fig. 10 shows that muscle tropomyosin was coprecipitated with the gels by low-speed centrifugation. Assuming that all the 42,000-dalton polypeptide in the gel was cell actin and that muscle tropomyosin was coprecipitated with the gels by binding with cell F-actin, quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis showed that the maximal amount of tropomyosin coprecipitated with cell F-actin in the gels was ~0.18 mol/mol of actin monomer. This value is consistent with the value of 0.15 mol tropomyosin/mol actin monomer reported for the binding of rabbit muscle tropomyosin with rabbit muscle F-actin (30, 38). This indicates that most of the 42,000-dalton polypeptide in the gels was cell F-actin.

FUNCTIONAL INVOLVEMENT OF ACTIN: Cell F-actin is the main protein component in the gels. Thus, to determine whether actin is functionally involved in the gelation, we used bovine pancreatic DNase I and CMB-S-1 as actin inhibitors.

DNase I binds very tightly with an equimolar

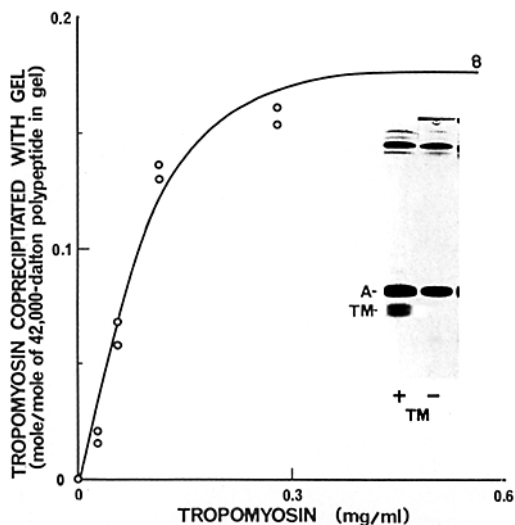


FIGURE 10 Coprecipitation of tropomyosin with the gels by low-speed centrifugation. For preparation of gels containing only two major protein components, ETC extracts were used after dialysis against buffered sucrose solution for 12 h in the absence of PMSF. Samples of extracts (4.2 mg/ml extract protein), in 1 ml of reaction mixture for gelation, were incubated for 30 min at 25°C for gelation in the presence or absence of rabbit muscle tropomyosin. The gelled extracts were broken by vigorous agitation in a Voltex mixer, and the broken gels were collected by low-speed centrifugation and washed twice with 2 ml of reaction mixture for gelation, as described for Fig. 9. The protein composition of the washed gels was analyzed by SDS-polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels. Other conditions were the same as for Fig. 1.

amount of G-actin (31, 32) and inhibits the polymerization of G-actin to F-actin (36). In addition, it depolymerizes F-actin to G-actin (22, 36). Thus, DNase I can block F-actin-dependent functions by forming an inactive equimolar G-actin-DNase I complex.

Fig. 11 shows the inhibition of gelation by DNase I. ETC extracts and DNase I were incubated together at 0°C for 30 min to allow the formation of a G-actin-DNase I complex, and then warmed to 25°C for gelation. The extent of gelation decreased linearly with increase in the DNase I concentration, and addition of 75 $\mu\text{g/ml}$ (2.4 μM) DNase I resulted in complete inhibition of gelation of ETC extracts (4.0 mg/ml extract protein).

To determine whether the inhibition of gelation by DNase I resulted from the formation of an equimolar cell G-actin-DNase I complex, we ex-

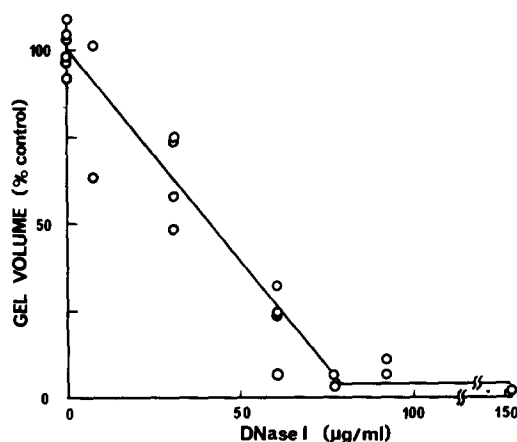


FIGURE 11 Inhibition of gelation by DNase I. ETC extracts (4.0 mg/ml extract protein), in 1 ml of reaction mixture, were incubated with various amounts of DNase I for 30 min at 0°C, and then for 30 min at 25°C for gelation. Other conditions were the same as for Fig. 1.

amined the neutralization of the inhibitory function of DNase I by muscle actin and the reversal of the inhibition by muscle actin. Fig. 12a shows that 196 $\mu\text{g/ml}$ (4.6 μM) muscle G-actin neutralized the inhibitory function of 148 $\mu\text{g/ml}$ (4.9 μM) DNase I when DNase I was preincubated with G-actin at 0°C for 10 min before its addition to the reaction mixtures for gelation. Fig. 12b shows that muscle G-actin also restored gelation after its inhibition by DNase I. The amount of actin required for complete recovery of the gelation inhibited by 296 $\mu\text{g/ml}$ (9.4 μM) DNase I was calculated as 585 $\mu\text{g/ml}$ (14 μM) by extrapolation of the results. The actin to DNase I molar ratio of 1.5 that is required for recovery of the gelation is consistent with the value of 1.0 for neutralization described above. These facts clearly show that DNase I inhibited the gelation by forming an equimolar cell G-actin-DNase I complex, and that muscle actin sustained the gelation as effectively as cell actin.

S-1 binds to F-actin in the absence of ATP, but dissociates from it on addition of ATP. CMB-S-1 is composed of equimolar amount of S-1A⁴ modified with CMB (CMB-S-1A) and S-1B not modified with CMB (52). In contrast to S-1, CMB-S-1A binds with F-actin very tightly and stoichio-

⁴ S-1A, S-1 without the initial burst of P_i -liberation; S-1B, S-1 with the P_i -burst. For further explanation, see Inoue, A., and Y. Tonomura. 1976. *J. Biochem. (Tokyo)*. 79:419-434.

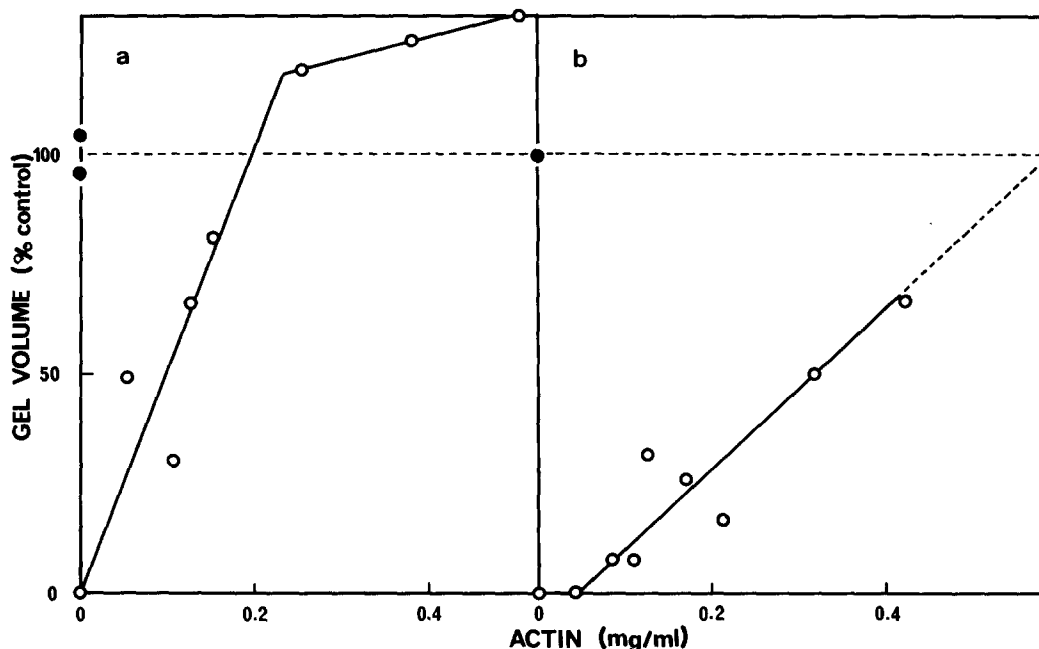


FIGURE 12 Neutralization by muscle actin of the inhibitory function of DNase I and reversal by muscle actin of inhibition of gelation by DNase I. (a) Neutralization of the inhibitory function of DNase I. DNase I and various amounts of rabbit muscle G-actin were incubated at 0°C for 10 min in the presence of 114 mM KCl, 2.8 mM ATP, 7.8 mM MgCl₂, 5 mM EGTA, and 0.2 mM PMSF to allow the formation of an equimolar G-actin-DNase I complex. After addition of the mixtures, ETC extracts (3.7 mg/ml extract protein), in 1 ml of reaction mixture, were incubated at 0°C for 30 min and then at 25°C for 30 min in the presence of 148 μg/ml DNase I and various amounts of muscle actin. Other conditions were the same as for Fig. 1. ○, Gelation in the presence of DNase I and various amounts of muscle actin; ●, gelation in the absence of both DNase I and muscle actin. (b) Reversal of the inhibition of gelation by DNase I. When ETC extracts (5.0 mg/ml extract protein), in 0.9 ml of reaction mixture, were incubated at 25°C for 30 min in the presence of 329 μg/ml DNase I and 0.5 mM PMSF, no gelation occurred. After addition of 0.1 ml of various amounts of rabbit muscle G-actin, the extracts in 1 ml of reaction mixture were further incubated at 25°C for 30 min in the presence of 296 μg/ml DNase I and various amounts of muscle actin. Other conditions were the same as for Fig. 1. ○, Gelation in the presence of DNase I and various amounts of muscle actin; ●, gelation in the absence of both DNase I and muscle actin.

metrically in a ratio of 1 mol CMB-S-1A/mol actin monomer, even in the presence of ATP. Thus, finally it occupies all the myosin binding sites of F-actin, and inhibits the ability of F-actin to interact with myosin. Because CMB-S-1 specifically binds with F-actin, it can be used as an actin-specific inhibitor (24, 52).

Fig. 13 shows the effects of CMB-S-1 and S-1 on the gelation. When reaction mixtures for gelation contained high concentrations of ATP,⁵ gelation of ETC extracts (8.5 mg/ml extract protein) was inhibited 50% and completely by 0.38 mg/ml

and 0.66 mg/ml of CMB-S-1, respectively, but only slightly by 1.9 mg/ml of S-1. This difference in the effects of S-1 and CMB-S-1 indicates that the inhibition by CMB-S-1 was caused by the tight binding of CMB-S-1A with cell F-actin, because the gelation was measured in the presence of high concentrations of ATP.

Inconsistent with the results of the actin-inhibitor experiments, the gelation activity of ETC extracts was partially or almost completely lost when cell F-actin was removed from the extracts by ultracentrifugation after polymerization of cell actin. The actin-deficient extracts, however, regained the full activity on addition of muscle actin. The extent of the gelation increased linearly with increase in the amount of muscle actin added.

⁵ At low concentrations of ATP, S-1 also inhibited the gelation. This inhibition, however, probably resulted from mere ATP deficiency.

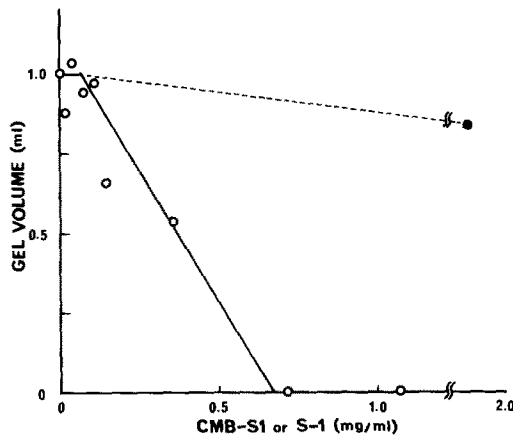


FIGURE 13 Effects of CMB-S-1 and S-1 on gelation. ETC extracts were dialyzed against 50 vol of buffered sucrose solution containing 0.25 mM ATP and 0.5 mM EGTA for 6 h to decrease the DTT concentration. The extracts (8.5 mg/ml extract protein), in 1 ml of buffered sucrose solution containing 55 mM KCl, 6 mM ATP, 7 mM MgCl₂, 0.5 mM EGTA, and 0.1 mM DTT, were incubated for 30 min at 25°C for gelation in the presence of various amounts of CMB-S-1 or S-1. Other conditions were the same as for Fig. 1. ○, CMB-S-1; ●, S-1.

GELATION OF ETC EXTRACTS RECONSTITUTED FROM ACTIN-BLOCKED OR ACTIN-DEFICIENT ETC EXTRACTS BY ADDITION OF MUSCLE ACTIN: To determine whether cell actin is responsible for the temperature dependence, cytochalasin sensitivity, or Ca²⁺ regulation of the gelation, we inactivated cell actin in ETC extracts by addition of excess DNase I, or removed it from the extracts by ultracentrifugation after its polymerization. We then reconstituted extracts with gelation activity by addition of muscle G-actin, and examined these actin reconstituted extracts for reversibility of temperature-dependent gel-sol transformation, cytochalasin-sensitivity, and Ca²⁺-regulation of gelation.

Irrespective of whether the extracts were reconstituted from actin-blocked or actin-deficient extracts, the actin reconstituted system behaved like the original extracts.

DISCUSSION

Quantitative Measurement

In this paper, we established a very simple quantitative method to measure the gelation or solation of cell extracts. With this method it became possible to study the gel-sol transformation of ETC extracts quantitatively and even to

examine the stoichiometry of actin in the gel-sol transformation. Therefore, this method is useful in studies of the interactions between protein components in the gel-sol transformation of cell extracts.

Protein Components

SDS-polyacrylamide gel electrophoresis showed that the main components of ETC extract gels had mol wt of 42,000, 48,000, 51,000, 89,000, 270,000, and >300,000 daltons (VLP). Washing the gels several times with gelation solution did not significantly alter the relative amounts of the major polypeptides. This indicates that these polypeptides are either structural components or associated with the true structural components. The 42,000-dalton polypeptide was identified as cell actin because it had the same mobility on SDS-polyacrylamide gel as muscle actin and bound with muscle myosin and muscle tropomyosin. However, the protein components and their functions remain to be established.

Functional Involvement of Actin

Bovine pancreatic DNase I inhibited gelation. Muscle G-actin neutralized the inhibitory function of DNase I when added in equimolar amount, and the addition of muscle actin also restored the gelation inhibited by DNase I. These facts indicate that DNase I inhibited the gelation by forming an equimolar G-actin-DNase I complex. The concentrations of free G-actin and F-actin, if any, are probably very low because in the presence of excess DNase I essentially all the cell actin is probably fixed as an equimolar complex. Therefore, inhibition of gelation by DNase I was caused by the lack of available free G- or F-actin.

CMB-S-1 (CMB-S-1A) inhibited the gelation in the presence of ATP, whereas S-1 did not. This differential inhibition of gelation indicates that CMB-S-1 inhibited the gelation as a result of the tight binding of CMB-S-1A with cell F-actin, thus suggesting the functional involvement of F-actin in the gelation. This view is consistent with the following facts: (a) The gelation depended on the conditions for polymerization of actin, namely, the presence of ATP, Mg²⁺, and KCl, and warming. (b) Removal of cell F-actin from ETC extracts by ultracentrifugation resulted in the loss of gelation activity. (c) Muscle tropomyosin bound stoichiometrically with cell actin in the gels, thus indicating that most of actin in the gels was F-actin. (d) The cyclic peptide phalloidin, which is a

toxic component of the toadstool *Amanitia phalloides*, induces polymerization of actin, and prevents depolymerization of F-actin (8, 33), scarcely affected the gelation (Ishiura and Okada, in preparation). Because phalloidin probably fixes actin in the polymerized state, this fact suggests that G-actin was not involved in the gelation. However, it is possible that another type of actin, distinct from G- or F-actin, might be involved in the gelation (cf. reference 7).

The content of functional actin in ETC extracts can be calculated from data on the inhibition of the gelation by CMB-S-1 or DNase I. If one assumes that 2 mol of CMB-S-1 (1 mol of CMB-S-1A) inhibits the function of 1 mol of actin monomer in the gelation, the results shown in Fig. 13 indicate that functional actin comprised ~1.4% of the total protein of ETC extracts. Similarly, if DNase I inhibits the function of actin in the gelation when added in equimolar amount, the results shown in Fig. 11 indicate that functional actin comprised ~2.5% of the total protein of the extracts. These values⁶ are consistent with each other, although quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis demonstrated that the 42,000-dalton component presumed to be cell actin comprised ~14%⁷ of the total stainable protein of ETC extracts. The band of 42,000-dalton polypeptide may contain denatured actin or a protein other than actin, because some of the polypeptide could not be coprecipitated with muscle myosin.

Interactions Between Actin, Myosin, and Gelation Protein

The inhibition of gelation by CMB-S-1 also indicates that when all the myosin-binding sites of

⁶ These values were calculated on the assumption that complete inhibition of gelation by the inhibitors was caused by a complete lack of functional actin. However, it is also possible that gelation could occur and (or) be detected in the present system when the functional actin concentration exceeded a critical point. Therefore, these values might be an underestimation.

⁷ This value might be an overestimation, because the content of functional actin (the 42,000-dalton polypeptide coprecipitated with muscle myosin) estimated by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis was two-three times greater than that calculated from data on the inhibition of the gelation by CMB-S-1 or DNase I.

F-actin are occupied by CMB-S-1A (24), it is difficult for a protein component that induces gelation of F-actin (gelation protein) to interact functionally with the actin. Thus, it is possible that binding of myosin with F-actin might prevent the functional interaction of the gelation protein with F-actin, and vice versa. In accordance with this possibility, Maruta and Korn reported that *Acanthamoeba* proteins that induce the gelation of F-actin inhibit actin-activated HMM-ATPase activity (37). Weihing reported that HMM inhibits the gelation of HeLa cell extracts (63). In addition, Condeelis and Taylor reported that treatments that inhibit the gelation and induce the solation of *Dictyostelium* extracts enhance the functional interaction of myosin and actin (contraction) in the extracts (7). However, in contrast to these observations, Stossel and Hartwig reported that actin-binding protein isolated from rabbit pulmonary macrophages, which causes the gelation of F-actin, does not affect the macrophage myosin-ATPase activity activated by actin and a cofactor protein (56). Therefore, further studies are necessary on the interactions between actin, myosin, and a gelation protein such as actin-binding protein.

Ca²⁺ Regulation

We showed that concentrations of 10^{-8} – 10^{-6} M free Ca^{2+} control the gelation. This concentration range is physiologically significant, because in animal cells the concentration of Ca^{2+} in the cytosol or free cytoplasm is in the range of 10^{-8} – 10^{-5} M (2, 3, 9, 29, 48, 49). Thus, it is possible that intracellular free Ca^{2+} controls the interaction between actin and gelation protein, namely, the assembly and disassembly of actin-containing filaments (microfilaments) in cells, and thereby the cell shape (cf. references 7 and 59). In fact, recently, Goshima et al. found that micromolar free Ca^{2+} controls the cell shape and rhythmical beating of cultured mouse cardiac cells (16).

In some nonmuscle cells, Ca^{2+} regulation of actin-myosin interactions seems to be carried out by the troponin-tropomyosin system (28, 40, 42; see also reference 6 for review). But the system has not yet been found in many nonmuscle cells. Ca^{2+} regulation of actin-myosin interaction via gelation protein might explain the Ca^{2+} regulation of nonmuscle cell contractility demonstrated in intact cells (4, 12, 50), cell models (23, 41), and isolated cytoplasm (10, 19, 25, 58, 60; see also references 6 and 21 for review). Condeelis and

Taylor have also proposed the same mechanism for Ca^{2+} regulation of cell contractility (7).

In reconstitution experiments, we demonstrated that even if cell actin of ETC extracts was replaced by muscle actin, the reconstituted extracts showed Ca^{2+} -regulated gelation. This suggests that cell actin is not directly involved in Ca^{2+} -regulation. Similar Ca^{2+} -regulated gelation of cell extracts has also been reported in sea urchin eggs (26) and *Dictyostelium* (7). However, it is unknown what component is the Ca^{2+} -binding protein responsible for Ca^{2+} regulation.

Temperature Dependence and Nucleotide Triphosphate Requirement

Reconstitution experiments demonstrated that reconstituted extracts in which cell actin was replaced by muscle actin showed temperature-dependent reversible gel-sol transformation as did the original extracts. This suggests that the temperature-dependent reversible G-F transformation of cell actin (13, 17), if any, is not the main cause of the temperature-dependent gel-sol transformation of ETC extracts, because rabbit skeletal muscle actin does not show significant reversible G-F transformation in response to temperature changes. Instead, a temperature-sensitive component other than actin may be responsible for the temperature dependence of the gelation. This possibility is also supported by the fact that addition of muscle F-actin did not cause gelation at 0°C and warming was always required for the functional interaction between F-actin and gelation protein. In this connection, it is important to note that the purified macrophage actin-binding protein solution shows a reversible turbidity change on alternate warming and cooling (56).

The gelation of ETC extracts depended on nucleotide triphosphates. The temperature dependence and the nucleotide triphosphate requirement suggest that assembly of actin-containing filaments (microfilaments) might depend on the hydrolysis of nucleotide triphosphates or the phosphorylation of a protein component involved in the gelation. However, the true substrate involved in the assembly is unknown, because ATP, GTP, ITP, and UTP were equally effective for sustaining the gelation, and the role of the substrate in the assembly is also unknown.

Cytochalasin Sensitivity

The reconstitution experiments also demonstrated that extracts which had been reconstituted

by addition of muscle actin showed cytochalasin-sensitive gelation. Low concentrations (5×10^{-8} – 10^{-7} M) of cytochalasins B and D inhibited the gelation, but earlier studies showed that these low concentrations of cytochalasin B do not markedly influence the viscosity, morphology, function, or polymerization of muscle actin (11, 33, 53, 54). The lack of influence of low concentrations of cytochalasin B has also been reported with macrophage actin (18). Furthermore, by assuming that functional actin comprised 1.4–2.5% of the total protein of ETC extracts, as discussed above, it was calculated from the results shown in Fig. 7 that the amount of cytochalasins required for the complete inhibition of gelation is 0.03–0.05 mol/mol of actin monomer, thus indicating a lack of stoichiometry between actin and cytochalasins. These facts suggest that a component other than actin might be responsible for the cytochalasin sensitivity of the gelation. The nature of the cytochalasin sensitive component has not been established, but it should be noted that Hartwig and Stossel demonstrated that low concentrations of cytochalasin B reversibly prevent the gelation of muscle actin by purified macrophage actin-binding protein (18). In addition, similar cytochalasin-sensitive gelation has also been reported with cell extracts of HeLa cells (62), *Acanthamoeba* (47), and *D. discoideum* (7), and, interestingly, these gels all contain a VLP apparently corresponding to the actin-binding protein.

Addendum

During the preparation of this manuscript, we learned that Mimura and Asano also observed Ca^{2+} -regulated cytochalasin-sensitive gelation of ETC extracts (39).

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