An Actin-depolymerizing Protein (Depactin) from Starfish Oocytes: Properties and Interaction with Actin

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ABSTRACT Physico-chemical properties and interaction with actin of an actin-depolymerizing protein from mature starfish oocytes were studied. This protein, which is called depactin, exists in a monomeric form under physiological conditions. Its molecular weight is ~20,000 for the native protein and ~17,000 for denatured protein. The Glu + Asp/Lys + Arg molar ratio of this protein is 1.55. The apparent pI of the denatured depactin is ~6.

The extent of actin polymerization is reduced by the presence of depactin; however, the rate of polymerization seems to be accelerated as measured spectrophotometrically at 238_{nm}. This effect is interpreted to indicate that depactin cut the newly formed filaments into small fragments, thereby increasing the number of the filament ends to which monomers are added. The apparent critical concentration of actin for polymerization, as determined by viscometry or flow birefringence measurement, is increased by the presence of depactin in a concentration-dependent manner. Raising the pH of the solution does not reverse the action of depactin. The molar ratio of actin and depactin, which interact with each other, is estimated to be 1:1 by means of a cross-linking experiment using a water-soluble carbodiimide. Depactin binds to a DNase I-Sepharose column via actin and is selectively eluted with 0.6 M KCl or 0.6 M KI. The association constant between actin and depactin is estimated, using the column, to be $2-3 \times 10^6\ M^{-1}$.

The content of depactin in the high-speed supernatant of the oocyte extract is determined to be 1%; this can act upon ~63% of the actin in the supernatant.

Actin is a monomeric globular protein at a low ionic strength, but it polymerizes in a manner similar to crystallization or condensation of gas to form a filament upon addition of salts in vitro (47). However, recent studies have shown that several nonmuscle cells contain a large pool of monomeric actin that corresponds to 50% or more of the total actin in the cell (7, 14, 35, 42, 43, 63).

Since actin purified from a crude monomeric actin fraction was competent to polymerize normally, the poor polymerizability of the actin in the crude fraction has been attributed to the coexistence of protein factors that inhibit polymerization of actin (14, 28, 35). Actually, the existence of proteins that form a stoichiometric complex with G-actin or depolymerize F-actin or inhibit the final extent of polymerization of actin has recently been demonstrated. In some mammalian tissue cells, a large part of the monomeric actin is complexed with a low molecular weight protein called profilin (5, 11). This complex is stable and actin does not polymerize upon addition of salts. A similar protein has been obtained from *Acanthamoeba castellanii* (52), from sea urchin eggs (33), and from a slime mold, *Physarum polycephalum* (48). Another low molecular weight protein that depolymerizes actin has been found in brain (1). Brain also contains a 94,000-mol-wt protein that inhibits actin polymerization in a stoichiometric manner and also depolymerizes actin (42). Actin polymerization inhibitors of 60,000–70,000 mol wt that do not depolymerize F-actin have been isolated from human granulocytes (55) and from leukemic myeloblasts (44). In *Physarum* a 43,000-mol-wt protein called fragmin is also present as a complexed form with G-actin (17). Fragmin fragments F-

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actin in a Ca^{2+}-dependent manner. A similar protein (40,000 mol wt) has been isolated from *Dictyostelium discoideum* (59). A protein from intestinal epithelial cells, called villin, severs F-actin and binds two actin monomers in the presence of Ca^{2+}, although it bundles F-actin in the absence of Ca^{2+} (8, 12, 13). These proteins may be responsible for the presence of the large monomeric actin pool. A group of proteins that bind to the barbed end of the actin filament thereby affecting the polymerization of actin (25, 50) may be partly responsible for this.

I have purified a new protein from starfish oocytes that inhibits the extent of actin polymerization and rapidly depolymerizes F-actin in a stoichiometric manner; both of these processes have been demonstrated by viscometric measurements (30) or by the DNase I inhibition assay (32). This paper describes its chemical and physico-chemical properties, and provides a detailed study of the interaction of this protein with actin. This protein has been called depactin (31, 32, 34). Some of the results of this study have been published in preliminary form (29, 31, 34).

MATERIALS AND METHODS

Buffer Solutions: "F-buffer" is a buffer solution that normally favors actin polymerization. For the present study it consisted of 0.1 M KCl, 1 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithiothreitol, and 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)[1] · NaOH buffer (pH 7.0–7.4). "G-buffer" is one that favors actin depolymerization. It consisted of 1 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) · NaOH, 0.2 mM ATP, 0.2 mM dithiothreitol, 1 mM NaN_3, and 0.05 mM $MgCl_2$ (pH 7.2).

Purification of Depactin: Starfish, *Asterias amurensis*, were obtained from Tokyo Bay or Akkeshi Bay. Oocytes were obtained by the use of 1-methyladenine according to Kanatani (22). Before the second meiotic division, the oocytes were dejellied at pH 5.0, washed once with ordinary sea water, washed again with cold 0.5 M glycerol, 0.2 M NaCl, and 10 mM $NaHCO_3$, and then packed at 2,000 *g* for 10 min. Oocytes were extracted as described previously for preparation of sea urchin egg extracts (35), except that MOPS buffer was used instead of 2-[*N*-morpholino]ethane sulfonic acid buffer. The extracts were centrifuged at 190,000 *g* for 2 h.

The actin-depolymerizing activity was measured as follows during the purification. Samples solution was mixed with F-actin (final actin concentration, 7 μM) in F-buffer (pH 7.4) and incubated at 25°C for 30 min. Liberated G-actin was determined by DNase I inhibition assay (4) as described previously (35).

Depactin was purified by a modification of the previously reported method (30). The modified method was designed to be able to start with a larger amount of the extract and to obtain higher yield. I will describe only the modified points in detail.

A saturated ammonium sulfate containing 10 mM PIPES · NaOH buffer (pH 6.85) was added to the high-speed supernatant of the extract to attain 65% saturation. Precipitates which formed were discarded after centrifugation at 20,000 *g* for 20 min. Solid ammonium sulfate was added to the supernatant up to 90% saturation. All precipitates floated on the top of the solution after centrifugation; these were collected, dissolved, and dialyzed against a large volume of 10 mM Tris · HCl (pH 8.2), 0.2 mM dithiothreitol, 0.5 mM *p*-tosyl-L-arginine methylester · HCl (TAME) with three changes of the outer buffer solution. Purification was further performed using a DEAE-cellulose column (0–0.25 M linear NaCl gradient elution) and a hydroxylapatite column (10–180 mM linear potassium phosphate buffer elution) (30), except that a single run on each column was carried out. Depactin was finally purified with a Sephadex G-75 column equilibrated with 0.1 M KCl, 0.2 mM dithiothreitol, 0.5 mM TAME, and 10 mM MOPS · NaOH buffer (pH 7.0), concentrated using Aquacide II-A (Calbiochem-Behring Corp., San Diego, CA) and dialyzed against 1 mM TES · NaOH buffer, 0.2 mM dithiothreitol, 0.2 mM TAME, 0.1 mM EGTA (pH 7.0).

Preparation of Actin: Actin was prepared from rabbit skeletal muscle as described by Spudich and Watt (58). This actin preparation was called conventional actin. Since this actin preparation has been known to contain small amounts of impurities (37, 49), it was further purified by gel filtration (51) using a Sephadex G-150 column equilibrated with G-buffer. Only the trailing half of the G-actin peak was used. Polymerization was usually induced by addition of 1/20 vol of 2 M KCl, 20 mM $MgCl_2$, 200 mM MOPS · NaOH buffer (pH 6.85) at 25°C. When the time course of the polymerization was studied, it was induced by addition of 1/31 vol of a solution of 625 mM KCl and 125 mM TES buffer (pH 7.0). In some actin polymerization experiments, F-actin was sonicated (Ohtake sonicator, Ohtake Seisakusho Ltd., Tokyo) at 30 W for 10 s and added as nuclei to G-actin solutions within 1 min after sonication.

Assay for Actin Polymerization or Depolymerization: Actin polymerization was monitored by one of the following three methods. The first is measurement of the increase in the ultraviolet absorption (19, 57). The measurement was carried out using a Shimadzu UV-300 spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan) at 238 nm. It should be noted that this method can monitor the polymerization of actin in the complete absence of shearing force. The second method is viscometry using an Ostwald type viscometer of 0.3 ml-capacity and out-flow time with water for 55 s at 25°C. This method exerts a shearing force (maximum velocity gradient for water at 25°C: 1,026 s^{-1}) on the sample solution during measurement. The third method is monitoring the change in the G-actin concentration by the DNase I inhibition assay. For actin depolymerization, either of the latter two methods was used.

The extent of polymerization at an equilibrium state was determined by viscometry, DNase I inhibition assay, or a flow birefringence measurement using an apparatus under a polarizing microscope (18) at a velocity gradient of 598 s^{-1}.

Preparation of Actin Filament End-blocking Proteins: A one-to-one complex of a 45,000-mol-wt protein and G-actin, which has been known to block the barbed end of the actin filament, was prepared from a sea urchin (*Hemicentrotus pulcherrimus*) egg extract by means of DNase I-affinity chromatography followed by gel filtration column chromatography using a Sephadex G-200 column (Hosoya, H. and I. Mabuchi, manuscript in preparation; 34). β-actinin was prepared from chicken skeletal muscle by Dr. K. Maruyama according to the method of Maruyama et al. (41).

Gel Electrophoresis: Prior to electrophoresis, samples were diluted with 8 M urea, 0.5% SDS, 0.1 M β-mercaptoethanol, 10 mM EDTA, and 20 mM Tris · HCl (pH 8.5) and boiled for 3 min.

Electrophoresis was carried out in a 15 or 12% acrylamide slab gel in the presence of SDS according to Laemmli (23). The gels were stained with 0.025% Coomassie Brilliant Blue dissolved in 10% (vol/vol) acetic acid and 25% (vol/vol) isopropanol and scanned with a Shimadzu CS-910 dual wavelength chromatoscanner. The peak of actin or depactin on a chart paper was cut and weighed to estimate the content of the protein, as described previously (35).

Molecular Weight Determination: Molecular weight of a native protein was determined by gel filtration chromatography using a Sephadex G-75 (Pharmacia Fine Chemicals Div., Pharmacia Japan Co. Ltd. Tokyo, Japan) column (1.9 × 50 cm) equilibrated with 0.1 M KCl, 0.5 mM dithiothreitol, 0.5 mM TAME, and 10 mM MOPS buffer (pH 7.0). Marker proteins used were ovalbumin (43,000 mol wt), bovine pancreas DNase I (31,000 mol wt), bovine erythrocyte carbonic anhydrase (29,000 mol wt), soybean trypsin inhibitor (20,000 mol wt), horse heart myoglobin (17,000 mol wt), and horse heart cytochrome *c* (13,000 mol wt), all from Sigma Chemical Co. (St. Louis, MO). The void and column volumes were determined using Blue dextran 2,000 (Pharmacia Fine Chemicals) and β-mercaptoethanol, respectively. Rabbit muscle phosphorylase *a* (95,000 mol wt) and BSA (68,000 mol wt), both from Sigma Chemical Co., were used in addition to the above proteins in the determination of the molecular weight of denatured proteins on SDS gels.

Isoelectric Focusing: Isoelectric focusing of proteins was carried out in a polyacrylamide gel rod (0.2 × 12.3 cm) at 500 V for 7 h. The composition of the gel was 4.85% acrylamide, 0.15% *N,N'*-methylene *bis*-acrylamide, 6.3% Pharmalyte (pH range, 4–6.5 or 5–8, Pharmacia Fine Chemicals), 2% Nonidet P-40, and 9.5 M urea, which was a combination of the composition described by the company and by O'Farrell (46). For the anolyte (lower solution), 10 mM DL-glutamic acid was used. For the catholyte (upper solution), 10 mM imidazole for pH 4–6.5 analysis or 10 mM monoethanolamine for 5–8 analysis was used.

Protein samples were diluted 10-fold with a solution of 9.5 M urea, 2% Nonidet P-40, 6.3% Pharmalyte, 0.1 M β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. Diluted samples were first boiled for 3 min, applied on the gel, and overlaid with 6 M urea and 3% Pharmalyte.

Amino Acid Analysis: The protein sample was hydrolyzed in 6 N HCl and 110°C for 24 h, and amino acids were analyzed with a Hitachi amino acid analyzer model 835 (Hitachi Ltd., Tokyo, Japan).

Protein Determination: Protein concentration was determined by the method of Lowry et al. (27) using BSA as a standard. To indicate the

[1] *Abbreviations used in this paper:* EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TAME, *p*-tosyl-L-arginine methylester·HCL; and TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

protein concentrations in molarity, we assumed the purity of rabbit actin to be 100%, and determined that of depactin for each preparation by densitometry of the SDS gel. The molecular weight of denatured depactin was assumed to be 17,000 (30).

Electron Microscopy: Samples were negatively stained with 1% uranylacetate on carbon-coated Formvar grids and viewed with a Hitachi HS-9 or JEOL JEM 100CX electron microscope at an accelerating voltage of 75 or 80 KV, respectively. Length of actin filaments was measured on printed micrographs using a Numonics digitizer model 1250 (Numonics Corp., Landsdale, PA).

Analytical Ultracentrifugation: A Beckman model E ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) equipped with Schlieren optics was used for sedimentation velocity experiments. The sedimentation coefficient of F-actin was measured at 25,980 rpm while that of the actin-depactin complex was measured at 50,740 rpm. Temperature was kept constant at 14.6°C.

RESULTS

Purification of Depactin

The fractionation profile and electrophoretogram of the active fraction at each purification step are shown in Figs. 1 and 2, respectively. The Sephadex G-75 fraction, namely the purified depactin, consisted of a single major protein according to SDS gel electrophoresis. The purity of depactin was estimated from the densitometric scan of the SDS gel to be in the range of 90–98%. The ratio of absorbance at 280 nm to that at 260 nm was >1.5, indicating little contamination by nucleic acids. The present purification procedure for depactin allowed a yield of 2 mg from 100 ml packed eggs, which is twice that of the previous procedure.

The content of depactin in the high-speed supernatant was estimated by densitometry of the SDS gel (Fig. 1) to be 1.01 (average of three determinations; range: 0.98–1.14%), while that of actin was 3.66% (average of three determinations; range: 3.21–4.03%).

Physical and Chemical Properties of Depactin

The subunit molecular weight of depactin has been estimated from the SDS gel electrophoresis to be 17,000 (30). The molecular weight of native depactin was estimated using the Sephadex G-75 column to be about 20,000 (Fig. 1). Therefore, this protein may exist as a monomer under physiological conditions.

Upon isoelectric focusing, the purified depactin fraction showed a single main band with two minor bands on the acidic side (Fig. 2 *g*). The isoelectric point of the main band protein was estimated to be 5.7–5.9 with pH 4–6.5 Pharmalyte or 5.8–6.1 with pH 5–8 Pharmalyte.

The amino acid composition of depactin is shown in Table I. This protein may be a little acidic due to a Glu + Asp/Lys + Arg ratio of 1.55. This is consistent with the isoelectric point as determined above. For comparison, the amino acid composition of sea urchin egg profilin (33) is given. Significant differences in the content of Glu, Gly, Ala, Val, Met, Leu, and Lys were observed between the starfish oocyte depactin and sea urchin egg profilin.

Effect of Depactin on the Polymerization of Actin

Polymerization of gel-filtered rabbit skeletal actin was monitored in either the absence or presence of shearing force on the actin solution. The A_{238nm} of the gel-filtered actin increased after addition of 20 mM KCl to make a slightly sigmoidal curve (Fig. 3*a*). The nucleation and the elongation steps in the actin polymerization (47) are not clearly separated by this

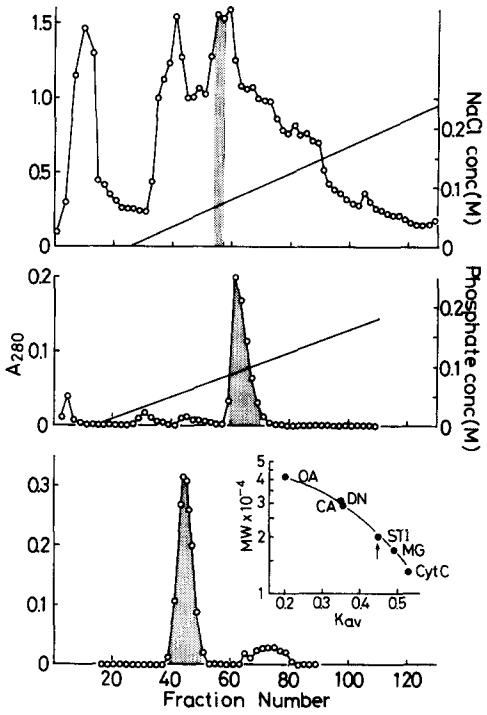

FIGURE 1 Fractionation of depactin by column chromatography. (*a*) DEAE-cellulose column chromatography. The 65–90% ammonium sulfate fraction (832 mg protein) was dialyzed against 10 mM Tris · HCl buffer (pH 8.2), 0.5 mM EGTA, 0.2 mM dithiothreitol, and 0.5 mM TAME and applied to a DE-52 (Whatman Laboratory Products, Inc., Clifton, NJ) column (2.5 × 30 cm). Proteins were eluted with 700 ml of a linear NaCl gradient of 0–0.25 M. (*b*) Hydroxylapatite column chromatography. The DE-52 fraction (15.6 mg protein) was dialyzed against 5 mM potassium phosphate buffer (pH 6.8), 0.5 mM dithiothreitol, and 0.5 mM TAME and applied to a hydroxylapatite (Biogel HTP, Bio-Rad Laboratories, Richmond, CA) column (1 × 5 cm). Proteins were eluted by 400 ml of a linear potassium phosphate buffer gradient of 5–180 mM. (*c*) Sephadex G-75 column chromatography. The hydroxylapatite fraction (5.6 mg protein) was dialyzed against 0.1 M KCl, 0.5 mM dithiothreitol, 0.5 mM TAME, and 10 mM MOPS buffer (pH 7.0) and applied to a Sephadex G-75 column (1.9 × 50 cm). (*Inset*) Molecular weight of the marker proteins are plotted against K_{av}. *OA*, ovalbumin (43,000 mol wt); *DN*, DNase I (31,000 mol wt); *CA*, carbonic anhydrase (29,000 mol wt); *STI*, soybean trypsin inhibitor (20,000 mol wt); *MG*, myoglobin (17,000 mol wt); and *CytC*, cytochrome c (13,000 mol wt). The arrow indicates the position of depactin. The shaded areas represent the active fractions saved for the next purification steps.

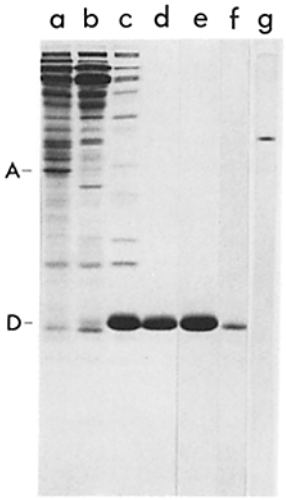

FIGURE 2 SDS gel electrophoresis and isoelectric focusing of depactin. (*a*) high-speed supernatant, 40 μg. (*b*) 65–90% ammonium sulfate fraction, 40 μg. (*c*) DE-52 fraction, 15 μg. (*d*) hydroxylapatite fraction, 6 μg. (*e*) Sephadex G-75 fraction, 6 μg. (*f*) Sephadex G-75 fraction, 1 μg. (*g*) isoelectric focusing of the Sephadex G-75 fraction, 1 μg. *A*, actin. *D*, depactin.

TABLE I

Amino Acid Composition of Depactin and Profilin

Amino acids	Starfish oocytes depactin			Sea urchin egg profilin‡
	Residues (17,000 *g*)	mole %*	SD	mole %
Asp	17	11.33	0.15	10.4
Thr	10	6.72	0.17	5.2
Ser	10–11	7.05	1.29	9.1
Glu	20	13.15	0.95	9.2
Gly	8	5.33	1.34	13.8
Ala	8–9	5.58	0.16	9.5
Pro	5–6	3.72	0.81	4.4
Cys	1	0.66	0.16	0.77
Val	11	7.28	0.62	4.7
Met	4	2.71	0.49	1.1
Ile	7	4.70	0.20	6.4
Leu	13	8.55	0.13	6.8
Tyr	4	2.83	0.33	2.6
Phe	5	3.50	0.14	3.6
Lys	17–18	11.63	0.69	7.4
His	3	2.17	0.56	3.1
Arg	5	3.12	0.20	2.0

* Average values from four determinations.

‡ From Mabuchi and Hosoya (33).

measurement. Actin supplemented with 50 μM $MgCl_2$ polymerized three to four times faster than the one supplemented with 0.1 mM $CaCl_2$ as stabilizing divalent cation. When depactin was present, the increase in the absorbance was similar to that in the control at first, but then it accelerated abruptly and terminated faster than that in the control, so that the sigmoidal shape of the time course was emphasized. With a larger amount of depactin, the extent of the acceleration was greater, and the total absorbance change was smaller although the onset of the abrupt increase in the absorbance did not seem to be affected significantly (Fig. 3*a*). Sonicated F-actin was used as nuclei to see the effect of depactin on the elongation of actin filaments. Materials were mixed with G-actin in one of the following two sequences: (*a*) 20 mM KCl, depactin, and F-actin fragments; or (*b*) KCl, F-actin fragments, and then depactin immediately. In both cases, the presence of depactin did not show a significant effect on the rate of the nucleated actin polymerization (Fig. 3*b*), although the final extent of the polymerization was as low as that without added nuclei. This result suggests that depactin has only a small effect, if any, on the initial elongation step of the actin polymerization. There was no difference in the rate of the nucleated actin polymerization when the ratio of depactin to actin was changed (0.009, 0.05, and 0.29) (not shown). Since in some of the above experiments the F-actin fragments were added to the mixture and mixed with a pasteur pipette ~1 min after the addition of depactin, the effect of pipetting on the actin polymerization was checked. The actin solution supplied with KCl but not with the nuclei or depactin was pipetted three times in a cuvette at various times after addition of KCl. No effect was observed after 1 min, although rapid increase in A_{238nm} was observed after 3 min (not shown), which confirmed the accelerating effect of the shearing force on the polymerization of actin (6, 40).

The polymerization of the same gel-filtered actin was also monitored under the same salt conditions by viscometry, using an Ostwald-type viscometer that exerts a shearing force

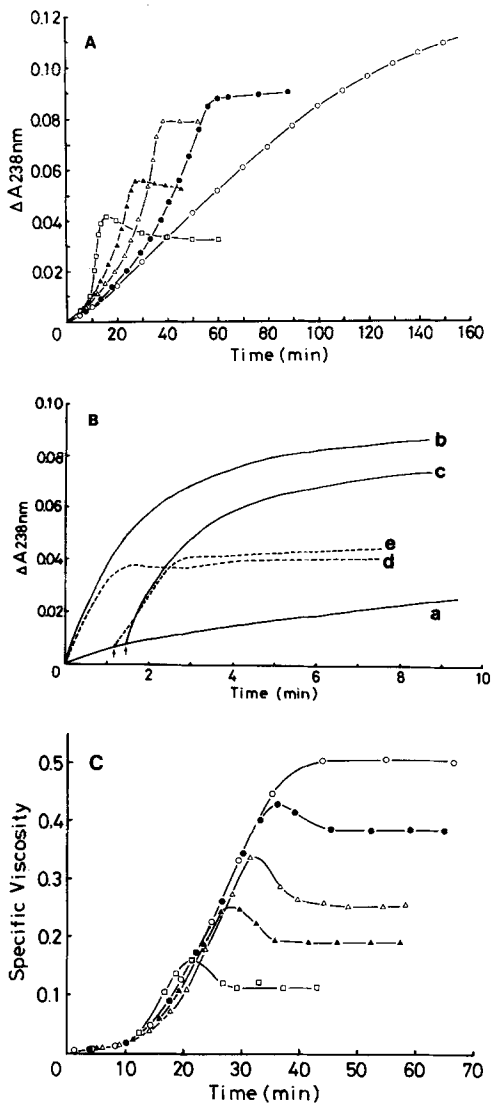

FIGURE 3 (*A*) Polymerization of actin measured by $A_{238\ nm}$. Gel-filtered G-actin (13.7 μM) in G-buffer was polymerized by the addition of 20 mM KCl in the absence (○) or presence (●, 2.0 μM; △, 3.0 μM; ▲, 4.0 μM; □, 5.9 μM) of depactin. (*B*) Polymerization of actin from added nuclei measured by ΔA_{238nm}. Effects of the addition of nuclei (final 1.0 μM sonicated F-actin) and of depactin (3.6 μM) on the 20 mM KCl-induced polymerization of the gel-filtered actin (10.8 μM) are shown. Materials were added to the G-actin solution in the following sequence: *a*, KCl; *b*, KCl and F-actin; *c*, KCl and F-actin (at the time indicated by the arrow); *d*, KCl, F-actin and then depactin immediately; and e, KCl, depactin, and then F-actin (at the time indicated by the arrow). (C) Polymerization of actin measured by viscometry. The same gel-filtered G-actin (13.6 μM) as in *A* was polymerized in an Ostwald-type viscometer by the addition of 20 mM KCl in the absence (○) or presence (●, 1.5 μM; △, 3.0 μM; ▲, 4.1 μM; □, 6.5 μM) of depactin and the change in the viscosity was measured.

on the sample solution. As shown in Fig. 3*c*, the control actin polymerized typically to fit the polymerization theory of actin of Oosawa's school (47)—that is, there was a lag of ~10 min, which is considered to represent a nucleation step, and then an abrupt increase in the viscosity, which is regarded as the elongation step. In the presence of depactin, the lag period did not seem to be affected; nor did the rate of elongation, although a little acceleration was observed with the highest depactin concentration. It should be noted that the polymer-

ization terminated in a shorter time than that measured by the increase in the UV-light absorbance. This again indicated that the shearing force accelerated the polymerization rate.

F-actin formed in the presence of depactin at an actin/depactin molar ratio of 1:0.8 was examined under an electron microscope. We observed F-actin filaments significantly shorter than the control actin (Fig. 4).

Effect of Depactin on F-actin

It has been shown that depactin depolymerizes actin quickly both by viscometric measurement (30) and by the DNase I inhibition assay (32). However, it is not yet known whether the depolymerization takes place from the ends of the actin filament or from the arbitral points on the filament. Therefore, we investigated the effect of depactin on actin filaments whose ends have been blocked by end-blocking proteins. As shown in Fig. 5, the rate of the actin depolymerization in the presence of both β-actinin and the 45,000-mol-wt protein-actin complex, which have been known to block the pointed (41) and the barbed ends, respectively, of the actin filament (34; H. Hosoya and I. Mabuchi, manuscript in preparation), did not change from that in the absence of the actin filament end-blocking proteins. The use of 5 μM cytochalasin B, which has been shown to block the barbed end (9, 24, 38), instead of the 45,000-mol-wt protein-actin complex gave almost the same result (not shown).

The effect of pH on the depolymerization of actin by depactin was studied. The G-actin concentration in the presence of depactin, as measured by the DNase I inhibition assay, was a little lower below pH 7.0 than above that level. The best depolymerization was observed at pH 8.5. On the other hand, the G-actin concentration in the absence of depactin (the critical concentration for polymerization) did not change significantly between pH 6 and 8.5 (Fig. 6).

To confirm the actin-depolymerizing activity of depactin by other criteria, we examined the mixture of F-actin and depactin by analytical ultracentrifugation, gel filtration chro-

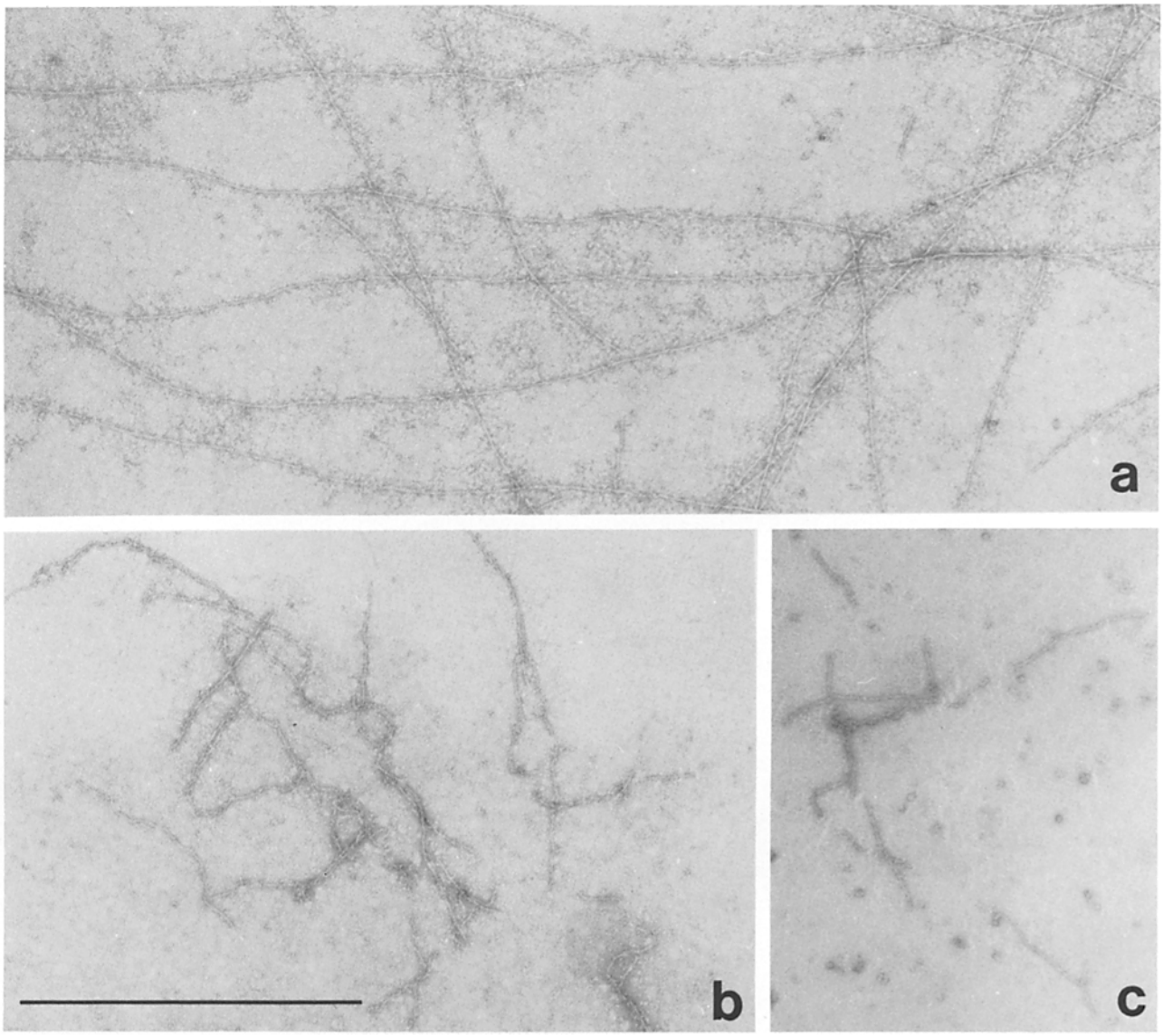

FIGURE 4 Electron micrographs of F-actin. (*a*) F-actin polymerized from gel-filtered G-actin (9.4 μM) by the addition of 0.1 M KCl and 1 mM $MgCl_2$. (*b*) Gel-filtered actin (7.0 μM) polymerized in the presence of 5.6 μM depactin. (*c*) Depactin (4.8 μM) added to the F-actin solution (9.4 μM). This specimen was prepared 20 min after addition of depactin. Bar, 1 μm. × 50,000.

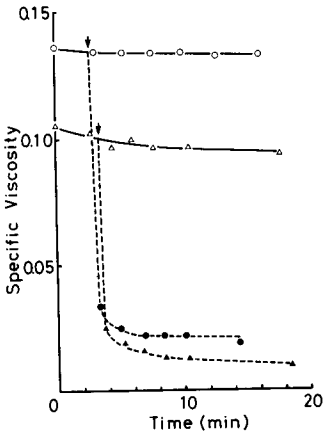

FIGURE 5 Effect of depactin on the viscosity of end-blocked actin filaments. Depactin (6.4 μM) was added either to F-actin (7.1 μM, circles) polymerized from gel-filtered actin in 75 mM KCl, 1 mM $MgCl_2$, and 10 mM MOPS buffer (pH 7.4) or to F-actin (7.1 μM, triangles) preincubated at 17°C for 1 h with 2.9 μg/ml β-actinin and 10 μg/ml 45,000-mol-wt protein-actin complex at the point indicated by the arrow. Viscosity of the solution was measured at 17°C. O, Δ: in the absence of depactin. ●, ▲: in the presence of depactin.

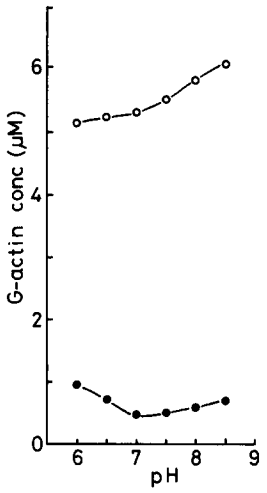

FIGURE 6 Effect of pH on the action of depactin. G-actin concentration in conventional actin solution (5.9 μM) in 0.1 M KCl and 1 mM $MgCl_2$ in the absence (●) or presence (6.5 μM, O) of depactin at various pH was estimated by the DNase I inhibition assay. pH 6–6.5, 25 mM 2-[N-morpholino]-ethanesulfonic acid buffer; pH 7–7.5, 25 mM MOPS buffer, pH 7.5–8, 25 mM TES buffer; and pH 8–8.5, 25 mM Tris buffer. Average values of four to six determinations are plotted.

matography, and electron microscopy. When the conventional F-actin (0.79 mg/ml) in F-buffer (pH 7.4) was centrifuged, one hypersharp sedimentation boundary of 47S, which represented F-actin boundary, appeared with a small polydisperse 8.2S boundary (Fig. 7). This 8.2S boundary was attributed to impurities in the conventional actin since such a boundary was not detected in the gel-filtered actin preparation. On the other hand, a mixture of this F-actin and the equimolar amount of depactin gave a small hypersharp F-actin boundary sedimenting at 78S and a slow sedimenting boundary of 4.2S, the latter of which was attributable to G-actin or actin-depactin complex. Note that we cannot estimate the particle size of F-actin by this experiment alone since sedimentation of F-actin shows very strong concentration dependency: we did not check the effect of protein concentration on the sedimentation velocity of F-actin.

A mixture of F-actin (21.3 μM) and depactin (21.2 μM) was applied to a Sephadex G-150 column (1.1 × 32 cm) that had been pre-equilibrated with F-buffer (pH 7.0). Co-elution of actin and depactin took place at the position where G-actin eluted, indicating that depactin depolymerized F-actin (not shown).

By a negative staining technique, generally short and often crooked actin filaments were observed with 9.4 μM actin plus 4.8 μM depactin (Fig. 4). The length of these filaments, however, was not uniform as it was in the case of fragmin-(17) or villin-induced shortening of the actin filaments (8, 12). Summation of the length of all the filaments observed in a unit area on a grid was carried out to quantify the effect of depactin. For 4.8 μM actin, it was 30.0 ± 4.0 μm/10 μm^2 (mean ± SD, n = 13). Upon addition of 3 μM depactin it became 4.2 ± 3.9 μm/10 μm^2 (n = 20).

Critical Concentration of Actin in the Presence of Depactin

The effect of depactin on the critical concentration for polymerization of actin was studied by means of flow birefringence measurement, viscometry, and DNase I inhibition assay. Mixtures of actin and depactin in F-buffer at pH 7.4 were kept standing for 1 d at 4°C and 1 d at 25°C prior to the measurements. The flow birefringence or viscosity of actin was extrapolated to an infinite dilution to obtain the critical concentration. In the experiment shown in Fig. 8, it was 0.6

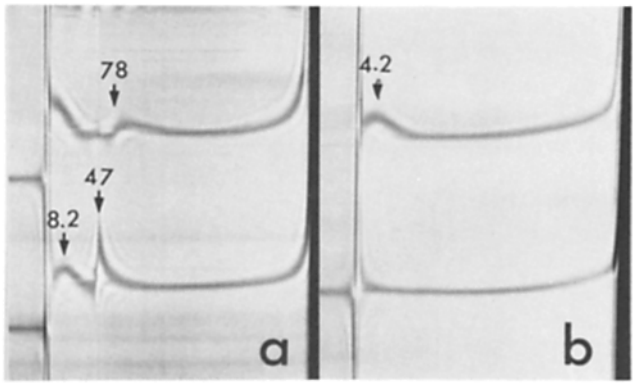

FIGURE 7 Sedimentation analysis of an actin-depactin mixture. Conventional F-actin (18.8 μM) in the absence (*lower profiles*) or presence (*upper profiles*) of depactin (17.7 μM) in F-buffer (pH 7.3) was centrifuged in an analytical ultracentrifuge at 14.6°C. (*a*) Taken at 8 min after reaching 25,980 rpm. (*b*) Taken at 7 min after reaching 50,740 rpm (after centrifugation for 14 min at 25,980 rpm). The sedimentation coefficient of each boundary is indicated.

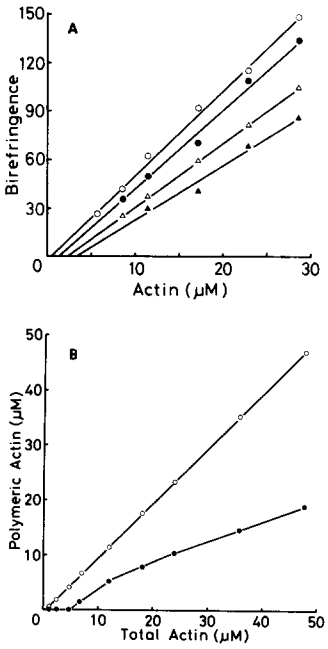

FIGURE 8 (*A*) Steady state birefringence of F-actin. Gel-filtered actin (5.7–28.5 μM) was polymerized in the absence or presence of depactin by the addition of 0.1 M KCl, 1 mM $MgCl_2$, and 10 mM MOPS buffer (pH 7.0) at 4°C for one day and at 25°C for another day, and then flow birefringence was measured. Molar ratios of depactin to actin are: O, 0; ●, 0.1; Δ, 0.2; ▲, 0.4. (*B*) Polymeric actin concentration in the actin-depactin mixture. Gel-filtered actin (1.2–47.6 μM) in the presence of the equimolar amount of depactin was polymerized as described above and the G-actin concentration was measured by the DNase I inhibition assay. Polymeric actin concentration (total actin concentration minus monomeric actin concentration) is plotted. O, in the absence of depactin. ●, in the presence of depactin.

μM in the absence of depactin. In the presence of depactin at a fixed depactin/actin molar ratio of 0.1, 0.2, or 0.4, the apparent critical concentration increased to 1.4, 2.5, or 3.5 μM, respectively (Fig. 8). A similar result was obtained by viscometry using an Ostwald-type viscometer (not shown). In both cases the slope of the lines in the presence of depactin became smaller, being dependent on the depactin/actin ratio. This may partly be because filaments were short in the presence of depactin. It also suggests that the G-actin concentration was not constant but might have increased with an increase in the depactin concentration. In other words, there might be an equilibrium between actin·depactin complex and actin and depactin. This was confirmed by the DNase I inhibition assay as will be described below.

A one-to-one mixture of actin and depactin in F-buffer was diluted and the monomeric actin concentration was determined by the DNase I inhibition assay. In Fig. 8, polymeric actin concentration is plotted against the total actin concentration for the sake of convenience. All the actin was in a monomeric form up to 4.8 μM actin (0.2 mg actin/ml), which may be considered as the critical concentration. Beyond this concentration, polymeric actin appeared and increased as the total actin concentration increased. However, the slope of the curve was significantly more gradual than that in the absence of depactin. The polymerizability of actin in the presence of depactin was very similar to that of the crude "monomeric actin fraction" of sea urchin egg reported previously (Fig. 3*b* in reference 35).

Binding of Depactin to Actin

A one-to-one mixture of F-actin and depactin was incubated at 25°C for 1 h and then applied to a DNase I-bound Sepharose 4B (Worthington Biochemical Corp., Freehold, NJ) column (26). After collection of the flow-through fraction, the proteins were eluted successively with 0.6 M KI and 3 M guanidine · HCl (Fig. 9). Almost all actin and a major part of depactin adsorbed to the column. The adsorbed depactin was eluted with 0.6 M KI, while actin was eluted with 3 M guanidine·HCl. KI could be replaced by KCl. A wash with 0.75 M guanidine · HCl prior to the elution with 3 M guanidine · HCl did not elute any detectable protein. When depactin alone was applied to the column, it did not adsorb to the column at all. Therefore, it was concluded that depactin bound to the column through its binding to actin. If we assume a very simplified model in which (*a*) the binding of a depactin molecule to an actin molecule in the F-actin causes the dropping out of the actin molecule as an actin-depactin complex and (*b*) depactin has the same affinity to the monomeric actin and actin molecules in the filament, the association constant between actin and depactin would be expressed as K_a = [actin·depactin complex]/[actin]·[depactin], which is [depactin]bound/[depactin]^{2}free for a one-to-one mixture system of actin and depactin. Therefore, if the concentrations of actin and depactin and the amount of depactin both in the flow-through fraction and in the KI eluates are known, the K_a of depactin and actin can be calculated. One experiment with the actin and depactin concentrations of 10 μM each gave the K_a of 2.8×10^6 M^{-1} (Fig. 9*I*). A similar K_a (2.3×10^6 M^{-1}) was obtained with the mixture made by the addition of depactin to a dilute actin solution (0.66 μM) of near the critical concentration for polymerization in F-buffer at a one-to-one ratio (Fig. 9*II*).

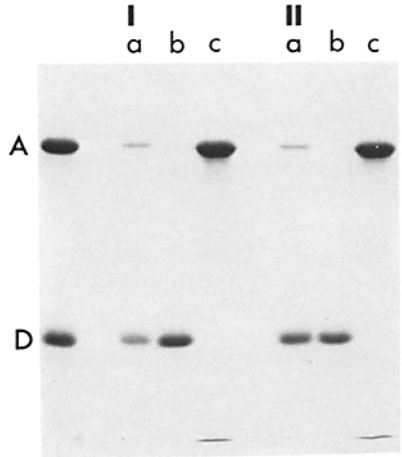

FIGURE 9 Binding of actin-depactin complex to the DNase I-Sepharose column. A mixture (0.5 ml) of F-actin polymerized from gel-filtered G-actin (final 10.0 μM) and depactin (final 10.0 μM) in F-buffer, pH 7.0 (*I*) or a mixture (7.5 ml) of F-ATP-monomer (0.66 μM) and depactin (0.66 μM) in F-buffer (*II*) was applied to a DNase I-bound Sepharose (Worthington Biochemical Corp.) column (0.2 ml) after incubation at 25°C for 1 h. The column was previously washed with 3 M guanidine · HCl, 10 mM MOPS buffer (pH 7.0), 0.2 mM ATP, 0.5 mM dithiothreitol and equilibrated with F-buffer. After obtaining the flow-through fraction (*a*), bound proteins were eluted successively with 0.6 M KI, 10 mM MOPS buffer, 0.2 mM ATP, and 5 mM dithiothreitol (*b*), and 3 M guanidine · HCl, 10 mM MOPS buffer, 0.2 mM ATP, and 0.5 mM dithiothreitol (*c*). Eluted proteins were dialyzed against H_2O containing 0.1 mM phenylmethylsulfonyl fluoride, freeze-dried, and electrophoresed as described in Materials and Methods. *A*, actin. *D*, depactin.

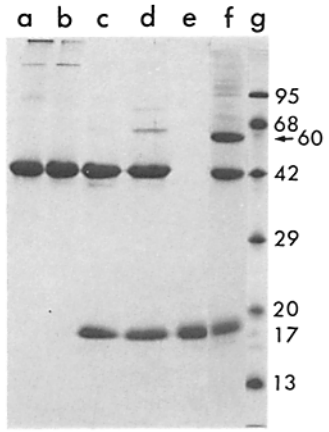

FIGURE 10 Cross-linking of actin and depactin. Proteins (14.1 μM gel-filtered actin and/or 16.2 μM depactin) were incubated in the presence of 15 mM EDC at 20°C for 2 h in F-buffer containing 50 mM MOPS buffer (pH 7.5) or in G-buffer, and then electrophoresed as described in Materials and Methods. *a*, actin alone in F-buffer; *b*, actin alone in G-buffer; *c*, actin plus depactin in F-buffer containing 0.6 M KI; *d*, actin plus depactin in F-buffer containing 1 M KCl; *e*, depactin alone in F-buffer; and *f*, actin plus depactin in F-buffer; *g*, marker proteins ($\times 10^{-3}$): phosphorylase *a* (95,000 mol wt); BSA (68,000 mol wt); actin (42,000 mol wt); carbonic anhydrase (29,000 mol wt); soybean trypsin inhibitor (20,000 mol wt); and cytochrome *c* (13,000 mol wt).

The binding of depactin to actin was alternatively studied by the use of a zero-length cross-linking reagent, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC). In 0.1 M KCl, 1 mM $MgCl_2$ and 50 mM MOPS buffer at pH 7.5, a new component of 60,000 mol wt appeared in the mixture of actin and depactin incubated in the presence of 15 mM EDC for 2 hr at 20°C, while such a component was very scarce in 1 M KCl and was hardly detected in 0.6 M KI (Fig. 10). This indicates that the two proteins are in contact in 0.1 M KCl at a molar ratio of 1, but are separated in 0.6 M KI or 1 M KCl. In addition, no significant cross-linking between actin molecules was observed in either G- or F-actin incubated in EDC. A similar result was obtained with 20 mM dimethylsuberimidate at pH 8.5, although the cross-linking efficiency was low compared to EDC (not shown).

DISCUSSION

Depactin rapidly reduces the viscosity of F-actin solution (30) and increases the monomeric actin concentration as measured by the DNase I inhibition assay (32). The depolymerization of actin was further confirmed by a sedimentation velocity measurement using an analytical ultracentrifuge, gel filtration chromatography, and electron microscopy. It was also confirmed by the fact that actin could not activate the heavy meromyosin ATPase activity in the presence of depactin (32). The interaction of these proteins was directly demonstrated to be one-to-one by a cross-linking experiment. Moreover, it was indicated that depactin changes the monomer-polymer equilibrium (30). These results lead us to speculate that depactin binds to any actin molecule in the F-actin and depolymerizes it by forming a one-to-one complex, or that it actively takes actin molecules away from one or both ends of the filament. The former seems to be more plausible since blocking of both ends to the actin filament by end-blocking proteins did not interfere with the depolymerization of actin by depactin.

From Fig. 3, it seems that the onset of the increase in both A_{238nm} and viscosity did not change significantly when the ratio of depactin to actin was increased. This suggests that the time required for the nucleation was not affected by depactin. On the other hand, the rate of the increase in A_{238nm} was enhanced by depactin. This could be due to the fact that depactin enhanced the polymerization rate by promoting the nucleation of actin, since the elongation of actin filaments from the added nuclei (Fig. 3 *b*) was not enhanced by depactin. This enhancement in rate was not seen by viscometry, although the final extent of polymerization was seen to be lower by both techniques. These results seem to be inconsistent. However, if we take into consideration that the viscometry using the Ostwald viscometer exerts a shearing force on the sample solution, which triggers actin polymerization (6, 40) several minutes after addition of KCl (this report), we have a reasonable explanation for the action of depactin on the actin polymerization: depactin may not influence the nuclei formation, which involves interaction of three to four G-actin molecules (47), but it increases the number of polymerization ends of the actin filaments by cutting the newly formed filaments into small fragments. The cut may occur as a result of binding of depactin to actin molecules in the filament and taking them away as actin-depactin complexes as discussed above. The shearing force exerted in the capillary viscometer may cut the newly formed filaments irrespective of the function of depactin, so that we may not be able to distinguish the effects of the force and of depactin by viscometry.

There is another way by which a factor promotes the nucleation of actin polymerization: the factor-actin complex itself becomes the nucleus, or the factor stabilizes the nucleus. This may result in the disappearance or shortening of the lag period because of the enhancement of the nuclei formation. Moreover, inhibition of the nucleated actin polymerization may occur at a certain concentration of the factor because of the blockade of one end of the actin filament. Therefore, the polymerization time course would change to hyperbolic from sigmoidal. A typical example of this type of modulation is found in the actin of a capping protein from *Acanthamoeba* (21) or of villin from intestinal epithelial cells (12). However, the time course of actin polymerization as modified by depactin was completely different, as discussed above; that is, the lag period did not seem to shorten and the rate of elongation of actin from the added nuclei was not inhibited by depactin at any concentrations. Therefore, this explanation is unlikely.

The critical actin concentration for polymerization was higher in the presence of depactin than in its absence and increased with an increase in the depactin/actin ratio. In a similar experiment, the critical actin concentration in the presence of very low concentrations of fragmin from *Physarum* plasmodium did not seem to change from that of the control under favorable conditions for polymerization (2 mM $MgCl_2$), which may be close to the present conditions (0.1 M KCl plus 1 mM $MgCl_2$), while it converged at a certain value (0.2 mg/ml) under less favorable conditions (0.3 mM $MgCl_2$), a value which is believed to be the critical concentration at the pointed end. This is considered one of the indications that fragmin binds to the barbed end of the actin filament (60). The present result with depactin indicates that depactin increased the monomeric actin concentration by forming a complex with actin rather than by binding to a low critical concentration end of the actin filament. This is consistent with the above discussion on the polymerization of actin. The binding of depactin to the actin monomers was actually demonstrated either by the chemical cross-linking experiment or by the DNase I-affinity chromatography.

There has been one strange observation among the effects of depactin on actin. Namely, the extent of viscosity drop of F-actin solution was not so great when depactin was added at a molar ratio to actin of 0.1–2 (30). On the other hand, a very low viscosity value might be expected for filaments randomly cut by such an amount of depactin, since the viscosity is very sensitive to the length of the filaments. This discrepancy may be explained if one supposes a cooperative depolymerization in which filaments once attacked by depactin became more susceptible to further attack by another depactin molecule than filaments that have not been attacked. This idea might be supported by the electron microscopic observations that the filaments after addition of depactin were generally short but not uniform.

The association constant of depactin to actin was estimated using a DNase I column. The K_a of depactin to actin was calculated to be $2–3 \times 10^6$ M^{-1}. Depactin also bound to monomeric actin in F-buffer as well as to actin molecules in the filament, which is called F-ATP-monomer (53), and is reported to be distinct from the G-actin in G-buffer. This value is similar to the one obtained by a competition experiment on actin molecule with heavy meromyosin (4.5×10^6 M^{-1}, reference 32) using the K_a of heavy meromyosin to actin to be 3×10^9 M^{-1} (15).

In conclusion, depactin accelerates the polymerization of actin, probably by cutting the newly formed filament to increase the number of the ends of the filament to which monomeric actin adds. It may depolymerize F-actin primarily by binding to actin molecules in the filament and taking them away from the filament by making a one-to-one complex. It is also capable of binding to free monomeric actin. There are several known proteins that are reported to be able to depolymerize actin: porcine brain modulator (45); actin-depolymerizing factors from plasma (16) and brain (1), which are distinct from each other; Gc-globulin from human serum (64); bovine pancreatic DNase I (20, 39); and profilin from *Acanthamoeba* (61, 62) from sea urchin eggs (33) or from *Physarum* (48). These proteins, except for the plasma and

brain actin-depolymerizing factors, may bind preferentially to free G-actin and may depolymerize F-actin by inactivation of the G-actin pool. On the other hand, the plasma and brain factors depolymerize actin quickly, which is similar to the action of depactin. The brain factor has a subunit molecular weight of 19,000, which is also similar to depactin. On the other hand, there is a significant difference between the brain factor and depactin; that is, tropomyosin has been reported to protect F-actin from the brain factor (3), although it did not protect F-actin from depactin (32). However, it is premature to discuss further the properties of these proteins since those of the plasma and brain factors have not been reported yet.

From the contents of depactin and actin in the high-speed supernatant estimated by densitometry of the SDS gel, the molar ratio of these proteins in the supernatant is 0.68:1. Taking the association constant of 2×10^6 M^{-1} into consideration, ~63% of the actin in the high-speed supernatant may be complexed with depactin.

In the case of sea urchin eggs, it has been shown that actin polymerization occurs shortly after fertilization to form microvillar actin bundles and networks underneath the plasma membrane (2, 10, 36, 56). It has been speculated that the polymerization is induced by raising the intracellular pH upon fertilization (2). These in vivo polymerization phenomena have not yet been demonstrated in starfish oocytes. Schroeder (54) has recently reported that spike-like projections emerge transiently on the surface of 1-methyladenine-treated starfish oocytes. These projections contain bundles of actin-like filaments as cores. It may be necessary to inactivate depactin to release actin from the actin-depactin complex and allow it polymerize in order to form these structures. In vitro experiments with purified proteins (present report) or with a crude sea urchin egg extract (35) showed that the action of depactin was not reversed by raising the pH. On the other hand, it has been shown that myosin or heavy meromyosin from skeletal muscle reversed the inhibition of depactin on actin in vitro (32). The natural direct regulator of the actin-depactin interaction may be another protein.

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