

A Re-examination of the Interaction of Vinculin with Actin

James A. Wilkins and Shin Lin

Department of Biophysics, The Johns Hopkins University, Baltimore, Maryland 21218

Abstract. Vinculin prepared by published procedures (i.e., Feramisco, J. R., and K. Burridge, 1980, *J. Biol. Chem.*, 255:1194–1199) contains contaminants that have been shown by Evans et al. (Evans, R. R., R. M. Robson, and M. H. Stromer, 1984, *J. Biol. Chem.*, 259:3916–3924) to reduce the low-shear viscosity of F-actin solutions. In this study we separated contaminants from conventional vinculin preparations by hydroxylapatite chromatography. We found that although the contaminants represented a small fraction ($\leq 5\%$) of the total protein in the conventional vinculin preparations, they were responsible for practically all of the filament capping and bundling activities previously attributed to vinculin. In addition, we exam-

ined the size of the molecule(s) responsible for the observed capping activity and found that its apparent molecular weight under denaturing conditions in sodium dodecyl sulfate (SDS) polyacrylamide gels fell within a broad range of 23,000–33,000. These results contrast with the observation that under nondenaturing conditions, the activity migrated in gel filtration columns at a position that corresponded to the Stoke's radius of a much bigger molecule. Since the migration of the activity in these chromatographic experiments is independent of the presence of vinculin, it is unlikely that the active protein associates with vinculin with high affinity under the conditions examined.

IN recent years, much attention has been focused on the protein vinculin, a 130-kD protein originally shown by Geiger to be localized at specialized sites on cellular membranes known as adhesion plaques (7). Based on its location at these areas where actin filament bundles appear to insert into the plasma membrane, he proposed that vinculin might link actin filaments to the membrane (7). This idea found support in subsequent studies which showed that vinculin preparations obtained according to published procedures affect assembly of actin filaments and lower the viscosity of F-actin measured by low-shear viscometric techniques (3, 8–10, 19), as well as cause bundling of actin filaments in vitro (8–10, 19). Recently, however, Evans et al. (5) reported that smooth muscle vinculin purified by a more extensive procedure does not seem to interact with F-actin as judged by its ability to reduce the low-shear viscosity of F-actin solutions. Instead, they found that a fraction derived from the vinculin preparations used in earlier studies contains potent activity in this assay. In this report we show that vinculin purified by steps of DEAE and hydroxylapatite chromatography has only a small effect on actin polymerization and the low-shear viscosity of actin solutions and no effect on the critical concentration of actin. Moreover, we demonstrate that the contaminants present in conventional vinculin preparations can adequately account for most of the actin-related activity previously attributed to vinculin (3, 8–10, 19). While this manuscript was in preparation, Rosenfeld et al. reported that vinculin purified from human platelets has little if any effect on the low-shear viscosity of actin solutions or on actin assembly (16).

Materials and Methods

Protein Preparations

Actin was prepared from acetone powder of rabbit skeletal muscle (17). For some experiments, G-actin was further purified on a 2.5×90 -cm column of Sephacryl S-200 equilibrated in 5 mM Tris-HCl, 0.2 mM CaCl_2 , 0.5 mM 2-mercaptoethanol, 0.2 mM ATP, pH 8.0 (buffer A).¹ The latter preparation was referred to as gel-filtered actin.

Vinculin was prepared by the method of Feramisco and Burridge (6) starting with 80 g of muscle from frozen chicken gizzards. Gizzards used in the preparations were obtained from a local poultry slaughterhouse, cleaned, and stored frozen at -70°C . The 37°C extract obtained from the membranes was fractionated with $(\text{NH}_4)_2\text{SO}_4$ as described (6). Ammonium sulfate precipitates from the 25–35% cut were suspended in and dialyzed against buffer B (20 mM Tris-acetate, 20 mM NaCl, 15 mM 2-mercaptoethanol, 0.1 mM EDTA, pH 7.6). After dialysis, the protein was loaded onto a 2.5×18 -cm column of Whatman DE52, DEAE cellulose (Whatman Inc., Clifton, NJ) equilibrated in buffer B. Fractions enriched in vinculin were pooled and dialyzed overnight against buffer B followed by concentration in an Amicon ultrafiltration apparatus (Amicon Corp., Danvers, MA) using a YM10 membrane. The final concentration of the vinculin ranged between 0.6 and 1.2 mg/ml. This preparation was referred to as DEAE-vinculin.

DEAE-vinculin was further purified by hydroxylapatite chromatography in the following way. 2 g of hydroxylapatite (Bio-Rad HTP, Bio-Rad Laboratories, Richmond, CA) was suspended in ~ 25 ml of a buffer that contained 20 mM NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.6 (HA buffer). The slurry was poured into a 1×10 -cm glass column, and the hydroxylapatite was allowed to settle. The column was then equilibrated with HA buffer by passing 10–15 column volumes of the buffer through the column. 5–6 mg of DEAE-vinculin in buffer B was then loaded onto the

¹ Abbreviations used in this paper: buffer A, a solution of 5 mM Tris-HCl, 0.2 mM CaCl_2 , 0.5 mM 2-mercaptoethanol, 0.2 mM ATP, pH 8.0; buffer B, a solution of 20 mM Tris-acetate, 20 mM NaCl, 15 mM 2-mercaptoethanol, 0.1 mM EDTA, pH 7.6; HA buffer, a solution of 20 mM NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.6.

column at which time fraction collection was begun. This was followed by washing of the column with 6–7 column volumes of HA buffer. The column was then eluted with a 60-ml linear gradient going from HA buffer to the same buffer that contained 0.3 M sodium phosphate (see Fig. 1).

Spectrin–band 4.1–actin complex was extracted from human erythrocytes at 37°C as previously described (12) and was used without further purification.

Gel Filtration Chromatography

A 1.0 × 50-cm column of Sephacryl S-200 (Pharmacia, Inc., Piscataway, NJ) equilibrated in buffer B was used to determine the relative Stoke's radius of DEAE-vinculin, HA-vinculin, and the active factor(s) present in DEAE-vinculin and in HA1. A 0.5-ml sample that contained 400 µg of DEAE-vinculin, 400 µg of HA-vinculin, or 30 µg of HA1 was loaded onto the column, and the proteins were eluted at a flow rate of 10 ml/h. The protein content of the 0.5-ml fractions was measured as described below. The activity of 0.1-ml aliquots of the fractions was measured by low-shear viscometry as described below.

Elution of Proteins from SDS Polyacrylamide Gels

15% SDS polyacrylamide gels were prepared and run according to the procedure of Laemmli (11). After electrophoresis, lanes that contained the HA1 or HA-vinculin were cut out and divided into 1-cm slices. The slices were placed in separate dialysis bags that contained 1 ml of 20 mM Tris, 150 mM glycine, pH 8.3. The bags were then placed in a Hoefer model TE-42 electrophoretic transfer unit (Hoefer Scientific Instruments, San Francisco, CA) that contained 20 mM Tris, 150 mM glycine, pH 8.3, and proteins were eluted from the gel slices for 4 h at an initial current of 0.75 A at room temperature. The bags were removed from the unit and were allowed to dialyze for 12 h against 2 liters of buffer B at 4°C. After dialysis, aliquots of the content in the dialysis bags were tested for their ability to lower the low-shear viscosity of F-actin as measured by falling ball viscometry.

Light Scattering Assay for Formation of Actin Filament Bundles

Light scattering intensity of actin solutions at an angle of 90° was measured in a Perkin-Elmer 650-10S recording spectrofluorometer (Perkin-Elmer Corp., Pomona, CA) with the excitation and emission wavelengths set at 400 nm and slits set at 2 nm. Gel-filtered actin was polymerized at a concentration of 100 µg/ml for 1 h in 0.75 ml of buffer A by adding MgCl₂ to a final concentration of 2 mM. The light scattering intensity of this solution was normalized to 0 units. An increase of one unit in scattering in Fig. 5 represents a 20-fold increase over the light scattering of F-actin alone in these assays. Aliquots of samples to be tested for bundle-inducing activity were then added to this solution, and the resultant light scattering intensity values were expressed as arbitrary units above baseline. In the absence of F-actin, none of the samples scattered light to a measurable degree under the conditions of the assay.

Detection of Actin Filament Bundle Formation by Electron Microscopy

10-µl aliquots of samples to be examined were placed on carbon-coated, glow-discharged copper-rhodium grids and allowed to stand for 1 min. Excess sample was blotted from the grid and replaced with 10 µl of a 1% aqueous solution of uranyl acetate. After 1 min the uranyl acetate solution was blotted from the grid, and the grid was allowed to dry. Specimens were viewed at 40 kV in a Philips 420 transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Low-shear Viscosity Measurements

Various protein fractions were tested for their effects on actin viscosity by falling ball viscometry (13). Samples were prepared in a final volume of 0.25 ml in buffer B that contained gel-filtered actin at a protein concentration of 0.5 mg/ml and the specified amounts of protein fractions (see figure legends). Polymerization was initiated by the addition of MgCl₂ and KCl to final concentrations of 2 mM and 50 mM, respectively. Duplicate samples were incubated for 1 h at 30°C unless otherwise specified. All measurements were made at an angle of 60°. Data points represent the average of two measurements, which always fall within 10% of each other. The apparent viscosity of the samples is expressed as a percentage of the viscosity of the control sample that contained only gel-filtered actin.

Assay for Actin Polymerization

Actin polymerization was followed by continuous recording of the increase in

fluorescence intensity of actin solutions (0.5 mg/ml final actin concentration), 5% of which had been labeled with pyrene according to published procedures (4). All measurements were made in a Perkin-Elmer 650-10S recording spectrofluorometer, with the wavelengths for excitation and emission set at 368 and 407 nm, respectively. The excitation and emission slits were set at 2 and 10 nm, respectively.

Critical Concentration Measurements

The critical concentration for actin polymerization was measured by a modification of the kinetic method described by Pollard (15). Polymerization of actin was measured spectrofluorometrically as described above. Samples (0.6 ml) were prepared in buffer A that contained various concentrations of gel-filtered actin (5% pyrene-labeled) in the presence of a fixed amount of spectrin–band 4.1–actin complex (20 µg) to act as nuclei. HA-vinculin or HA1 was added at the concentrations indicated in the figure legend. At time zero, MgCl₂ and KCl were added to final concentrations of 2 and 50 mM, respectively, and initial rates of polymerization were determined. A plot of the initial rates of polymerization versus the initial actin monomer concentration gives a straight line with a slope proportional to the association rate constant, k_+ , and the critical concentration, C_0 , given by the X-intercept, according to the equation $k_- = k_+ \times C_0$.

Measurement of Protein Concentration

Concentration of G-actin was determined spectrophotometrically using an extinction coefficient of 0.637 mg⁻¹ cm² at 290 nm. Concentrations of other proteins were determined by the dye binding method of Bradford (2).

Results

The recent study by Evans et al. (5) raises the question of whether any of the previously reported effects of vinculin preparations on actin can be attributed to vinculin itself. If some or all of these effects are due to the presence of a highly active contaminant in the vinculin preparations, it would be of interest to characterize this component and determine whether it associates with vinculin in a specific way. To answer these questions, we further purified the vinculin preparation (herein referred to as DEAE-vinculin) described by Feramisco and Burridge (6) by chromatography on a hydroxylapatite column. Fig. 1A shows a typical elution profile obtained from such an experiment. A small amount of protein loaded onto the column flowed through without binding to the hydroxylapatite, while most of the protein, which consisted primarily of vinculin, was eluted from the column at ~0.1 M sodium phosphate.

Fractions from the column were then tested for their ability to decrease the low-shear viscosity of actin solutions in falling ball viscometers (13). Those fractions that contained protein not bound to the column were the only ones that contained significant activity, causing drastic decreases in the viscosity of F-actin solutions. The rest of the fractions, which included those that contained vinculin eluted from the column by the salt gradient, had no measurable activity at the concentrations used in this assay. The active fractions and those that contained vinculin were pooled (designated as HA1 and HA-vinculin, respectively), dialyzed against buffer B, and concentrated by ultrafiltration with Amicon YM 10 filters.

Fig. 1B shows the result of electrophoretic analysis of these two fractions and the material before hydroxylapatite chromatography (i.e., DEAE-vinculin) in SDS polyacrylamide gels. Although most of the activity was present in HA1, it is clear that it contained no vinculin. Instead, this fraction consisted primarily of components with apparent molecular weights <45 kD (see below). Because the activity in HA1 had previously been found to co-migrate with vinculin on the DEAE-column and a gel filtration (Sephacryl 6B) column

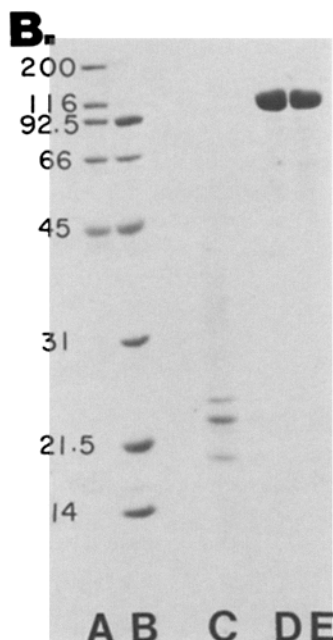
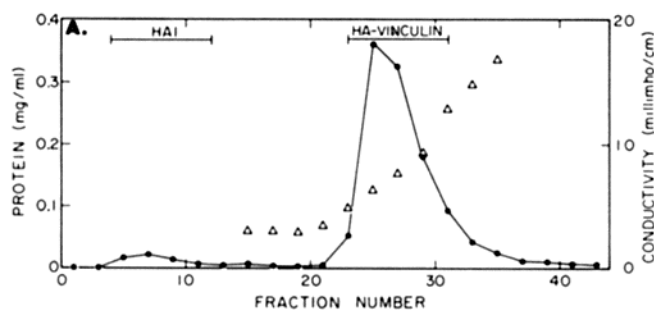


Figure 1. Purification of DEAE-vinculin by hydroxylapatite chromatography. (A) Vinculin prepared from chicken gizzard by the method of Feramisco and Burrige (6) was loaded onto a hydroxylapatite column, and collection of 2-ml fractions was started immediately. After a washing step, the column was eluted with a linear gradient of sodium phosphate (see Materials and Methods). Protein content (●) and salt concentrations measured as conductivity (Δ) of the fractions are indicated here. Fractions 4–12, which contained the first protein peak collected before the start of the salt gradient, were pooled and designated HA1. Fractions 23–31 from the second protein peak were pooled and designated HA-vinculin. HA1 and HA-vinculin were dialyzed overnight against buffer B, concentrated by ultrafiltration, and used in subsequent experiments. (B) Electrophoretic analysis of pooled fractions from the hydroxylapatite chromatography step. The 10% SDS polyacrylamide slab gel was prepared as described by Laemmli (11) and stained with Coomassie Blue. A and B, standards with molecular weights given in kilodaltons; C, HA1, 14 μg; D, HA-vinculin, 14 μg; E, DEAE-vinculin, 14 μg.

(19), we wanted to determine whether this reflects a tight association of the active components with vinculin before hydroxylapatite chromatography. In a series of chromatographic experiments, we looked at the migration rate of the various fractions on a Sephacryl S-200 column in buffer B with or without 0.1 M KCl. Qualitatively similar data were obtained under both conditions: DEAE-vinculin and HA-vinculin migrated with the same mobility on this column (Fig. 2). We also found a peak of activity (measured as the ability to decrease the low-shear viscosity of F-actin) that migrated close to the vinculin peak when DEAE-vinculin was

chromatographed on the column. When HA1 was chromatographed on the column, a peak of activity was detected at a position similar to that observed in the DEAE-vinculin experiment. These results indicate that the similar migration rates of vinculin and of the active material reflect a similarity in Stoke's radius, rather than a tight association of the two molecules under the conditions used in the chromatography experiments.

Since HA1 represents such a small percentage ($\leq 5\%$) of the total protein in the DEAE-vinculin preparation, the identification of the protein(s) responsible for the inhibitory activity described above has been difficult. However, we were able to establish that the active material in HA1 is extraordinarily heat stable (i.e., activity is stable to treatment at 90°C for 5 min) and that it is definitely a protein (i.e., its activity was abolished by treatment with 170 μg/ml trypsin for 30 min at room temperature). We also attempted to identify its molecular weight by first fractionating HA1 on 15% SDS polyacrylamide gels, followed by measuring the activity of materials eluted from slices of the gel. Fig. 3A shows the activity profile of the eluates from the different gel slices; the total amount of activity recovered from all of the slices was estimated to be ~50%. Fig. 3B shows a Coomassie Blue-stained gel similar to the one used for elution. Activity of HA1 migrated as a broad peak that corresponded to an approximate molecular weight range between 23,000 and 33,000 (Fig. 3C). In a control experiment in which HA-vinculin was fractionated on such a gel, no activity could be eluted from any of the gel slices. Active fractions from HA1-containing gels were sensitive to trypsin treatment, which indicates again that a protein was responsible for the observed activity. In addition, we showed that when the active material from the gel slices was chromatographed in a gel filtration column in the manner described in Fig. 2, an activity peak was eluted at the same position as that observed with samples of HA1 or DEAE-vinculin. We also tried mixing experiments in which the active

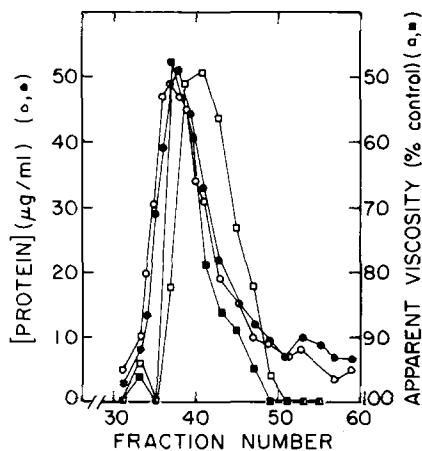


Figure 2. Gel filtration chromatography of DEAE vinculin, HA-vinculin, and HA1. The samples were chromatographed on a Sephacryl S-200 column equilibrated with buffer B, and the protein content and activity (measured as ability to lower the low-shear viscosity of actin) of the fractions were determined as described in Materials and Methods. ○, protein profile for DEAE-vinculin; □, activity profile for DEAE-vinculin; ●, protein profile for HA-vinculin; ■, activity profile for HA1. Protein content of HA1 fractions and activity level of HA-vinculin fractions were below the limit of detection of this experiment.

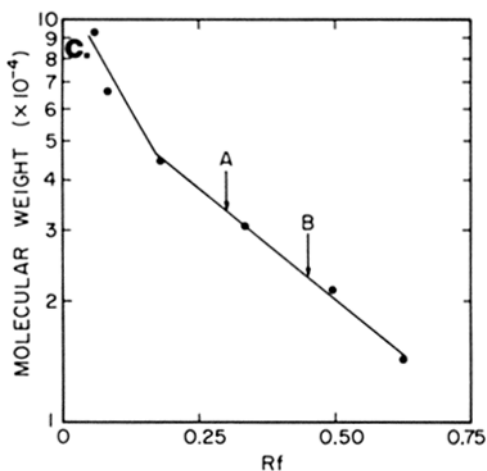
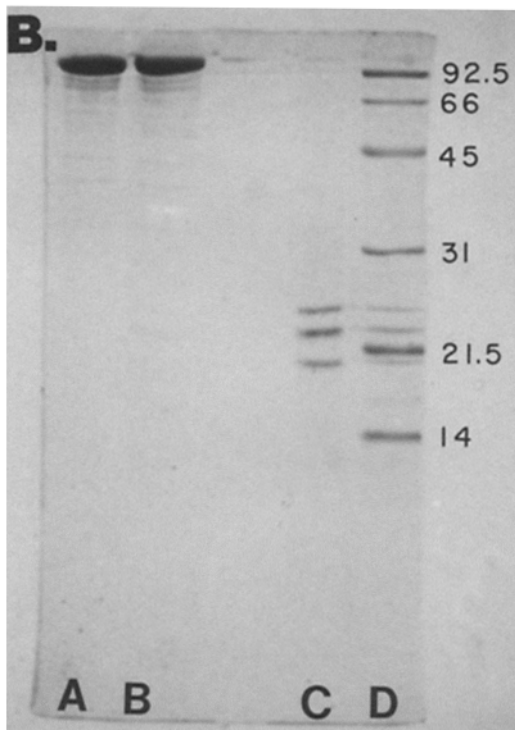
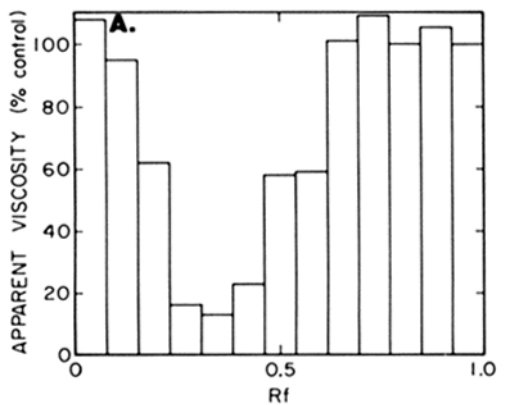


Figure 3. Elution of inhibitory activity from SDS polyacrylamide gel. (A) A 15% SDS polyacrylamide slab gel was run as described by Laemmli (11). Slices from the lanes that contained HA1 (a total of 42 μg protein) were cut out and electrophoretically eluted as described in Materials and Methods. After dialysis against buffer B, equal

material recovered from the gel slices was incubated with HA-vinculin in buffer B with or without 0.1 M KCl for 2–18 h at room temperature or at 4°C. Under no conditions did we see a shift in the mobility of the inhibitory activity on the gel filtration column (data not shown).

Previous studies on DEAE-vinculin preparations have led to the hypothesis that vinculin caps the barbed end of actin filaments in a cytochalasin-like manner (19). Because a decrease in the low-shear viscosity of actin solutions could be explained by formation of filament bundles (8–10) as well as reduction of filament length due to capping of filament ends, we decided to use more specific assays to study the interaction of DEAE-vinculin, HA-vinculin, and HA1 with F-actin. In the following series of experiments, we measured the effect of the protein preparations on nucleated actin polymerization under conditions in which filament elongation occurs exclusively at the barbed end and spontaneous nucleation is negligible (12). As shown in Fig. 4A, both DEAE-vinculin and HA1 exhibited inhibitory activity at low concentrations, with the former having significantly higher specific activity. In comparison, HA-vinculin had much less activity than either of the other two preparations. Similar results were obtained when polymerization of actin in the presence of these three fractions was measured with an Ostwald viscometer (data not shown).

Fig. 4B shows a quantitative comparison of the specific activities of the three fractions. Since the inhibition of the initial rate of polymerization is approximately a linear function of inhibitor concentration, one can use the data to compare the specific activities of the three fractions and to estimate whether all of the activity in the DEAE-vinculin preparation can be recovered in HA1. The result of such a calculation for a representative experiment in which the three fractions were derived from the same starting material is shown in Table I. We have chosen in this instance to define one unit of inhibitory activity as that amount required to inhibit nucleated actin polymerization by 50% under the conditions stated in the legend to Fig. 4. It is apparent from the values presented in Table I that taking into account the yield of the hydroxylapatite chromatographic step, most of the inhibitory activity present in the DEAE-vinculin preparation was recovered in the HA1 fraction.

In addition to the experiment described in Table I, we also performed mixing experiments in which various amounts of HA-vinculin were mixed with HA1. We found that this had no effect on the activity of HA1, which indicates that vinculin does not modulate the activity of HA1 in any measurable way (data not shown).

To further examine the interaction of vinculin and HA1 with actin, we measured the effects of these proteins on the critical concentration of actin assembly. For these measure-

aliquots of the eluates were tested for their ability to reduce the low-shear viscosity of gel-filtered actin. (B) Coomassie Blue-stained gel of proteins prepared essentially as described in A. A, 14 μg of HA-vinculin; B, 14 μg of DEAE-vinculin; C, 8 μg of HA1; D, molecular weight standards with values given in kilodaltons. (C) Semi-log plot of molecular weight versus relative distance of migration of protein bands. The closed circles represent the molecular weight standards shown in B. The peak of activity shown in A migrated between arrows A and B, which correspond to an apparent molecular weight range of 23,000–33,000.

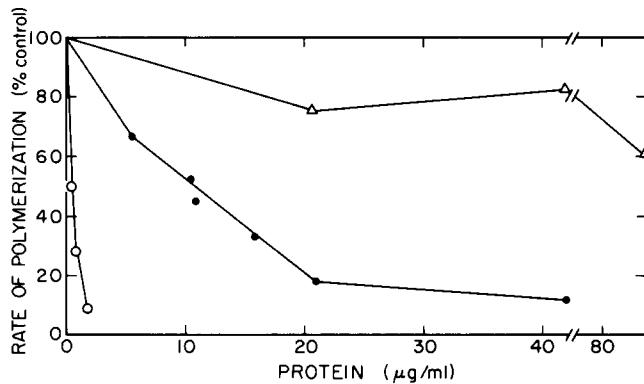
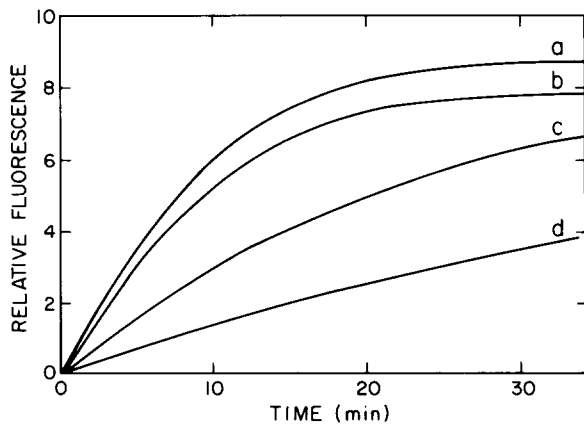


Figure 4. (A) Effect of various protein fractions on nucleated actin polymerization. Samples contained pyrene-labeled actin (0.5 mg/ml; 5% labeled), stabilized actin nuclei in the form of spectrin-band 4.1-actin complex (20 μg protein), and the indicated amounts of additional proteins, in 0.6 ml of buffer A. At time zero, MgCl_2 was added to a final concentration of 0.4 mM, and the polymerization that followed was monitored as an increase in the fluorescence of the sample. *a*, control with no additions; *b*, 30 $\mu\text{g}/\text{ml}$ HA-vinculin; *c*, 31 $\mu\text{g}/\text{ml}$ DEAE vinculin; *d*, 7.8 $\mu\text{g}/\text{ml}$ HA1. Under the conditions of this experiment, there was negligible polymerization of actin in the absence of the spectrin-band 4.1-actin complex. (B) Quantitative comparison of the effect of DEAE-vinculin, HA-vinculin, and HA1 on nucleated actin polymerization. Various amounts of DEAE-vinculin (\bullet), HA-vinculin (Δ), or HA1 (\circ) were tested for their ability to inhibit the initial rate of nucleated actin polymerization in assays performed as described in the legend to Fig. 3. The polymerization rate was linear under these conditions for at least 5 min. For these assays, all proteins had been dialyzed into buffer A.

ments we used an assay similar in principle to that described by Pollard (15), in which initial rates of polymerization are measured at different actin concentrations in the presence of fixed numbers of preformed actin nuclei. In this assay a plot of the initial rate of polymerization versus actin concentration under conditions in which no self-nucleation occurs yields both the on- and off-rate constants for assembly and the critical concentration (given by the X-intercept) (15). Measurements were made and plots were constructed as shown in Fig. 5. The critical concentration measured in this assay for actin was $\sim 0.2 \mu\text{M}$ in buffer A with 2 mM MgCl_2 and 50 mM KCl at pH 8.0. This value did not change in the presence of 117 $\mu\text{g}/\text{ml}$ HA vinculin. HA1, on the other hand, at a concentration of 6.6 $\mu\text{g}/\text{ml}$, raised the critical concentration to 0.4 μM and altered the slope of the line, which indicates a decrease in both on- and off-rate constants for actin assembly,

Table I. Quantitation of Activity of DEAE-Vinculin, HA1, and HA-Vinculin by Their Effects on Initial Rates of Actin Polymerization

Fraction	Specific activity $U/\mu\text{g protein}$	Total protein μg	Total activity
DEAE-Vinculin	0.16	4,410	700
HA1	3.60	130	457
HA-Vinculin	0.016	2,500	40

Specific and total capping activity present in DEAE-vinculin, HA-vinculin, and HA1. The data from Fig. 4 were used to determine specific and total capping activities present in the three fractions. 1 U of capping activity is defined as that amount of a given fraction required to reduce the initial rate of polymerization by 50% under the conditions given in the legend to Fig. 3. The specific activity values were then multiplied by the total protein in the fractions to give total activities. Protein yield, 60%. Activity yield, 71%.

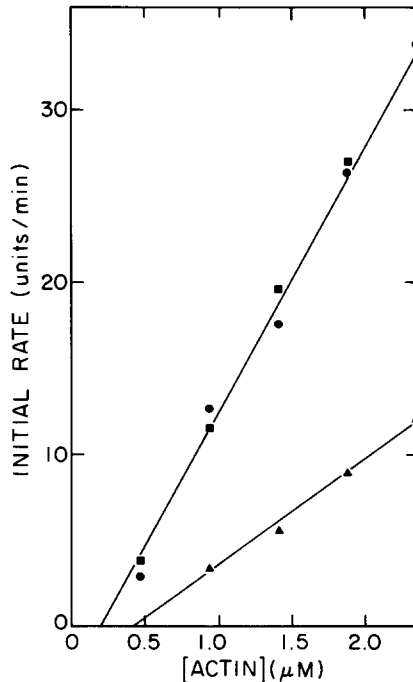


Figure 5. Effect of HA-vinculin and HA1 on the critical concentration of actin. Samples (0.6 ml) contained the indicated amounts of gel filtered actin (5% pyrene-labeled) in buffer A with 2 mM MgCl_2 and 50 mM KCl and the following: \bullet , no additions; \blacksquare , 117 $\mu\text{g}/\text{ml}$ HA vinculin; \blacktriangle , 6.6 $\mu\text{g}/\text{ml}$ HA1. Initial rates of polymerization were measured as described in Materials and Methods. Values shown represent the means of two determinations in the case of HA vinculin and HA1 and three determinations for the control. Linear regression analysis gave an intercept of 0.2 μM for the control and HA-vinculin samples and 0.4 μM for the HA1 samples.

a feature expected for a protein that blocks actin assembly at the barbed end of the filament (e.g., 18). The increase in the critical concentration is dependent upon the affinity of the proteins in HA1 for the barbed end of the filaments and thus on the concentration used in this particular experiment, as noted recently by Wanger and Wegner for bovine brain capping protein (18).

Because of the recent report (5) of the ability of highly purified vinculin preparations to increase the low-shear viscosity of F-actin solutions, we investigated whether HA-vinculin would also produce a similar effect. Various amounts of HA-vinculin were added to G-actin, and the viscosity of

the resultant solutions was measured after polymerization of the actin under different salt conditions (Fig. 6). Alternatively, vinculin was added to the actin after polymerization, and viscosity was measured when it had reached a constant level after an overnight incubation. We found that in all of the conditions tested, HA-vinculin consistently decreased the low-shear viscosity of the actin samples.

We and others (8-10, 19) had reported earlier that DEAE-vinculin preparations cause actin filaments to aggregate into bundles. In view of the present results on filament elongation and low-shear viscosity, it was of interest to determine if vinculin and/or the contaminants present in the DEAE-vinculin preparation were responsible for filament bundle formation. To quantify bundling activity, we used a light scattering assay which is both sensitive and rapid. Such an assay was recently used to measure the bundling of actin filaments induced by the addition of myosin S-1 (1). Electron microscopic studies showed that formation of increasing numbers of actin filament bundles is correlated to increases in the amount of light scattered by these structures (see below). In this study, F-actin solutions were mixed with increasingly higher concentrations of DEAE-vinculin, HA-vinculin, or HA1, and light scattering of these solutions was measured at a 90° angle at a wavelength of 400 nm. As shown in Fig. 7 we found that HA-vinculin had little or no effect on the light scattering of F-actin solutions. HA1, on the other hand, was extraordinarily effective even at low concentrations in increasing light scattering of F-actin solutions. Scattering changes

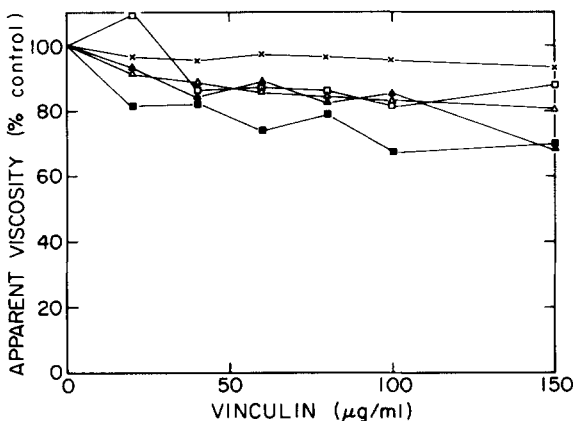


Figure 6. Effect of HA-vinculin on the low-shear viscosity of actin. Two sets of samples (0.25 ml) contained gel filtered G-actin (0.125 mg) and the indicated amounts of HA-vinculin in buffer B. After actin polymerization was initiated by the addition of 2 mM MgCl₂ (▲) or 2 mM MgCl₂ + 50 mM KCl (△), samples were immediately drawn into microcapillary pipets and allowed to incubate for 1 h at 30°C. This was followed by measurement of low-shear viscosity as described in Materials and Methods. Two other sets of samples were prepared starting with actin polymerized in 2 mM MgCl₂ (■), or 2 mM MgCl₂ and 50 mM KCl (□). The F-actin (final concentration of 0.5 mg/ml) was added to the indicated levels of HA-vinculin in buffer B that contained the same concentration of MgCl₂ or MgCl₂ and KCl. Samples were drawn into pipets and allowed to incubate for 12 h before low-shear viscometric assay. One other set of samples was assayed as described by Evans et al. (5). HA-vinculin was dialyzed overnight into 1 mM NaH₂CO₃, pH 7.5. Various concentrations of this protein were then added to G-actin solutions in 1 mM EGTA, 1 mM ATP, 10 mM Imidazole, pH 7.5. Polymerization was initiated by raising the salt concentration of the samples to 2 mM MgCl₂. The viscosity of the samples was determined after incubation for 15 min at 30°C (×).

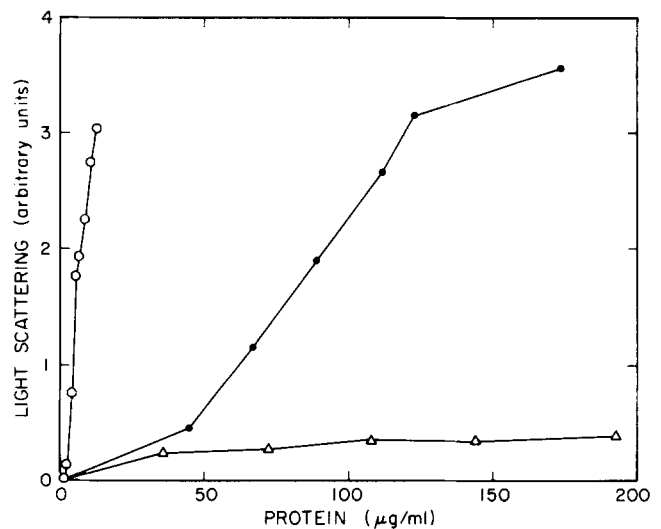


Figure 7. Effects of DEAE-vinculin, HA-vinculin, and HA1 on actin filament bundle formation as measured with a light scattering assay. Each of three samples contained gel-filtered actin (100 µg/ml) polymerized in buffer A and 2 mM MgCl₂ at room temperature for 1 h. To these samples were added increments of HA1 (○), DEAE-vinculin (●), or HA-vinculin (△), to reach increasingly higher concentrations of these proteins. After each addition, the samples were mixed gently, and filament bundle formation was measured as an increase in light scattering at 90° at a wavelength of 400 nm as described in Materials and Methods. The final volume in the samples was increased by no more than 14% due to the addition of the proteins.

took place immediately upon addition of HA1 and were complete within 1 min. The light scattering of a 100 µg/ml solution of F-actin was increased 60-fold by the addition of HA1 to a final concentration of <20 µg/ml. DEAE-vinculin could increase light scattering, but its specific activity was significantly lower than that of HA1. The results of this experiment were confirmed by electron microscopy (Fig. 8). Consistent with the light scattering measurements, addition of HA1 to F-actin led to formation of filament bundles similar to those described previously (8-10, 19). Addition of HA-vinculin, on the other hand, caused formation of a rather loose network of actin filaments that apparently caused no change in light scattering of the solutions (see above). Such networks were marked by an apparent decoration of globular structures of a size similar to that expected for vinculin. The results of these studies indicate that the ability of the DEAE-vinculin preparation to induce formation of large, tightly packed actin filament bundles can be attributed to the contaminants present in HA1 rather than to vinculin itself.

Discussion

We and others had previously reported that vinculin preparations obtained from chicken gizzard smooth muscle (i.e., DEAE-vinculin) by the method of Feramisco and Burridge (6) have the ability to (a) decrease the low-shear viscosity of F-actin solutions (3, 9, 10, 19), (b) inhibit the rise in viscosity measured at high shear rates observed during actin polymerization (3, 9, 10, 19), and (c) induce formation of bundles of actin filaments as observed by electron microscopy (8-10, 19). The first two observations have been interpreted as a result of vinculin capping of the barbed end of F-actin (3, 19), or inducing the formation of filament bundles (8-10). Although there is no question about the validity of all three of

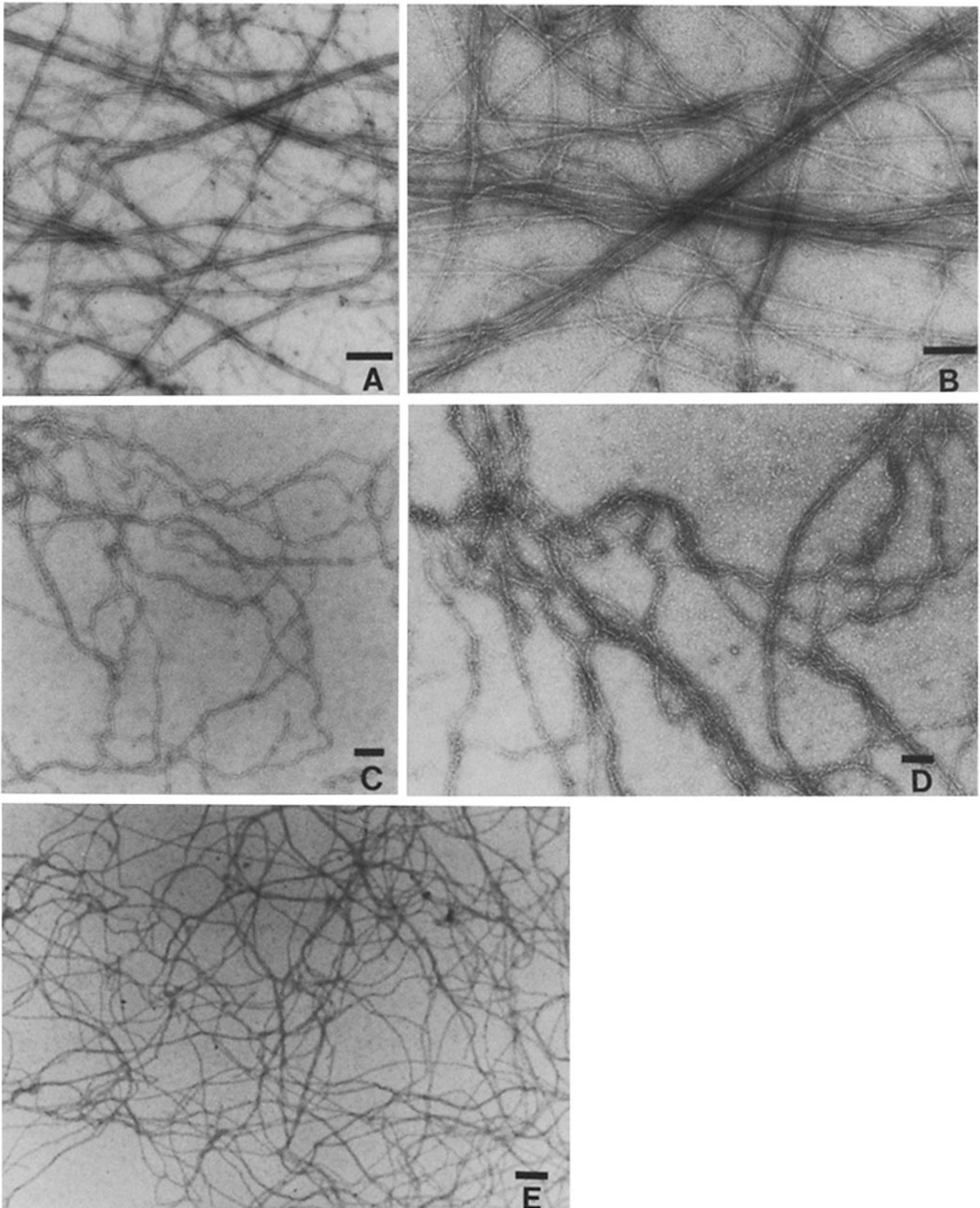


Figure 8. Electron microscopic analysis of actin filament bundles. Samples that contained 100 $\mu\text{g}/\text{ml}$ of gel-filtered G-actin in buffer A were polymerized for 1 h at room temperature by the addition of MgCl_2 and KCl to final concentrations of 2 and 50 mM, respectively. The resultant F-actin solution was then divided into three aliquots. Each aliquot was diluted to a final F-actin concentration of 20 $\mu\text{g}/\text{ml}$ with buffer A that contained 2 mM MgCl_2 and 50 mM KCl. The following protein preparations were then added to the desired final concentrations followed by mixing: (A and B) HA1, 5 $\mu\text{g}/\text{ml}$; (C and D) HA-vinculin, 30 $\mu\text{g}/\text{ml}$; (E) control, no additions. Samples were incubated overnight and prepared for electron microscopy as described in Materials and Methods. Bars in A, C, and E, 0.2 μm . Bars in B and D, 0.1 μm .

these observations, it appears that the previous conclusions that all of these effects are caused by vinculin is wrong. As initially suggested by Evans et al. (5) and more quantitatively documented by the present study, contaminants present in the DEAE-vinculin preparation at a level of 5% or less appear to have been responsible for both the filament bundling and barbed-end capping activities reported previously (3, 19). This contaminating protein(s) is separable from vinculin by either carboxymethyl cellulose (5), or hydroxylapatite chromatography (Fig. 1), but not by DEAE-chromatography or by gel filtration chromatography (Fig. 2). Although the exact nature of these proteins remains to be determined, it is clear that they are highly active bundling and capping factors. It has not been determined, however, whether the same protein is responsible for both bundling and capping activities associated with the HA1 preparation described here.

It is evident from the experiment on electrophoresis of HA1 on SDS polyacrylamide gels that the active material does not migrate as a sharp peak. Indeed, the peak of eluted activity corresponded to a broad molecular weight range between 23,000 and 33,000, covering two of the three major bands stained by Coomassie Blue (see Fig. 3B). This is in contrast to the result of fractionation of HA1 by gel filtration chromatography. Since the inhibitory activity migrates in these columns with a Stokes' radius similar to that of vinculin, one must conclude that the active molecule(s) is multimeric or that it is highly asymmetric in shape.

Our measurements showed that at substoichiometric levels, DEAE-vinculin further purified by hydroxylapatite chromatography (HA-vinculin) had a small but measurable effect in many of the assays used in this study. These results could reflect residual contamination of HA-vinculin by active material similar to that found in HA1, and/or a low level of filament capping and bundling activity intrinsic to vinculin itself. It is of interest to note that Evans et al. (5) found that a vinculin preparation purified by carboxymethyl cellulose chromatography causes small increase in the low-shear viscosity of F-actin, whereas our HA-vinculin preparation lowers the viscosity under the same experimental conditions. This difference in results can probably be attributed to a difference in the nature of the vinculin preparations used in the two laboratories.

Our electron micrographs, which suggest possible interaction of HA-vinculin with actin, are reminiscent of published micrographs prepared with and without ferritin-labeled antibodies, which indicate binding of vinculin to the sides of actin filaments (8). In addition, HA-vinculin appears to induce formation of loosely packed networks of actin filaments (Fig. 8). Whether there is any connection between these observations and the low level of filament capping and bundling activity of the HA-vinculin remains to be seen. In any case, because the total amount of activity in the HA1 fraction came close to the inhibitory activity in DEAE-vinculin, one can safely conclude that the major part of the effects on actin filament assembly and bundle formation that had previously been attributed to vinculin are caused by the contaminants in the vinculin preparations used in those studies (3, 8–10, 19).

In conclusion, it is now clear from this and other studies (5, 14, 16) that substoichiometric amounts of vinculin of higher purity than those used in previous studies have little or no effect on actin assembly and on the low-shear viscosity of F-actin solutions. Because of its location in cellular adhe-

sion plaques as well as at adherens junctions in other cell types, vinculin was thought to be a linkage protein between actin filaments and cellular membranes. In view of our present understanding of vinculin-actin interaction, such a role for vinculin must be seriously questioned. It should be noted, however, that the apparent weak interaction of vinculin with actin under the experimental conditions used here does not necessarily exclude the possibility of strong associations between these proteins *in vivo*. Such associations can only be understood in the context of all of the components of an entire junctional region such as the adhesion plaque. Similarly, the cellular function of the actin filament capping and bundling factor(s) in HA1 is presently unclear. Whether or not these factors are involved in adhesion plaque structure and function remains to be determined.

We gratefully acknowledge Dr. Jacob Varkey for invaluable assistance with electron microscope experiments and Dr. David Cribbs for many helpful suggestions and insightful comments during the course of this work.

This work was supported by a research grant from the National Institutes of Health (AM-33115).

Received for publication 11 March 1985, and in revised form 6 November 1985.

References

- Ando, T., and D. Scales. 1985. Skeletal muscle myosin subfragment-1 induces bundle formation by actin filaments. *J. Biol. Chem.* 260:2321–2327.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Burridge, K., and J. R. Feramisco. 1982. Alpha-actinin and vinculin from non-muscle cells: calcium-sensitive interactions with actin. *Cold Spring Harbor Symp. Quant. Biol.* 46:587–597.
- Cooper, J. A., S. B. Walker, and T. D. Pollard. 1983. Pyrene actin: documentation of the validity of a sensitive assay for actin polymerization. *J. Muscle Res. Cell Motil.* 4:253–262.
- Evans, R. R., R. M. Robson, and M. H. Stromer. 1984. Properties of smooth muscle vinculin. *J. Biol. Chem.* 259:3916–3924.
- Feramisco, J. R., and K. Burridge. 1980. A rapid purification of alpha-actinin, filamin, and a 130,000 dalton protein from smooth muscle. *J. Biol. Chem.* 255:1194–1199.
- Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193–205.
- Isenberg, G., K. Leonard, and B. M. Jockusch. 1982. Structural aspects of vinculin-actin interactions. *J. Mol. Biol.* 158:231–249.
- Jockusch, B. M., and G. Isenberg. 1981. Interactions of alpha-actinin and vinculin with actin: opposite effects on filament network formation. *Proc. Natl. Acad. Sci. USA.* 78:3005–3009.
- Jockusch, B. M., and G. Isenberg. 1982. Vinculin and alpha-actinin: interaction with actin and effect on microfilament network formation. *Cold Spring Harbor Symp. Quant. Biol.* 46:613–623.
- Laemmli, U. K. 1970. Change of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680–685.
- Lin, D. C., and S. Lin. 1979. Actin polymerization induced by a motility-related high affinity cytochalasin binding complex from human erythrocyte membranes. *Proc. Natl. Acad. Sci. USA.* 76:2345–2349.
- MacLean-Fletcher, S., and T. D. Pollard. 1980. Viscometric analysis of the gelation of *Acanthamoeba* extracts and purification of two gelatin factors. *J. Cell Biol.* 85:414–428.
- Otto, J. 1983. Vinculin: interactions with actin, with itself, and with a 220,000 dalton vinculin binding protein. *J. Cell Biol.* 97:282a. (Abstr.)
- Pollard, T. D. 1983. Measurement of rate constants for actin filament elongation in solution. *Anal. Biochem.* 134:406–412.
- Rosenfeld, G. A., D. C. Hou, J. Dingus, I. Mega, and J. Bryan. 1985. *J. Cell Biol.* 100:669–676.
- Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.
- Wanger, M., and A. Wegner. 1985. Equilibrium constant for binding of an actin filament capping protein to the barbed end of actin filaments. *Biochemistry.* 24:1035–1040.
- Wilkins, J. A., and S. Lin. 1982. High affinity interaction of vinculin with actin filaments *in vitro*. *Cell.* 28:83–90.