SPECTRIN PLUS BAND 4.1 CROSS-LINK ACTIN

Regulation by Micromolar Calcium

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ABSTRACT

A low-salt extract prepared from human erythrocyte membranes forms a solid gel when purified rabbit muscle G- or F-actin is added to it to give a concentration of ~1 mg/ml. This extract contains spectrin, actin, band 4.1, band 4.9, hemoglobin, and several minor components. Pellets obtained by centrifugation of the gelled material at 43,000 *g* for 10 min contain spectrin, actin, band 4.1, and band 4.9. Although extracts that are diluted severalfold do not gel when actin is added to them, the viscosity of the mixtures increases dramatically over that of G-actin alone, extract alone, or F-actin alone at equivalent concentrations. Heat-denatured extract is completely inactive. Under conditions of physiological ionic strength and pH, formation of this supramolecular structure is inhibited by raising the free calcium ion concentration to micromolar levels. Low-salt extracts prepared by initial extraction at 37°C (and stored at 0°C) gel after actin is added to them only when warmed, whereas extracts prepared by extraction at 0°C are active on ice as well as after warming. Preincubation of the 37°C low-salt extract under conditions that favor conversion of spectrin dimer to tetramer greatly enhances gelation activity at 0°C. Conversely, preincubation of the 0°C low-salt extract under conditions that favor conversion of spectrin tetramer to dimer greatly diminishes gelation activity at 0°C. Spectrin dimers or tetramers are purified from the 37° or 0°C low-salt extract by gel filtration at 4°C over Sepharose 4B. The addition of actin to either purified spectrin dimer (at 32°C) or tetramer (at 0°C or 32°C) results in relatively small increases in viscosity, whereas the addition of actin to a high-molecular-weight complex (HMW complex) containing spectrin, actin, band 4.1, and band 4.9 results in dramatic, calcium-sensitive increases in viscosity. These viscosities are comparable to those obtained with the 37° or 0°C low-salt extracts. The addition of purified band 4.1 to either purified spectrin dimer (at 32°C) or purified spectrin tetramer (at 0°C) plus actin results in large increases in viscosity similar to those observed for the HMW complex and the crude extract, which is in agreement with a recent report by E. Ungewickell, P. M. Bennett, R. Calvert, V. Ohanian, and W. B. Gratzer. 1979. *Nature (Lond.)*. **280**:811–814. We suggest that this spectrin-actin-band 4.1 gel represents a major structural component of the erythrocyte cytoskeleton.

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/80/05/0361/16 $1.00
Volume 85 May 1980 361–376

The peripheral membrane proteins, spectrin and actin, are thought to comprise a cytoskeletal meshwork underlying the human erythrocyte membrane (11, 21, 30, 34, 47, 50). Changes in the state of organization of these cytoskeletal structures and their associations with the membrane are postulated to be responsible for the shape and deformability of the erythrocyte (12, 30, 34, 48, 47, 50), the distribution of surface markers (31) and intramembrane particles (11), and the regulation of membrane protein mobility (13). Observations concerning the differential extractability of the membrane components have led to the development of an operational definition of the membrane cytoskeleton and have suggested that its structural integrity is partially independent of its associations with the membrane (30, 50). Extraction of the majority of the lipids and integral membrane proteins from ghosts (30, 50) or intact erythrocytes (47) with nonionic detergents produces insoluble, discrete, structures, which retain the form and size of the cell and appear as anastomosing meshworks in the electron microscope (30, 47, 50). The membrane polypeptides that remain after detergent extraction include spectrin, actin, band 4.1, band 4.9, band 2.1 (the membrane attachment site for spectrin [4, 29, 60]), and minor components (30, 47, 50).[1] In contrast, membranes that are fragile and easily fragmented into small vesicles are generated by treatments that solubilize spectrin, actin, band 4.9, and small amounts of other components from the membranes (12, 24, 34, 50). In this paper, we will be concerned with the reconstitution of the erythrocyte cytoskeleton in vitro from these solubilized components.

Actin appears to provide the basic structural framework for the extensive cytoskeletal meshwork and gel structures in motile cells, probably as short actin filaments cross-linked by other cytoskeletal components (7, 23, 51, 52). Actin may have an analogous function in the erythrocyte cytoskeleton inasmuch as purified spectrin does not self-associate beyond the tetrameric state (41, 56), and band 4.1 and 2.1 bind specifically to spectrin in solution without cross-linking the spectrin molecules into higher-order aggregates (54). On the other hand, variable amounts of a high-molecular-weight complex that contains spectrin, actin, band 4.1, and band 4.9 can be separated from dimeric or tetrameric spectrin and monomeric actin, which are also present in the low-ionic-strength extracts from erythrocyte membranes (8, 9, 27, 39). The capacity of erythrocyte actin to self-associate into filaments (46, 53) is similar to that of muscle and other nonmuscle actins (7, 23, 51, 52) and suggests that actin self-association may provide the mechanism by which these cytoskeletal proteins form an extensive cross-linked structure underlying the erythrocyte membrane.

There are several studies suggesting that spectrin and actin can associate in vitro (8, 21, 30, 37, 38, 53). Tilney and Detmers (53) describe an interaction between spectrin-containing, low-ionic-strength extracts and added rabbit muscle actin detectable by high-shear viscometric techniques. However, the specific components involved in the formation of these supramolecular structures and the factors that regulate their interactions were not identified. The observations of Pinder et al. (37) that such low-ionic-strength extracts stimulated actin polymerization also were interpreted to support a spectrin-actin interaction. However, it now appears that this actin-polymerizing activity is the result of the presence of F-actin seeds in the active preparations (5, 6, 8, 17, 27, 39).

Thus, there is no direct evidence for a cross-linked spectrin-actin complex that could account for the structural and viscoelastic properties of the erythrocyte membrane. We report here that a low-ionic-strength extract that contains spectrin, actin, band 4.1, and band 4.9 is capable of interacting with actin to form a solid gel in vitro. Using a low-shear viscometric technique (16) to quantitate these interactions, we find that spectrin, actin, and band 4.1 are all required for the optimal formation of these supramolecular structures. Furthermore, the formation of these structures is inhibited by raising the free calcium ion concentration to micromolar levels, a mechanism that could operate to control cell shape and deformability in vivo.

This work was first presented at the Annual Meeting of the American Society for Cell Biology in November, 1978 (14). While this manuscript was in preparation, Brenner and Korn (6) reported that pure spectrin is active in cross-linking actin, and Ungewickell et al. (55) reported that band 4.1 is required for the formation of a spectrin-actin complex.

MATERIALS AND METHODS

Materials

Chemicals were obtained as follows: EGTA, PIPES, dithiothreitol (DTT), and Tris from Sigma Chemical Co., St. Louis,

[1] Steck's nomenclature for the erythrocyte membrane proteins is used (50).

Mo.; glycerol from Fisher Scientific Co., Pittsburgh, Pa.; ATP from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; Sepharose 4B from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.; Aquacide II-A from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif..

Outdated whole human blood drawn into acid-citrate-dextrose was obtained through the Northeastern Regional Red Cross, and was used within 3–5 wk of drawing.

Stainless steel balls for viscometry (0.64 mm in diameter, density 10.2 mg/mm^3, grade 10, material 440-C, from the Microball Co., Peterborough, N. H.) were supplied by the courtesy of T. Pollard, Johns Hopkins School of Medicine, Baltimore, Md.

Preparation of Proteins

LOW-SALT EXTRACT: Ghost membranes were prepared from erythrocytes (3), washed once in 0.3 mM sodium phosphate buffer, pH 7.6 at 0°C, and then extracted into 1–2 volumes of the same buffer at 37°C as described by Bennett and Branton (3). The high-speed supernate from this step is hereafter referred to as the 37°C low-salt extract (Fig. 1, lane *b*). For the initial gelation experiments, the low-salt extract was left in the 0.3 mM phosphate buffer and stored on ice until it was tested for gelation activity. For the viscosity experiments, the low-salt extract was dialyzed into 0.5 mM DTT, 0.5 mM NaN_3, 2.0 mM PIPES, pH 7.0, and clarified at 100,000 *g* for 30 min, unless otherwise indicated. In experiments in which the actin cross-linking activity of the low-salt extract is compared with that of the purified components, 20 mM KCl was included in the dialysis buffer described above. No significant differences were observed in the activity of the low-salt extract, whether or not KCl was present in the dialysis buffer.

FRACTIONATION OF THE LOW-SALT EXTRACT: Spectrin dimers were separated from a high-molecular-weight complex, erythrocyte actin, and low-molecular-weight components (see Results) by chromatography at 4°C on Sepharose 4B according to published methods (42, 56). The 37°C low-salt extract was made 2.5 mM in sodium phosphate, pH 7.6, 20 mM KCl, 0.5 mM DTT, 0.5 mM NaN_3 and loaded onto a Sepharose 4B column (2.5 × 90 cm) previously equilibrated with the same buffer. The fractions were monitored for protein at A_{280} and then assayed for actin cross-linking activity by low-shear viscometry (see below). Protein peaks with activity were pooled, concentrated 5–6-fold by dialysis against dry Aquacide II-A, and then dialyzed overnight at 0°C into 20 mM KCl, 0.5 mM DTT, 0.5 mM NaN_3, and 2.0 mM PIPES, pH 7.0. After clarification for 30 min at 100,000 *g* at 2°C, they were assayed for actin cross-linking activity as described below. The activity of fractions tested directly from the column is stable for several days, but decays rapidly after the fractions are pooled, concentrated, and dialyzed. Although SDS polyacrylamide gel electrophoresis does not reveal any obvious proteolysis, addition of 100 μg/ml phenylmethylsulfonyl sulfide in 0.5% ethyl alcohol, 0.2 mM EGTA, and 0.1 mM $MgCl_2$ is effective in partially arresting this decay.[2]

Spectrin tetramers were purified from a 0°C low-salt extract of erythrocyte membranes by chromatography on Sepharose 4B (42, 56). Membranes were washed once in 0.3 mM sodium phosphate, pH 7.6, 0.5 mM NaN_3 at 0°C, resuspended to 1.5 times their volume, and sonicated for 60 s in an ice bath. After dialysis for 18–20 h at 0°C against the same buffer, the membranes were sonicated again and centrifuged for 30 min at 250,000 *g* at 2°C. The supernate that contained spectrin (0°C low-salt extract) was fractionated at 4°C as described above.

ACTIN: G-actin was prepared from an acetone powder of rabbit skeletal muscle with a single cycle of polymerization and sedimentation from 0.8 M KCl (49). The actin was stored at −20°C as the lyophilized powder with 2 mg sucrose/mg of protein, and before use it was resuspended to 8–10 mg of protein/ml in 2 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT and dialyzed against the same buffer overnight at 0°C. The actin was clarified by centrifugation at 100,000 *g* for 30 min and used within 48 h. The purity of the actin was judged by SDS gel electrophoresis (Fig. 1, lane *e*).

Viscosity Measurements

Viscosity was measured by the use of a low-shear falling sphere viscometer similar to the one described by Griffith and Pollard (16). Unless otherwise indicated, assay conditions were 50 mM KCl, 20 mM PIPES, pH 7.0, 0.5 mM DTT, 4.0 mM EGTA, and 0.2 mM $CaCl_2$, with protein concentrations, incubation times, and temperatures as stated in the figure legends. Samples were drawn up into 100-μl micropipettes, sealed with Plasticene at one end, and then incubated in a water bath at the appropriate temperature. After incubation for the indicated times, the micropipette was placed at an angle between 20 and 80° from the horizontal, and the time for a small stainless steel ball to fall between two points was measured. These times can be converted into viscosities because 1/velocity is directly proportional to the viscosity of the solution (16). A calibration curve for each angle is constructed using a series of glycerol solutions (0–100%) with known viscosities (determined using a Cannon-Manning semimicro viscometer). Viscosities in centipoise (cp) are reported relative to the viscosity of the buffer solution, which is 1 cp. All samples were measured in triplicate and averaged. Measurements are reproducible within 20%. In general, reproducibility tends to decrease as solutions become more viscous. We were not able to measure viscosities greater than ~400–500 cp because our calibration curves do not extend beyond these values, and because the ball does not fall steadily and reproducibly under these conditions. (This corresponds to falling times of between 3 and 4 min at an angle of 80° for a distance of 6.1 cm.) We converted the raw data into viscosities assuming we were measuring Newtonian fluids (1, 16). Actually, the preparations change in time from Newtonian to non-Newtonian fluids. Therefore, the falling sphere viscometer was used only as a semiquantitative assay of the relative consistency of the solutions. Moreover, measurements of viscosity are not accurate indicators of gelation because these gels are viscoelastic solids and are perturbed more or less vigorously by the method employed (1, 16, 52).

RESULTS

Gelation of the Low-salt Extract

Addition of rabbit muscle G- or F-actin (to ~1 mg/ml) at 0°C to the 37°C low-salt extract (0.3–1.0 mg/ml) followed by warming of this mixture to 32°C results in the formation of a gel that does not flow out of an inverted test tube. Actin alone, extract alone, or heat-treated extract (15 min, 53°C) plus actin does not gel. Once formed, the

[2] Suggested by W. R. Hargreaves, J. M. Tyler, and E. J. Luna (Harvard Biological Laboratories, Harvard University, Cambridge, Mass.) to inhibit proteolysis. Personal communication.

gel does not solate when cooled on ice, even over a period of several hours.

In addition to spectrin, the 37°C low-salt extract contains some endogenous actin (42,000 daltons), band 4.1 (88,000 daltons), band 4.9 (45,000 daltons), a polypeptide in the region of band 7 (~29,000 daltons), some hemoglobin, and minor components[3] (Fig. 1, lane *b*). At an extract concentration of 1.0 mg/ml, the concentrations of the spectrin and the actin are ~0.6–0.7 mg/ml and ~0.1 mg/ml, respectively, as determined by Coomassie Blue staining. The concentrations of the other components are more variable and are difficult to determine because they are each <0.05 mg/ml. In a preliminary attempt to determine which of these components might interact with rabbit muscle actin to form a gel, we centrifuged the gel for 10 min at 43,000 *g* and compared the supernates and pellets by SDS polyacrylamide gel electrophoresis (Fig. 1). After addition of exogenous actin and warming, most of the actin, spectrin, band 4.1, and band 4.9 are pelletable (Fig. 1, lane *j*). A comparison of this pellet with the supernate suggests that simple trapping of components in the gel matrix is unlikely because most of the hemoglobin in the extract remains in the supernate (Fig. 1, lane *i*). Actin alone does not pellet under these conditions (Fig. 1, lanes *f* and *g*). In the absence of added actin, the low-salt extract does not gel, and, thus, is not pelletable (Fig. 1, lanes *c* and *d*). The SDS polyacrylamide gel profile of the gelled material also demonstrates that none of the components are proteolysed during the gelation process (Fig. 1, lanes *i* and *j*).

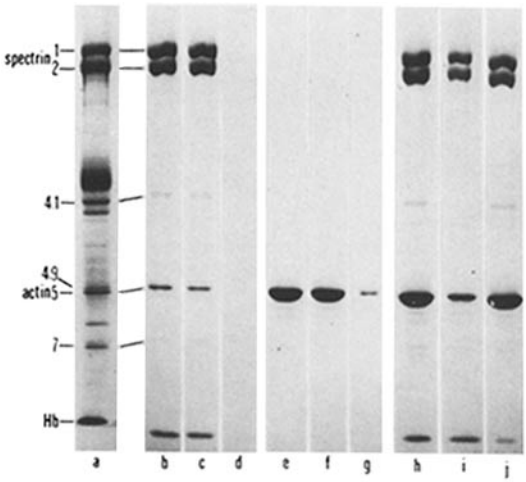

FIGURE 1 SDS polyacrylamide gels of (*a*) erythrocyte membranes, (*b–d*) 37°C low-salt extract, (*e–g*) actin, and (*h–j*) 37°C low-salt extract plus actin. (*b*, *e*, and *h*) Samples before centrifugation. (*c*, *f*, and *i*) Supernates after centrifugation. (*d*, *g*, and *j*) Pellets. Rabbit muscle G-actin (final concentration 0.80 mg/ml) was mixed with the 37°C low-salt extract (final concentration 0.60 mg/ml) in a buffer containing 50 mM KCl, 4.0 mM EGTA, 0.2 mM $CaCl_2$, 0.5 mM NaN_3 and 20 mM PIPES (final concentrations) and adjusted to pH 7.0 with a pH meter. Samples were incubated in Sorvall 2-ml centrifuge tubes (DuPont Instruments–Sorvall, DuPont Co., Newtown, Conn.) at 32°C for 4 h and then centrifuged for 10 min at 43,000 *g* at 2°C. After the supernates had been carefully removed, the pellets were resuspended to the initial volume (determined gravimetrically). Parallel samples (equal volumes) of supernates and pellets were then electrophoresed according to Laemmli (26) on SDS polyacrylamide, 5–15% linear gradient slab gels with a 5% stacking gel. The similarity in molecular weight of band 4.9 to actin and the large excess of actin make it difficult to demonstrate the presence of band 4.9 in the pellet of the gelled material. However, heavier sample loading and longer electrophoresis times (not shown) reveal that band 4.9 is indeed concentrated in the pellet along with the spectrin, band 4.1, and actin (lane *j*).

Quantitation of Gelation

The low-shear viscometric technique described by Griffith and Pollard (16) was used as a semiquantitative assay to characterize the interactions between actin and the 37°C low-salt extract. It should be stressed, however, that this technique assays only apparent viscosity and not the formation of gels. Although the ability of the solution to flow down the side of an inverted test tube enables us to distinguish qualitatively between gels that fragment easily during flow and those that cohere and are more resistant to disruption (see reference 52 for references to other workers who have used this technique), these differences are not measurable by the falling sphere viscometer because the ball does not fall down the micropipette in either case. Therefore, extracts were diluted severalfold to generate structures within the measuring range of this technique. Implicit in this approach is the idea that the cross-linking of actin to generate viscous solutions is controlled by the same factors governing the cross-linking of actin to generate very highly cross-linked or gelled structures.

Fig. 2 illustrates the dependence of the viscosity on the concentration of the extract. At 0.30 mg/

[3] Molecular weights are calculated with standard methods by SDS polyacrylamide gel electrophoresis on 5–15% slab gels according to Laemmli (26).

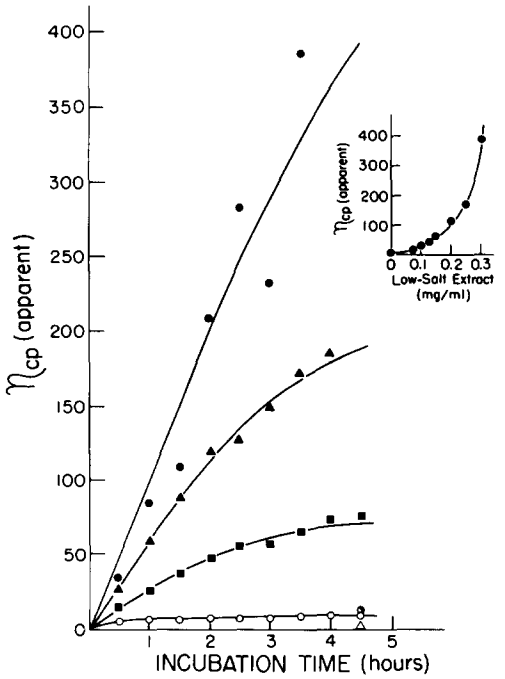

FIGURE 2 Increase in viscosity over time of the 37°C low-salt extract plus actin, at (●) 0.30 mg/ml, (▲) 0.25 mg/ml, (■) 0.15 mg/ml, (○) 0 mg/ml, low-salt extract, or (◔) 0.20 mg/ml low-salt extract heated 10 min at 60°C. (△) 0.20 mg/ml extract in the absence of added actin. Rabbit muscle G-actin (final concentration 0.80 mg/ml) was mixed with various concentrations of the 37°C low-salt extract at 0°C under the gelation conditions described in the legend to Fig. 1. Samples were incubated at 32°C for the times indicated, and then the viscosity was measured. (*Inset*) Viscosities attained as a function of extract concentration after incubation for 3.5 h at 32°C.

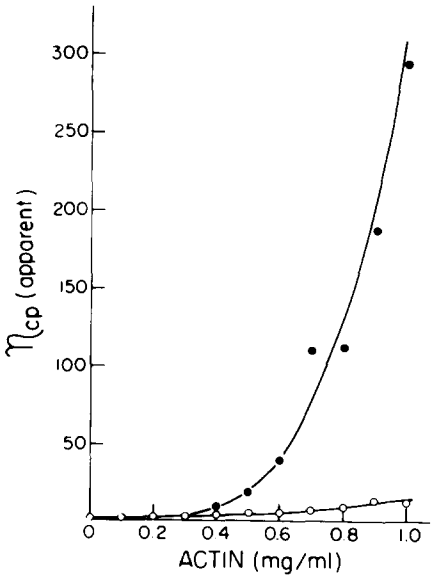

FIGURE 3 Effect of actin concentration on the viscosity of the 37°C low-salt extract. Rabbit muscle G-actin at the final concentrations indicated was mixed with the 37°C low-salt extract (final concentration 0.20 mg/ml) as described in the legend to Fig. 1. Samples were incubated at 32°C for 4 h, and then the viscosity was measured. (●) Low-salt extract plus actin. (○) Actin.

ml of extract, the viscosity of the 37°C low-salt extract plus G-actin increases dramatically to ~350 cp after 3.5 h of incubation at 32°C (Fig. 2). After 5 h the ball no longer falls through the viscous solution, even at an angle of 80° from the horizontal. The addition of still higher concentrations of extract results in even more dramatic initial increases in viscosity. However, the extent of the increases in viscosity (or the degree of gelation) can only be assessed qualitatively by the ability of the solution to flow down the side of an inverted test tube. The increases in viscosity are dependent on the low-salt extract concentration in a highly cooperative manner (Fig. 2, *inset*). In the absence of added low-salt extract, the viscosity of the actin, which is initially in the monomeric state, increases to 4–5 cp (Fig. 2). This viscosity is equivalent to that of the same concentration of F-actin, suggesting that under the conditions of this assay (50 mM KCl, pH 7.0, 32°C) the actin polymerizes.[4] In the absence of added actin, the viscosity of the low-salt extract, at all concentrations tested (up to 1.0 mg/ml), is not significantly greater than that of the buffer (Fig. 2, △). Heat-denatured extract is completely inactive (Fig. 2, ◔)

The interaction of the 37°C low-salt extract with actin is also dependent on the actin concentration in a highly cooperative manner (Fig. 3). The lowest actin concentration that supports both gelation and these dramatic increases in viscosity is similar to that observed in other systems (0.4–0.5 mg/ml) (see reference 52 for a review). Greater concentra-

[4] The low-shear viscosity of polymerized muscle actin (F-actin) varies appreciably from preparation to preparation and can be as high as 40 cp. (This is true for actin polymerized under the standard assay conditions [50 mM KCl, 20 mM PIPES, pH 7.0, 4 mM EGTA, 0.2 mM $MgCl_2$] as well as in 0.1 M KCl, 2 mM $MgCl_2$.) However, the relative increases in viscosity of the low-salt extract with actin over that of the actin alone are relatively independent of the absolute viscosity of the actin.

tions of low-salt extract (up to 0.50 mg/ml) result in greater viscosities only at actin concentrations >0.40 mg/ml. Thus, the endogenous actin concentration in the 37°C low-salt extract as routinely prepared is too low (~0.1–0.2 mg/ml) to induce the formation of supramolecular structures detectable by these viscometric techniques (see Discussion).

An actin concentration of 0.80 mg/ml and a 37°C low-salt extract concentration between 0.15 and 0.25 mg/ml is used for the experiments reported in this paper. To assess the influence of various factors on the extent of the interactions, an incubation time of 4 h at 32°C was routinely used because the increases in viscosity begin to plateau at this time (Fig. 2). The effect of various factors on the rate was assessed by measuring the viscosity after a 1-h incubation, at which time the increases in viscosity are still linear. Although the activity of extracts varies somewhat from preparation to preparation, the general characteristics of the interaction with actin, as described below, are very similar.

Ionic Conditions

The interactions between the 37°C low-salt extract and actin are critically dependent on the ionic conditions. In the presence of calcium (0.02–0.2 mM), the increases in viscosity of the 37°C low-salt extract plus actin exhibit a pH optimum of ~6.0 (Fig. 4) and are inhibited by raising the KCl concentration above ~10–20 mM. Some variability in this optimum is observed (±0.2 pH units), depending on whether the extract is tested directly in the 0.3 mM sodium phosphate extraction buffer or is first dialyzed into other buffers. Neither the extract nor the actin alone exhibits such increases in viscosity (Fig. 4).

In contrast, if the free calcium ion concentration is reduced to below $\sim 10^{-7}$ M with EGTA (see below), significant increases in viscosity of the same concentration of 37°C low-salt extract plus actin also occur at pH 7.0, with a KCl optimum between about 20 and 50 mM (Fig. 5).[5] The data presented for both the KCl dependence (Fig. 5) and the pH dependence (Fig. 4) were obtained after a 4-h incubation at 32°C. However, the shapes of the curves are identical after only a 1-h incubation at 32°C, suggesting that both the rate and extent of the interactions are affected similarly by these parameters. The experiments reported in Figs. 1–3 and in the remainder of this paper were performed at pH 7.0 in 50 mM KCl, with a free calcium ion concentration of $\sim 10^{-8}$ M, unless otherwise indicated.

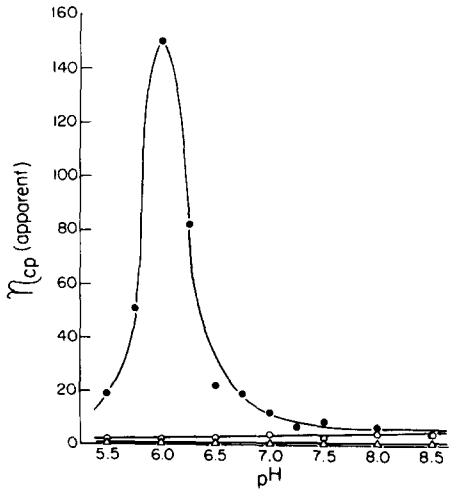

FIGURE 4 pH dependence of the increase in viscosity of the 37°C low-salt extract plus actin. Rabbit muscle G-actin (final concentration 0.80 mg/ml) was mixed with the 37°C low-salt extract (final concentration 0.20 mg/ml) in the 0.3 mM sodium phosphate extraction buffer. KCl, stock sodium phosphate solutions of the appropriate pH, and DTT were added to final concentrations of 50, 20, and 0.5 mM, respectively. The samples were then adjusted with a pH meter to the indicated pH and incubated at 32°C for 4 h in the micropipettes before the viscosities were measured. The shape of the pH curve was the same in the absence of KCl, except the same increases in viscosity were obtained with lower concentrations of extract. Below pH 6.0, the decreases in viscosity of the extract plus actin correlate with increases in turbidity of the solution, until, at pH 5.5, the protein is completely precipitated. The shape of this pH curve is reminiscent of the abrupt rise in opalescence that has been observed just before the protein of spectrin-containing low-salt extracts precipitates when the pH is decreased to ~pH 5.5 (15). (●) Low-salt extract plus actin. (○) Actin. (△) Low-salt extract.

Increasing $MgCl_2$ concentrations (from 0.1 to 5.0 mM) have small and somewhat variable effects on the interaction of the 37°C low-salt extract with actin (data not shown). The effect of MgATP (0.1–5.0 mM) is extremely variable, in some extracts

[5] Preliminary experiments at pHs between 6.5 and 8.0 indicate that the general relationship illustrated in Fig. 6 between the increases in viscosity and the pH is also true in the presence of EGTA. However, it is difficult to interpret the effect of pH under these conditions because the affinity of EGTA for calcium decreases dramatically as the pH is decreased (2).

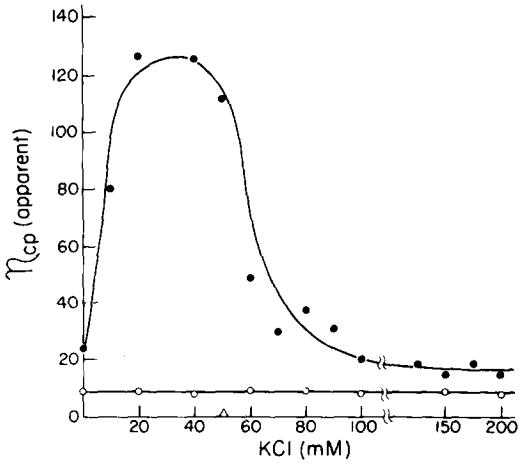

FIGURE 5 KCl dependence (at pH 7.0 in the presence of EGTA) of the increases in viscosity of the 37°C low-salt extract plus actin. Rabbit muscle G-actin (final concentration 0.80 mg/ml) was mixed with the 37°C low-salt extract (final concentration 0.20 mg/ml) as described in the legend to Fig. 1. Samples were incubated at 32°C for 4 h. Significant increases in viscosity over that of actin alone were also achieved at KCl concentrations above 50 mM when more extract was used. (●) Low-salt extract plus actin. (○) Actin. (△) Low-salt extract.

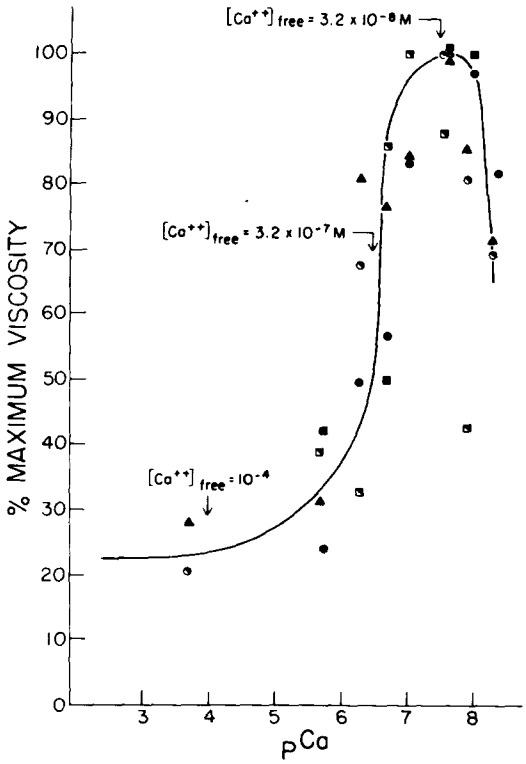

FIGURE 6 Dependence of the viscosity of the 37°C low-salt extract plus actin on the free calcium ion concentration, expressed as pCa. Each set of symbols represents values from a different experiment; results from five separate extracts are presented here. The measured viscosities from each experiment were converted into percent of maximum viscosity attained so that results obtained with the various extracts could be compared directly. Rabbit muscle G-actin (final concentration 0.80 mg/ml) was mixed with the 37°C low-salt extract (final concentration 0.20 mg/ml) in an assay volume of 0.3 ml. Stock Ca^{++}/EGTA solutions (1.25–49.88 mM $CaCl_2$, 50 mM EGTA, pH 7.0, i.e., Ca^{++}/EGTA ratios ranging from 0.025 to 0.998) were diluted into the assay mixture to give final calcium concentrations ranging from 0.1 to 3.99 mM at an EGTA concentration of 4.0 mM. The final concentrations of other components in the assay mixture were 20 mM PIPES, pH 7.0, 50 mM KCl, and 0.5 mM DTT. The buffering capacity of the PIPES was verified by mixing larger volumes of one series of samples and checking the pH directly with a pH meter. Samples were incubated at 32°C for 4 h, and then the viscosities were measured. The free calcium ion concentrations were calculated using an association constant of 4.79×10^6 M^{-1} (2).

inhibiting, and in others enhancing, both the rate and extent of the increases in viscosity (data not shown).

Calcium Sensitivity

The increases in viscosity at pH 7.0 of the 37°C low-salt extract plus G-actin are optimal at free calcium ion concentrations between 10^{-8} and 10^{-7} M (Fig. 6). The viscosities are greatly inhibited by free calcium ion concentrations above $\sim 5 \times 10^{-7}$ M and are only partially inhibited by free calcium ion concentrations below $\sim 10^{-8}$ M (Fig. 6). Neither the viscosity of the actin alone nor that of the 37°C low-salt extract alone is significantly affected by these changes in the free calcium ion concentration. The data in Fig. 6 were obtained after a 4-h incubation at 32°C; very little difference as a function of the free calcium ion concentration is observed if the viscosity is measured after only a 1-h incubation period, suggesting that the free calcium ion concentration influences the extent rather than the rate of the interaction between the 37°C low-salt extract and actin. Some variability is observed both in the degree of calcium sensitivity of each extract and in the free calcium ion concentration at which the increases in viscosity are maximal (compare the various symbols in Fig. 6). This variability might result, in part, from

differential extraction of the cytoskeletal components inasmuch as the free calcium ion concentration is not controlled in the standard methods for preparation of the ghosts and low-salt extract. (Standard methods for ghost and low-salt extract preparation do not include EGTA [3, 50].)

Centrifugation of Low-salt Extract-Actin Gels

To correlate the information obtained by low-shear viscometry with that obtained by a technique capable of measuring gelation of actin plus higher concentrations (up to 0.80 mg/ml) of low-salt extract, we centrifuged the 37°C low-salt extract-actin mixture for 10 min at 43,000 *g* (Fig. 1, Table I). Conditions that inhibit the interaction of dilute 37°C low-salt extract with actin as measured by low-shear viscometry (free calcium ion concentration above $\sim 5 \times 10^{-7}$ M, or 200 mM KCl) also reduce the amount of pelletable protein from extract-actin gels (Table I). The polypeptide composition of the pellets is as pictured in Fig. 1, lane *j*. No obvious changes were observed in the relative proportions of the low-salt extract proteins or actin under the several conditions tested. Only a small amount of either the 37°C low-salt extract or the actin alone pellets under optimal gelation conditions (50 mM KCl, pH 7.0, $[Ca^{++}]_{free} = 10^{-8}$ M) (Fig. 1 and Table I).

Influence of the State of Spectrin Self-association on Actin Cross-linking

Low-salt extraction of erythrocyte membranes at 37°C predominantly yields spectrin dimers, whereas low-salt extraction at 0°C predominantly yields spectrin tetramers (42, 56). Under the appropriate conditions, spectrin tetramers and dimers are interconvertible, with the relative proportions of each species apparently determined by a thermodynamic equilibrium (56). At 37°C and low ionic strength, spectrin is almost completely dimeric (42, 56). The tetrameric association state is increasingly favored as the temperature is lowered and the ionic strength is raised to physiological levels (56). Dissociation of tetramer to dimer (at 37°C in low ionic strength) is rapid, occurring within minutes, whereas association of dimer to tetramer occurs over a period of several hours (at 30°C in 0.15 M KCl) (56). At 0°C, however, no interconversion of tetramers and dimers occurs, even after several days (42, 56).

Low-salt extracts prepared at 0°C cross-link actin at both 0° (Fig. 7) and 32°C (not shown), whereas low-salt extracts prepared at 37°C cross-link actin only at 32°C (Fig. 2) and are completely inactive at 0°C (Fig. 7). The increases in viscosity of 37°C extract-actin mixtures measured at 32°C occur much more slowly than the increases in viscosity of 0°C extract-actin mixtures measured at 0°C (cf. Figs. 2 and 7) or at 32°C (not shown). Also, significantly lower concentrations of 0°C extracts are effective in inducing extensive increases in viscosity of actin at 0°C as compared to the effective concentrations of 37°C extracts at 32°C (Figs. 2 and 7). This is also true when the actin cross-linking activity of the 0°C extracts is measured at 32°C (not shown). Preincubation of 0°C extracts at 37°C in low-ionic-strength buffer greatly diminishes their actin cross-linking activity at 0°C (Fig. 8). Conversely, preincubation of 37°C

TABLE I

Centrifugation of Low-salt Extract-Actin Gels

	KCl	$[Ca^{++}]_{free}$	Total protein	
			Pellet	Supernate
	mM	*M*	%	%
Low-salt extract	50	1×10^{-8}	65.5	34.1
plus actin	50	2×10^{-6}	17.7	82.3
	200	1×10^{-8}	46.0	54.0
Low-salt extract	50	1×10^{-8}	11.2	88.7
Actin	50	1×10^{-8}	13.8	86.2

Rabbit muscle G-actin (final concentration 0.80 mg/ml) was mixed with the 37°C low-salt extract (final concentration 0.80 mg/ml) as described in the legend to Fig. 1, with KCl and free calcium ion concentrations as specified. Samples were then incubated and centrifuged at 43,000 *g* for 10 min as described in the legend to Fig. 1. Protein concentrations were determined according to the method of Lowry et al. (28) for the supernates and the pellets after they had been resuspended to the initial volume. Samples from each were also electrophoresed as described in the legend to Fig. 1. The results in this table are from one representative experiment.

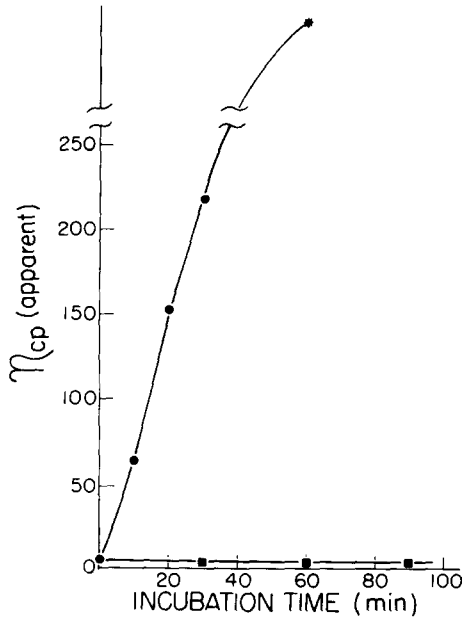

FIGURE 7 Increases in viscosity at 0°C of (●) a 0°C low-salt extract plus actin compared with (■) a 37°C low-salt extract plus actin. Rabbit muscle G-actin was at a final concentration of 0.80 mg/ml, the 0°C low-salt extract at a final concentration of 80 μg/ml, and the 37°C low-salt extract at a final concentration of 0.20 mg/ml. Assays were performed as described in the legend to Fig. 6 at a free calcium ion concentration of $\sim 10^{-8}$ M (Ca^{++}/EGTA = 0.05), except that samples were incubated at 0°C for the indicated times before the viscosities were measured. (*) At this time, the viscosity of the 0°C extract was not measurable by this technique.

extracts at 29.5°C in 0.10 M KCl greatly enhances their actin cross-linking activity at 0°C (Fig. 8). Although other explanations may be possible for the differences in activity between the 37° and 0°C preparations, these observations are all consistent with the possibility that spectrin tetramers are required for actin cross-linking.

Identification of Components Causing Gelation

Fractionation of the 37° or the 0°C low-salt extract by Sepharose 4B chromatography yields two spectrin-containing protein peaks, both of which are active in inducing increases in viscosity of actin (Fig. 9 *a* and *b*). The protein peak from both extracts that elutes first is apparently a high-molecular-weight complex (HMW complex) of spectrin, actin, band 4.1, and band 4.9 (Fig. 9 *a* and *b*, peak *A*; Fig. 10, lanes *b* and *e*). The other peak consists of purified spectrin dimer (Fig. 9 *a*, peak *B*; Fig. 10, lane *c*) or purified spectrin tetramer (Fig. 9 *b*, peak *B*; Fig. 10, lane *f*). The HMW complex from either the 37° or the 0°C extract is much more active than the purified spectrin dimer (Fig. 9 *a*) or tetramer (Fig. 9 *b*). However, a simple comparison of the viscosities attained by the HMW complexes, the spectrin dimer or tetramer, and the unfractionated low-salt extracts plus actin is not meaningful due to the nonlinear dependence of the increases in viscosity on the extract concentration (Fig. 2, *inset*). Therefore, we pooled the

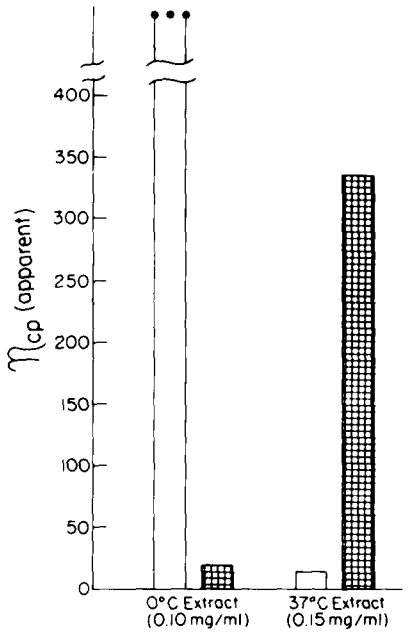

FIGURE 8 Effects of preincubation on the viscosities at 0°C of the 0°C and 37°C low-salt extracts plus actin. To promote conversion of tetramers to dimers, the 0°C extract was preincubated at 37°C for 30 min (42, 56) in a buffer containing 2.0 mM PIPES, pH 7.0, 0.5 mM DTT, and 0.5 mM NaN_3 before actin was added. To convert spectrin dimers to tetramers (56), the 37°C extract was preincubated at 29.5°C for 2 h in a buffer containing 0.10 M KCl, 2.0 mM PIPES, pH 7.0, 0.5 mM DTT, and 0.5 mM NaN_3. The 0°C extract was at a final concentration of 0.10 mg/ml, the 37°C extract at 0.15 mg/ml, and the rabbit muscle G-actin at 0.80 mg/ml. The assay conditions were 50 mM KCl, 20 mM PIPES, pH 7.0, 0.2 mM $CaCl_2$, 4.0 mM EGTA, and 0.5 mM DTT. Assays were performed as described in the legend to Fig. 6, except that samples were incubated at 0°C for 90 min before the viscosities were measured. (*) The viscosity of this sample was not measurable by this technique. (*bar*) Not preincubated. (*crosshatched bar*) Preincubated.

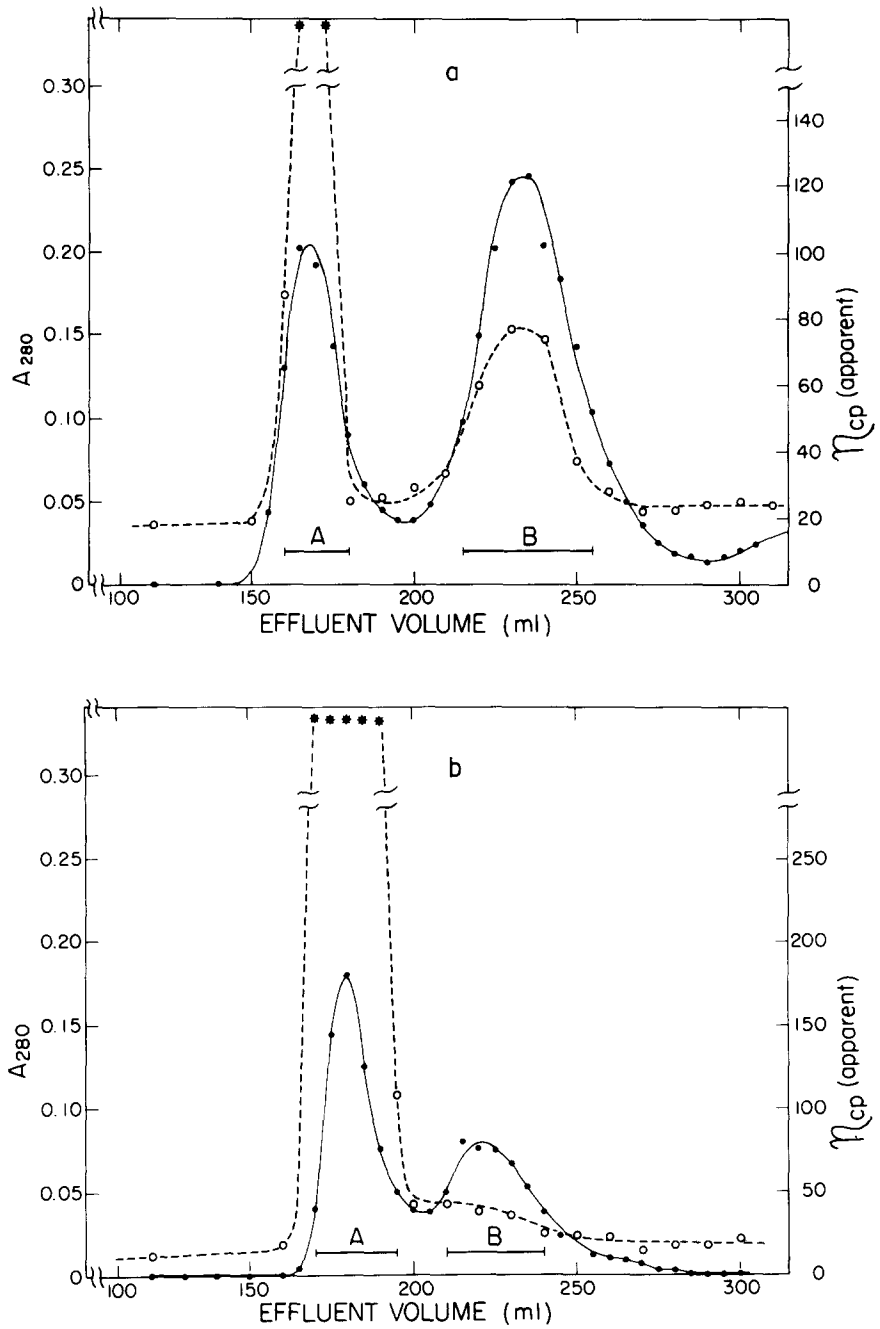

FIGURE 9 Gel filtration on Sepharose 4B of (*a*) the 37°C low-salt extract and (*b*) the 0°C low-salt extract. (●) A_{280} of fractions. (○) Viscosity of fractions plus actin. (*a*) Peak *A*, HMW complex; Peak *B*, spectrin dimer. (*b*) Peak *A*, HMW complex; Peak *B*, spectrin tetramer. Low-salt extracts were fractionated at 4°C as described in Materials and Methods. The columns were loaded with (*a*) 24 mg and (*b*) 10 mg of protein. The interaction of the fractions with actin was assayed by low-shear viscometry as described in Materials and Methods and in the legend to Fig. 6, with a Ca^{++}/EGTA ratio of 0.05. Rabbit muscle G-actin was at a final concentration of 0.80 mg/ml, and the same volume (176 μl) of each fraction was present in the assay mixture (final volume, 300 μl). Samples were incubated at 32°C for 4 h. Only the first half of the column profile is presented because the fractions without spectrin eluting later (including erythrocyte actin, a component in the region of band 7, and hemoglobin) had no detectable effect on the viscosity of added rabbit actin. Peak fractions were pooled for concentrating (see Materials and Methods) as indicated. (*) A viscosity not measurable by this technique.

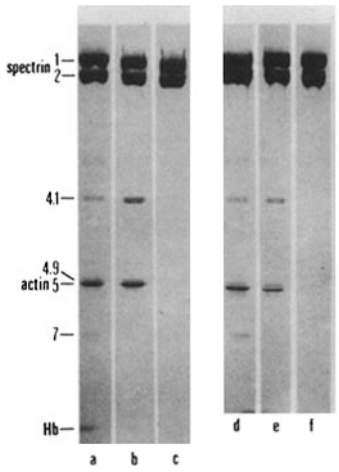

FIGURE 10 SDS polyacrylamide gels of (*a*) 37°C low-salt extract before fractionation, (*b*) HMW complex isolated from the 37°C extract, (*c*) spectrin dimer, (*d*) 0°C low-salt extract before fractionation, (*e*) HMW complex isolated from the 0°C extract, (*f*) spectrin tetramer. The extracts and purified fractions were prepared and isolated as described in Materials and Methods and in the legend to Fig. 9. The samples used for electrophoresis were taken directly from the peak fractions of the respective columns. Samples obtained from pooled, concentrated, dialyzed, and clarified fractions were indistinguishable. Samples in lanes *a–c* were from a single slab gel (5–15% linear gradient, performed according to Laemmli [26]), as were the samples in lanes *d–f*.

separate column fractions, concentrated them, and compared the concentration dependence of each with that of the low-salt extract plus actin (Fig. 11 *a* and *b*). The concentration dependence of the increases in viscosity of the HMW complex isolated from a 37°C extract plus actin is similar to the concentration dependence of the 37°C low-salt extract plus actin (Fig. 11 *a*). The interaction of the purified spectrin dimer with actin is much reduced and may be qualitatively different (Fig. 11 *a*). The interaction of the purified spectrin tetramer with actin is also much reduced in comparison with the interaction of the 0°C low-salt extract and the HMW complex isolated from a 0°C extract with actin (Fig. 11 *b*).

As we observed with the 37° and 0°C extracts, the HMW complex isolated from a 37°C extract is only active at 32°C, whereas the HMW complex isolated from a 0°C extract is active at both 32° and 0°C. Although the activity of the two preparations is not strictly comparable when measured under different conditions of temperature, it is worth pointing out that the HMW complex from a 0°C extract is considerably more active per milligram per milliliter in cross-linking actin at 0°C than is the HMW complex from a 37°C extract at 32°C (compare Fig. 11 *a* with Fig. 11 *b*). The interactions of all fractions and both extracts with actin are inhibited by free calcium ion concentrations above ~5×10^{-7} M (Fig. 12). (Representative data for the 37°C low-salt extract and column fractions are presented in Fig. 12).

Effect of Band 4.1 on the Viscosity of Spectrin-Actin Mixtures

The results presented above suggest that the presence of one or more of the additional components in the HMW complex (band 4.1, band 4.9, or endogenous actin) is also required for actin cross-linking. Addition of purified band 4.1 (54) to spectrin tetramer-actin mixtures results in dramatic increases in viscosity comparable to those observed for the HMW complex (Fig. 13). Note that the viscosity of the spectrin tetramer (50 μg/ml) plus actin in the absence of added band 4.1 is only 10 cp (Figs. 11 and 13). Heat-denatured band 4.1 (5 min, 65°C) is completely inactive. If heat-denatured spectrin tetramer is used in the spectrin-actin-band 4.1 mixture, the viscosity is equivalent to that of the actin alone (Fig. 13). In the absence of spectrin, band 4.1 also has no effect on the viscosity of the actin. Purified band 4.1 causes similar extensive increases in the viscosity of spectrin dimer-actin mixtures, but only if the mixtures are incubated at 32°C (data not shown), a temperature which favors association of spectrin dimers to form tetramers (56). The interaction of purified spectrin plus band 4.1 with actin is also inhibited by free calcium ion concentrations above ~5×10^{-7} M (data not shown).

DISCUSSION

This study describes the cross-linking and gelation of actin in vitro by spectrin plus band 4.1. We suggest that these proteins interact to form supramolecular structures that may represent part of the extensive cytoskeletal meshwork underlying the erythrocyte membrane in vivo. The formation of these structures is inhibited by raising the free calcium ion concentration to micromolar levels, a mechanism which could operate in vivo to control erythrocyte shape and deformability. Our observations lend support to earlier suggestions (37, 46, 53) that spectrin is functionally similar to actin-gelling factors isolated from other nonmuscle cells (for reviews, see references 7, 23, 51, and 52), several of which are also calcium sensitive. Our

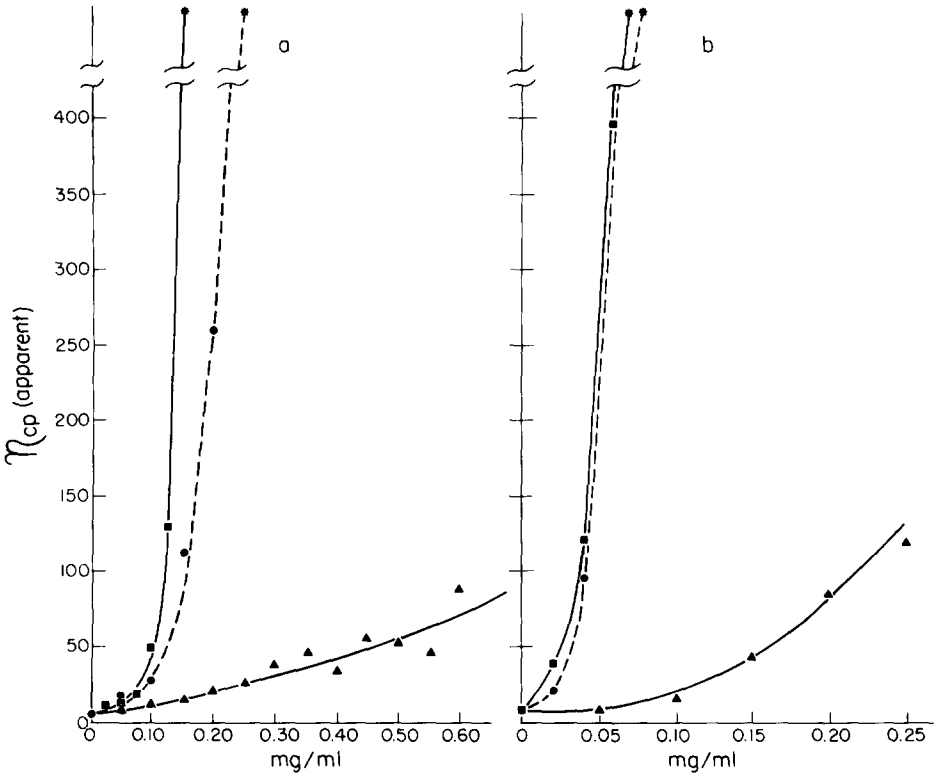

FIGURE 11 Comparison of (*a*) the interactions with actin of (●) unfractionated 37°C low-salt extract, (■) HMW complex isolated from the 37°C low-salt extract, and (▲) purified spectrin dimer and (*b*) the interactions with actin of (●) unfractionated 0°C low-salt extract, (■) HMW complex isolated from the 0°C low-salt extract, and (▲) purified spectrin tetramer. The HMW complexes and spectrin dimer or tetramer were isolated and concentrated as described in Materials and Methods and in the legend to Fig. 9. Rabbit muscle G-actin was at a final concentration of 0.80 mg/ml. The low-salt extract, the HMW complex, and the spectrin dimer or tetramer concentrations were as indicated. Assays were performed as described in the legend to Fig. 6 at a free calcium ion concentration of $\sim 10^{-8}$ M (Ca^{++}/EGTA = 0.05). Samples from the 37°C extract were incubated at 32°C for 4 h, and samples from the 0°C extract at 0°C for 90 min. (*) At these concentrations of HMW complex and low-salt extract, the viscosities were not measurable by this technique. Upon clarification of the concentrated and dialyzed fractions from the HMW complex isolated from the 0°C low-salt extract (Fig. 9 *b*, peak *A*), up to 50% of the total protein was recovered in a large, clear, gellike pellet containing spectrin, actin, band 4.1, and band 4.9 (see reference 30). (This was also characteristic of the 0°C low-salt extract itself after dialysis into the PIPES buffer in preparation for the viscometric assays.) The actin cross-linking activity of the supernate (*b*), which also contained spectrin, actin, band 4.1, and band 4.9, was significantly lower than before centrifugation. When the pellet was homogenized and resuspended and then assayed, it was also very active in cross-linking actin. In contrast, there was no visible pellet after centrifugation of the purified and concentrated spectrin tetramer. Thus, the actin cross-linking activities of the HMW complex and low-salt extract (*b*) are underestimated with respect to the purified tetramer. (Centrifugation of the 37°C extract, the HMW complex isolated from the 37°C extract, or the spectrin dimer produced insignificant pellets whose activities were not tested.)

work thus extends Tilney and Detmer's initial observations of actin cross-linking by low-salt extracts containing spectrin (53), and provides a molecular basis for the viscoelastic properties of the erythrocyte membrane (12, 24, 34). Our work also suggests that the gelation observed by Pinder et al. (38) of cytoskeletons made by detergent extraction of intact erythrocytes is due to the formation of a highly cross-linked spectrin-actin-band 4.1 complex.

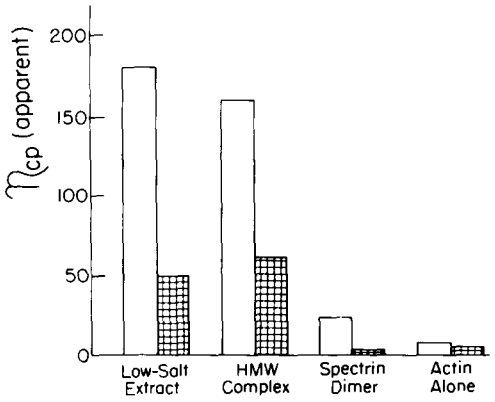

FIGURE 12 Calcium sensitivity of the interaction of actin with the 37°C low-salt extract, the HMW complex isolated from the 37°C low-salt extract, and purified spectrin dimer. The 37°C low-salt extract, the HMW complex, and the spectrin dimer were prepared as described in Materials and Methods and the legend to Fig. 9. Rabbit muscle G-actin was at a final concentration of 0.80 mg/ml, the low-salt extract at 0.20 mg/ml, the HMW complex at 0.20 mg/ml, and the spectrin dimer at 0.30 mg/ml. Assays were performed as described in Materials and Methods and in the legend to Fig. 6 at a free calcium ion concentration of $\sim 10^{-8}$ M (*bar*) or $\sim 2 \times 10^{-6}$ M (*crosshatched bar*) (Ca^{++}/EGTA = 0.05 and 0.9, respectively). Samples were incubated at 32°C for 4 h before viscosity measurements were made.

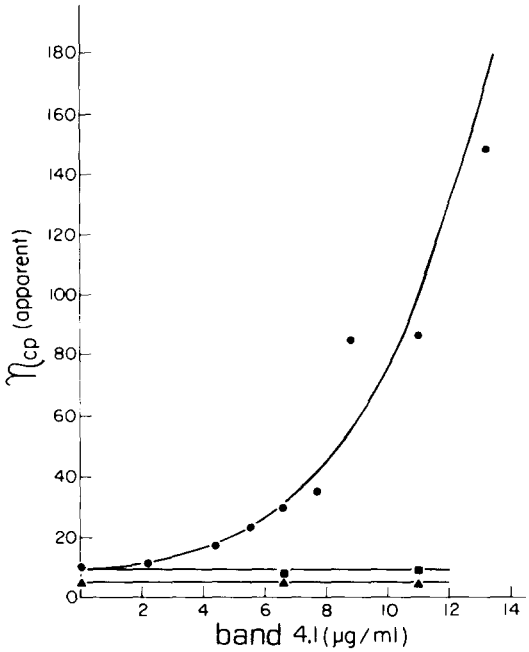

FIGURE 13 Effect of increasing concentrations of band 4.1 on the viscosity of purified spectrin tetramer plus actin. Band 4.1, kindly provided by W. R. Hargreaves, was purified as described by Tyler et al. (54). Spectrin tetramer was purified as described in Materials and Methods and in the legend to Fig. 9. Rabbit muscle G-actin was at a final concentration of 0.80 mg/ml, spectrin tetramer at a final concentration of 50 μg/ml, and band 4.1 was at the indicated concentrations. The assays were performed as described in the legend to Fig. 6 at a free calcium ion concentration of $\sim 10^{-8}$ M (Ca^{++}/EGTA = 0.05). Samples were drawn up into the micropipettes immediately after all three components had been added to the assay mixture, and were incubated at 0°C for 90 min before the viscosities were measured. (●) Spectrin tetramer plus band 4.1 plus actin. (■) Spectrin tetramer plus heated band 4.1 (5 min, 65°C) plus actin. (▲) Heated spectrin tetramer (5 min, 65°C) plus band 4.1 plus actin.

Components of the In Vitro Cytoskeleton

Although spectrin self-associates in solution to form tetramers (42, 56) and also apparently associates with band 4.1 in a specific complex (54), the addition of actin is required to reconstitute supramolecular structures in vitro. Our results suggest that spectrin tetramer is probably the active form of spectrin. Preparations containing predominantly spectrin dimers are only active in cross-linking actin under conditions favoring conversion of dimers to tetramers (32°C, 50 mM KCl). However, preparations containing predominantly spectrin tetramers are active at both 32° and 0°C. The long time-course for actin cross-linking by extracts containing dimers (at 32°C; see Fig. 2) is similar to the long time-course reported for dimer conversion to tetramers (56). The lower activity of these preparations as compared with the 0°C extracts that contain tetramers suggests that not all of the dimers undergo conversion to tetramers during the 32°C incubation with actin. This suggestion is consistent with previous observations that the association of dimers to tetramers is not complete under similar conditions (56).

The presence of band 4.1 significantly enhances spectrin-actin cross-linking and gelation. This observation is in agreement with the report of Ungewickell et al. (55) that band 4.1 is required for the formation of a high-molecular-weight complex between spectrin tetramer and actin. In contrast, two other recent reports suggest that purified spectrin tetramer is active in cross-linking actin in the absence of band 4.1 (reference 6 and footnote 6). We did, in fact, observe a relatively small amount

[6] Cohen, C. M., and C. Korsgren. Submitted for publication.

of actin cross-linking activity that copurifies with the spectrin tetramer and dimer (see Figs. 9 and 11). It is possible that this weak activity could result from contamination of the spectrin tetramer of dimer peak by the extremely active high-molecular-weight spectrin-actin-band 4.1-band 4.9 complex (HMW complex), or from small quantities of a band 4.1-spectrin complex (54) co-eluting with the spectrin. In fact, if concentrated preparations of purified spectrin dimer or tetramer are grossly overloaded and electrophoresed on SDS polyacrylamide slab gels according to Laemmli (26), trace amounts of band 4.1 are frequently visible (unpublished observations). On the other hand, it is also possible that the weak activity we observed for purified spectrin tetramer may represent a qualitatively different (perhaps lower affinity) interaction between spectrin and actin. Direct studies of spectrin-actin binding would aid in distinguishing between these alternatives.

Structure of the In Vitro Cytoskeleton

It is possible to draw only certain tentative conclusions regarding the actual nature of the supramolecular structures assayed by viscometry. The final viscosity attained by spectrin-actin-band 4.1 mixtures is much greater than that of F-actin alone, and under conditions under which we detect extensive increases in viscosity, the actin is present in great excess over the spectrin and band 4.1. This suggests that the structure detectable by low-shear viscometry consists of actin filaments cross-linked by spectrin plus band 4.1. However, our data do not allow us to determine whether band 4.1 acts to cross-link the spectrin to the actin or to cross-link spectrin molecules (which have independently bound to the actin) to each other, thus cross-linking the actin, or whether it acts by some other, more complex mechanism. Certainly, spectrin-actin-band 4.1 complexes formed at the spectrin tetramer:actin:band 4.1 ratios (1:3.5–4:2) that exist in the erythrocyte (unpublished data and references 30 and 50) might be qualitatively different from those formed in the presence of large amounts of excess actin, although they both might be manifested macroscopically as a gel. Moreover, cytoskeletal structures assembled in the plane of the membrane might be quite different from structures assembled in the absence of these constraints in solution. If these proteins are confined to the inner surface of the erythrocyte membrane in a volume not exceeding 10% of the total cell volume, their concentrations would be ~20 mg/ml of spectrin, 3.5–4 mg/ml of actin, and 4 mg/ml of band 4.1.[7] In this context, it is worth noting that there is at present no direct evidence regarding whether actin in the erythrocyte is in the G or the F state. The absence of identifiable actin filaments in thin sections of unperturbed ghost membranes (53) or in negatively stained images of the detergent cytoskeletons (30, 47, 50) could be explained by the presence of randomly oriented and highly cross-linked short actin filaments (see references 7, 51, and 52 for reviews). Other techniques, such as yield-strength measurements, strain birefringence, fluorescence spectroscopy, and electron microscopy (51, 52), will be required to elucidate the structure of this spectrin-actin-band 4.1 gel. It would be particularly interesting to compare the physical parameters that have been used to characterize the viscoelastic properties of the intact erythrocyte membrane (12, 24, 34) with similar measurements on spectrin-actin-band 4.1 gel structures formed in vitro.

Calcium Sensitivity

The inhibition of spectrin-actin-band 4.1 cross-linking and gelation by free calcium concentrations in the micromolar range is analogous to the effect of calcium on the interaction of actin with actin-gelling factors isolated from other nonmuscle cells (for reviews, see references 7, 23, 51, and 52). The free calcium ion concentration at which spectrin-actin-band 4.1 interactions are half-maximally inhibited ($2–3 \times 10^{-7}$ M) is strikingly similar to the free calcium ion concentration at which erythrocyte deformability is significantly decreased (1×10^{-7} M) (24). The biphasic shape of the calcium curve suggests that there might be more than one calcium-regulated event in the interaction of the spectrin- and band 4.1-containing extract with actin. Because the interaction of both the HMW complex and purified spectrin with actin is calcium sensitive, and because we cannot eliminate the possibility that the purified spectrin is contaminated with trace amounts of band 4.1, our experiments provide no clues regarding the location of the calcium-sensitive interaction(s) in this complex, multicomponent system.

[7] These concentrations are calculated both from our data and from data supplied by Steck (50); 10% of the cell volume corresponds to a layer about 600 Å thick under the cell membrane. Thus, the concentrations given may be somewhat underestimated.

It is interesting that a crude spectrin extract from erythrocyte membranes has been reported to contain high-affinity binding sites for calcium (25). The variability we observe in the degree of calcium sensitivity of the increases in viscosity could perhaps be related to the extent of phosphorylation of spectrin or some other component in the extract (38). Although we do not observe any proteolysis, our results do not rule out the possibility that a calcium-activated protease could contribute to the inhibition of spectrin-actin-band 4.1 gelation.

Cytoskeletal Control of Erythrocyte Shape and Deformability In Vivo

Erythrocyte shape and deformability are critically dependent on the level of intracellular calcium (10, 22, 24, 25, 30, 33, 34, 35, 40, 57). The cellular calcium concentration in fresh erythrocytes is between 5 and 15 μM (19, 34, 58), and indirect evidence suggests that the intracellular free calcium concentration may be <1 μM (44). There is some evidence suggesting that erythrocyte deformability in vivo may be regulated by calcium fluxes in the micromolar range (22, 24, 33, 40) similar to actin-based cytoskeletal and contractile systems in other cells (see references 7, 23, and 52 for reviews). These cell-shape and deformability changes are fully reversible (10, 43, 57), and should be distinguished from the irreversible changes induced by incorporation of millimolar quantities of calcium, which are suggested to be instrumental in erythrocyte senescence (for reviews, see references 30 and 34).

However, some of the effects of calcium on cell shape and deformability can not be easily explained by simple dissociation of a spectrin-actin band 4.1 gel underlying the membrane. Observations of ATP and calcium-dependent cellular contraction and stiffening of ghost membranes suggest that more active contractile processes may be involved (33, 40, 59). The energy-dependent shape changes and endocytosis described for erythrocyte ghosts may also be significant in this regard (35, 36, 45, 48). Earlier proposals that interactions between spectrin, actin, and associated proteins might represent the erythrocyte version of an actomyosin contractile apparatus (18, 21, 32, 46, 57) are worth reevaluating in light of our observation of a micromolar calcium-regulated interaction between spectrin, actin, and band 4.1. An erythrocyte version of the solation-contraction coupling mechanism suggested for motile cells (20, 52) could be responsible for erythrocyte shape and deformability changes.

Certainly, the regulation of erythrocyte shape and deformability involves processes other than the interactions among spectrin, actin, and band 4.1 (30, 34). It is, for example, possible that dissociation of a spectrin-actin-band 4.1 gel may modify the interaction of spectrin with its membrane attachment site, band 2.1 (4, 29, 60), and alter the interaction of other membrane proteins with the spectrin, actin, or band 4.1. Band 4.9, which copurifies with the high-molecular-weight spectrin-actin-band 4.1 complex may also be involved in the control of erythrocyte membrane properties. Thus, complete reconstitution of the erythrocyte cytoskeleton will almost certainly require components in addition to spectrin, actin, and band 4.1.

The authors would like to thank D. Branton and the members of his laboratory (including C. M. Cohen, S. R. Goodman, E. J. Luna, W. R. Hargreaves, and J. M. Tyler) for their material and intellectual contributions to this work. We are particularly grateful to W. R. Hargreaves for his generous gift of purified band 4.1 and for the enthusiasm and critical spirit of E. J. Luna. We would also like to acknowledge the influence on our thinking of disussions with W. Gratzer while this work was in progress. We also thank L. Griffith and T. D. Pollard for detailed explanations of the construction and use of the falling sphere viscometer, and L. Tanasugarn for building our unit. We would also like to thank G. Guidotti, J. Heiple, S. Hellewell, S. Virgin, Y.-L. Wang, A. Strauch, and J. LaFountain for their critical comments.

This work was completed in partial fulfillment of the requirements for the Ph.D. in the Department of Biology at Harvard University by Velia Fowler and was supported by National Science Foundation and National Institutes of Health predoctoral fellowships to Velia Fowler, a grant from the National Institute of Arthritis, Metabolism and Digestive Diseases (AM 18111) to D. L. Taylor and, in part, by NSF (PCM-77-13962) and NIH (HL 17411) grants to D. Branton.

Received for publication 12 September 1979, and in revised form 18 December 1979.

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