Partial Purification and Characterization of

an Actin-bundling Protein,

Band 4.9, from Human Erythrocytes

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ABSTRACT Band 4.9 (a 48,000-mol-wt polypeptide) has been partially purified from human erythrocyte membranes. In solution, band 4.9 polypeptides exist as trimers with an apparent molecular weight of 145,000 and a Stokes radius of 50 Å. Electron microscopy shows that the protein is a three-lobed structure with a radius slightly greater than 50 Å. When gel-filtered rabbit muscle actin is polymerized in the presence of band 4.9, actin bundles are generated that are similar in appearance to those induced by "vinculin" or fimbrin. The bundles appear brittle and when they are centrifuged small pieces of filaments break off and remain in the supernatant. At low band 4.9 to actin molar ratios (1:30), band 4.9 lowers the apparent steady-state low-shear falling ball viscosity by sequestering filaments into thin bundles; at higher ratios, the bundles become thicker and obstruct the ball's movement leading to an apparent increase in steady-state viscosity. Band 4.9 increases the length of the lag phase and decreases the rate of elongation during actin polymerization as measured by high-shear Ostwald viscometry or by the increase in the fluorescence of pyrene-labeled actin. Band 4.9 does not alter the critical actin monomer concentration.

We hypothesize that band 4.9, together with actin, erythrocyte tropomyosin, and spectrin, forms structures in erythroid precursor cells analogous to those formed by fimbrin, actin, tropomyosin, and TW 260/240 in epithelial brush borders. During erythroid development and enucleation, the actin filaments may depolymerize up to the membrane, leaving a membrane skeleton with short stubs of actin bundled by band 4.9 and cross-linked by spectrin.

Band 4.9 along with spectrin, actin, and band 4.1 are the main constituents of the erythrocyte membrane skeleton (see references 5, 17, 38, and 70 for recent reviews). Of the four skeletal components, band 4.9 is the only one that has not been formally studied. The belief that band 4.9 is a minor component of the membrane (39) may be due to its electrophoretic proximity to actin, which stains darkly on an SDS polyacrylamide gel, and to its exquisite sensitivity to proteolysis. A careful determination of the relative amount of band 4.9 in ghost membranes by either gel scanning or by the quantitative elution of Coomassie Blue stain with pyridine reveals that there is one band 4.9 polypeptide for every spectrin tetramer or about one band 4.9 polypeptide for every five actin monomers (see Fig. 1) (5). Values for the distribution of most erythrocyte membrane proteins based on Coo-

massie Blue–staining coincide closely with a very recent evaluation by amino acid analysis (79).

Information about band 4.9 has accumulated during studies of other red cell components. Unlike the other skeletal proteins, band 4.9 is phosphorylated by a kinase that is both cAMP-dependent and membrane-bound (25, 40, 76, 89). Band 4.1's kinase is also cAMP-dependent but it is not membrane-bound (48, 49, 73) and spectrin's kinase is cAMP-independent (26, 42, 87). Band 4.9's degree of phosphorylation is higher in sickle cells than in normal cells (23). This increased level of phosphorylation is apparently a result of the higher calcium levels found in sickle cells, since band 4.9 phosphorylation can be manipulated in normal cells with calcium and ionophore A23187 (53). These references to band 4.9 phosphorylation are intriguing because changes in red cell

shape have long been associated with the phosphorylation of some as yet undetermined membrane component or components.

The gel electrophoretic profiles of hereditary elliptocytic membrane skeletons presented in the studies of Mueller and Morrison (65), Tchernia et al. (88) and Feo et al. (28) reveal that in addition to the lower level of band 4.1 reported by these researchers, the levels of band 4.9 are also diminished. In addition, Holdstock and Ralston (46) and Ralston and Crisp (75) have reported that red cell cytoskeletons fall apart when treated with p-chloromercuribenzenesulfonic acid. Because one of the protein components to which the p-chloromercuribenzenesulfonic acid attaches is band 4.9, those authors suggested that the trigger site of p-chloromercuribenzenesulfonic acid–induced disruption might be band 4.9. They concluded that band 4.9 is important in stabilizing the erythrocyte membrane skeleton (46; but see also reference 47).

To study band 4.9, we partially purified this protein and investigated its interactions with the other erythrocyte cytoskeletal components. We discovered that band 4.9 is a trimeric actin-bundling protein that interacts with actin to form filaments similar in appearance to those induced by other known actin-bundling proteins.

MATERIALS AND METHODS

Partial Purification of ^{32}P-radiolabeled Band 4.9: Freshly drawn (within 72 h) normal human blood was obtained from the Northeast Regional Red Cross Program (Boston, MA). The erythrocytes, washed in labeling buffer (20 mM imidazole, 15 mM glucose, 1 mM MgCl$_2$, 50 mM NaCl, 5.6% sucrose [pH 7.4]) were incubated at 30% hematocrit with 0.5 mM cAMP, 0.01% penicillin-G and streptomycin, and 50 μCi/ml ^{32}P-orthophosphoric acid for 12 h at 37°C to label band 4.9 and other membrane components (89). The cells were again washed in labeling buffer and ghosts were prepared in 5 mM NaPO$_4$, 1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride (pH 7.6) (22) (Fig. 1 a). Washing and lysing erythrocytes in the presence of 5 mM phosphate buffer supplemented with 1 mM Mg does not increase the amount of band 4.9 in the final ghosts (80). After a brief incubation in phosphate-buffered saline (155 mM NaCl, 5 mM NaPO$_4$, 1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride [pH 7.6]) to remove band 6 (54), the membranes were washed twice in 5 mM NaPO$_4$, 1 mM EGTA (pH 8.0) and extracted with 0.5% Triton, 7.5 mM NaPO$_4$, 1 mM EGTA (pH 8.0) containing the following protease inhibitor cocktail: 2 mM diisopropylfluorophosphate, 10 U/ml Trasylol, 10 μg/ml benzamidine, 2 μg/ml antipain, and 1 μg/ml each of leupeptin, chymostatin, and pepstatin A. After 20 min on ice, the Triton-insoluble membrane skeletons were sedimented at 39,000 g for 30 min, washed once with 7.5 mM NaPO$_4$ buffer (pH 8.0) containing 0.5% Triton, and then once in the same buffer without Triton (Fig. 1 b). The membrane skeletons were dissociated by incubation in 10 vol of 3 mM Tris, 0.5 mM EDTA, 2 mM dithiothreitol (pH 8.5) with the protease inhibitor cocktail but without diisopropylfluorophosphate for 45 min at 37°C. The material was centrifuged at 130,000 g for 45 min to remove any insoluble material and the supernatant was made 20 mM in Tris (pH 8.3) and loaded at 30 ml/h onto a DEAE-Sephacel column (2.5 × 35 cm) pre-equilibrated in 20 mM Tris, 20 mM KCl, 0.5 mM dithiothreitol, 1 mM EGTA, 0.02 % NaN$_3$ (pH 8.3). The column was developed with a 500-ml linear 20–300 mM KCl gradient in the equilibration buffer. The use of Triton X-100, EDTA, and other reagents in our preparations made the column elution profile monitored by ultraviolet light useless. SDS polyacrylamide gels (Fig. 1, c and d) showed that band 4.9 eluted as 160 mM KCl entered the top of the column, whereas band 4.1, spectrin, and actin began to elute as 220 mM KCl entered the column. Band 4.9 was concentrated by precipitation with 80% ammonium sulfate at pH 7.0 and centrifugation at 10,000 g for 45 min in siliconized COREX tubes. The pellet was brought up in 1–2 ml of 100 mM KCl, 20 mM Tris, 1 mM EGTA, 1 mM dithiothreitol, 0.02 % NaN$_3$ (pH 8.3), and dialyzed versus this buffer to remove residual ammonium sulfate.

Actin Purification: Actin was prepared from rabbit skeletal back and leg muscles according to the method of Spudich and Watt (84) and gel-filtered on Sephadex G-150 as described by MacLean-Fletcher and Pollard (63). It was stored at 4°C by dialysis versus G-actin buffer (2 mM Tris, 0.2 mM ATP, 0.2 mM CaCl$_2$, 0.5 mM dithiothreitol, 0.02% NaN$_3$ [pH 8.3]).

R$_s$ Determination for Band 4.9: Purified band 4.9 was chromatographed at 2.75 ml/h on a Sephadex G-150 column (1.0 × 60 cm) equilibrated with 100 mM KCl, 20 mM Tris, 1 mM EGTA, 0.5 mM dithiothreitol, 0.02% NaN$_3$ (pH 7.6). Standard proteins were rabbit muscle aldolase (R$_s$ = 48.1 Å), bovine serum albumin (R$_s$ = 35.5 Å), ovalbumin (R$_s$ = 30.5 Å), and pancreatic chymotrypsinogen A (R$_s$ = 20.9 Å). A plot of log R$_s$ versus elution volume fit a straight line with a correlation coefficient of 0.98.

Viscometry Measurements: High-shear Ostwald capillary viscometry was performed as previously described (18) using a Cannon viscometer no. 150 (Cannon Instrument Co., State College, PA) immersed in a 25°C water bath. The sample size was 0.6 ml. Low-shear falling ball viscometry was performed as described (74) and the data were plotted as the seconds required for the ball to fall 1 cm at an angle of 80°.

Pyrene-labeled Actin Fluorescence: Pyrene-labeled actin was prepared according to the method of Kouyama and Mihashi (56) as modified by Cooper et al. (19). Measurements of fluorescence were made in an SLM 4800 spectrofluorometer (SLM Instruments, Inc., Urbana, IL) interfaced with a Commodore 2001 Series computer. A thermostatted cuvette holder maintained the cuvette (0.3 × 0.3 cm, quartz) and sample (140 μl) at 25°C. The excitation and emission wavelengths were 365 and 407 nm, respectively. To minimize fluorescence bleaching, we used an excitation slit width of 0.5 nm and we took measurements at 2 to 3 min intervals. The emission slit width was 8.0 nm. Under these conditions, measurements were not affected by light scattering. This was verified by measuring unlabeled actin samples as well as dilute ghost suspensions. Fluorescence-depleted imidazole Grade III (Sigma Chemical Co., St. Louis, MO) was used as a pH buffer.

Microscopy: Light photomicrographs were taken with a Leitz microscope equipped with epifluorescent optics. Negatively stained preparations of actin and actin/band 4.9 complexes were prepared as described (18) and examined in a Philips EM301 electron microscope at 80 kV.

Other Methods: SDS polyacrylamide slab gels (9.0 × 6.5 cm) were run according to Laemmli (57) with a 10% separation gel and a 6% stacking gel. Relative dye content of gel bands was determined according to the pyridine elution method (27). Protein concentrations were measured according to the Bradford dye-binding assay (4) using bovine serum albumin as a standard.

RESULTS

Partial Purification of Band 4.9

Regardless of method, obtaining milligram quantities of band 4.9 is difficult because this polypeptide is very sensitive to proteolysis and it comprises only 1% of the total erythrocyte ghost protein. A number of methods for isolating relatively pure (greater than 89%) band 4.9 preparations completely free of spectrin, actin, and band 4.1 were developed. Two basic approaches involved either eluting band 4.9 from spectrin- and actin-depleted inside-out vesicles (as in the purification of ankyrin and band 4.1) (95) or extracting band 4.9 from membrane skeletons derived from Triton-extracted ghosts. The former approach yielded dephosphorylated band 4.9 (data not shown). Although this observation raised some interesting questions concerning the role phosphorylation might play in band 4.9's cytoskeletal interactions (see Discussion), it was felt that the initial band 4.9 studies should use material that included phosphorylated band 4.9. Band 4.9 was therefore isolated from the membrane skeletons which contained spectrin, actin, band 4.1, and band 4.9 (Fig. 1). Because the band 4.9 in these skeletons remained bound to the spectrin-actin-band 4.1 complex under physiological salt conditions, it was, by definition, judged to be the active binding form.

Of a variety of agents known to disrupt cytoskeletal protein interactions including charged sulfhydryl modifiers (46, 75), polyphosphates (77, 78), and molar concentrations of Tris (21), incubating Triton-insoluble membrane skeletons at 37°C in low ionic strength, EDTA-containing, alkaline buffer most effectively freed band 4.9 from the membrane skeleton (see Materials and Methods). This method was specifically de-
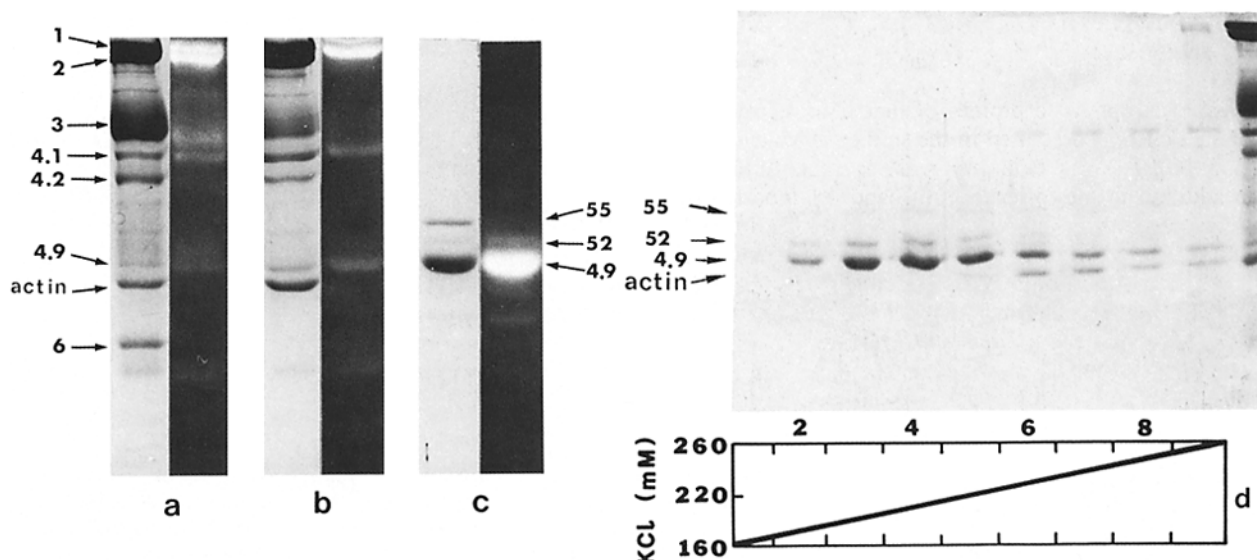
FIGURE 1 Partial purification of band 4.9. SDS polyacrylamide gels stained with Coomassie Blue show the protein composition at different stages during purification (a, b, and c; left in each pair) and autoradiograms (a, b, and c; right in each pair). Shown are (a) ghosts, (b) Triton X-100–insoluble membrane skeletons, and (c) partially purified band 4.9. See text for details. Note that in a the radiolabeled band in the actin region is band 4.9, not actin, as has sometimes been assumed. Although it appears in a and b that both bands 1 and 2 of spectrin are radiolabeled, the upper labeled band is ankyrin (band 2.1) which runs above band 2 in the Laemmli gel system (90). A typical DEAE-Sephacel column elution pattern (d) shows band 4.9 elutes as 160 mM KCl enters the column (lane 1 and graph of corresponding KCl gradient, below). Polypeptide bands are numbered according to Steck (86).

signed to denature actin and favor the breakdown of spectrin tetramers to dimers.

With this purification protocol, ~2–3 mg of band 4.9 was routinely obtained from one unit of blood (>40% of the theoretical maximum yield). The preparation, completely free of spectrin, actin, and band 4.1, was at least 89% pure as judged from scans of Coomassie-stained, SDS polyacrylamide gels. The principal contaminants were polypeptides with molecular weights of 55,000, which accounted on a mole basis for up to 9% of the protein in the purified preparations, and 52,000, which accounted for about 2% (Fig. 1, c and d). The presence of these components raised the possibility that they were responsible for some or all of the actin-binding effects presented in the following sections. But the 55,000-mol-wt polypeptide did not bind to actin in any of the sedimentation assays presented below. It did not appear to be related to or complexed with band 4.9 because it eluted off the DEAE-Sephacel column slightly before band 4.9 (Fig. 1 a) and eluted off the Sephadex G-150 sizing column just after band 4.9 (data not shown). Whereas band 4.9 was phosphorylated, the 55,000-mol-wt polypeptide did not appear to be phosphorylated (Fig. 1 c). The 55,000-mol-wt polypeptide may in fact be related to band 4.1 since it competed with band 4.1 for binding to spectrin (personal communication, Dr. Larry T. Mimms). On the other hand, the 52,000-mol-wt polypeptide appears to be related to band 4.9: it did bind to actin, it co-purified precisely with band 4.9 on both ion exchange and gel filtration columns, and it was phosphorylated (Fig. 1 c). Recent experiments indicate that the 52,000-mol-wt component, but not the 55,000-mol-wt contaminant, cross-reacts with a polyclonal antibody to DEAE-purified band 4.9 further purified by eluting only the polypeptide at the band 4.9 position from the SDS polyacrylamide gel (J. Hung, personal communication). Further work to purify and characterize band 4.9 is in progress but our current view, based on the antibody cross-reactivity and on a limited series of two dimensional maps, is

that the 52,000-mol-wt polypeptide is a closely sequence-related form of band 4.9.

Band 4.9 Exists as a Trimer

In solution, band 4.9 is a trimer with an apparent molecular weight of 145,000 and a Stokes radius of 50 Å as determined by gel filtration on Sephadex G-150 (see Materials and Methods) and chemical cross-linking by copper-phenanthroline catalyzed oxidation (Fig. 2). Low-angle, rotary-shadowed images showed that the protein is a three-lobed structure with a radius slightly greater than 50 Å (Fig. 3).

Sedimentation Properties and Critical Monomer Concentration of F-actin in the Presence of Band 4.9

We have not detected any binding between band 4.9 and purified spectrin under a variety of conditions. But when band 4.9 was mixed with gel-filtered rabbit muscle G-actin and placed in polymerizing conditions, band 4.9 molecules bound to and sedimented with the actin filaments (Fig. 4). Furthermore, more actin remained in the supernatant of the samples containing band 4.9 than in the supernatant of the samples polymerized in the absence of band 4.9 (Fig. 4). Since this inhibition of actin sedimentation was completely prevented by pretreating band 4.9 with trypsin (81), the phenomenon appeared to be a protein-mediated event that required intact band 4.9 molecules.

The additional actin in the supernatant of band 4.9–containing samples could be accounted for if band 4.9 molecules capped the "barbed" ends of the actin filaments, raising the critical monomer concentration to the higher value of the "pointed" end as does villin, gelsolin, and other capping proteins (8, 20, 35, 50, 55, 64, 98). Alternatively, band 4.9 molecules might have prevented actin polymerization by
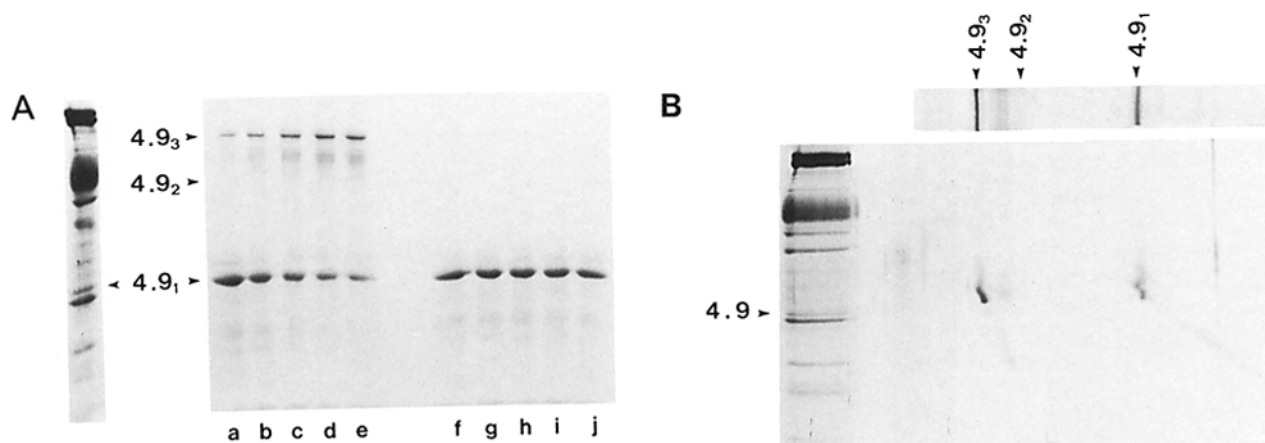
FIGURE 2 Copper/phenanthroline–catalyzed cross-linking of purified band 4.9. (A) One-dimensional analysis of cross-linked products. Samples of band 4.9 in 100 mM KCl, 50 mM triethanolamine (pH 8.0) with (f–j) or without (a–e) 5 mM p-chloromercuribenzenesulfonic acid were incubated for 1.5 h at 25°C with the following concentrations, in μM, of CuSO₄/o-phenanthroline: (a and f) 0/0; (b and g) 1.7/3.4; (c and h) 17/34; (d and i) 170/340; (e and j) 1,700/3,400. The reaction was stopped by the addition of sufficient concentrated EDTA to make the sample 2 mM in EDTA. The samples were then dissolved in sample buffer without reduction and electrophoresed as in Fig. 1. Note the disappearance of band 4.9 and concomitant increase in higher molecular weight products with increasing copper/phenanthroline in lanes a–e. Inhibition of cross-linking by the sulfhydryl modifier p-chloromercuribenzenesulfonic acid shows that cross-links are produced by sulfhydryl oxidation (lanes f–j). The principal cross-linked product corresponds to the molecular weight of a band 4.9 trimer (145,000). A light band, presumably a partially cross-linked trimer, is barely visible at the band 4.9 dimer position (96,000). Cross-linked products corresponding to molecular weights between 110,000 and 130,000 are believed to result from trimers containing pieces of proteolyzed band 4.9 molecules. Note that a small amount of purified band 4.9 appears to have already been oxidized into a cross-linked product in the absence of copper/phenanthroline (lane a). This may be related to Liu and Palek's observations (62, 68) that band 4.9 forms spontaneous sulfhydryl cross-links into higher molecular weight complexes in ATP-depleted erythrocytes that lack reducing power. (B) Two-dimensional analysis of cross-linked products shows that the high molecular weight complexes comprise band 4.9. A gel lane corresponding to lane e was incubated in gel sample buffer containing 5% 2-mercaptoethanol for 15 min at 25°C and run on a second gel to determine the composition of each Cu/P-produced complex. A replicate gel of the first dimension is placed at the top of the slab and a ghost sample applied directly on the second-dimension gel is shown at the left for reference. Note that the 145,000-mol-wt putative cross-linked band 4.9 is reduced to band 4.9, and the 110,000–130,000 molecular weight complexes break down to band 4.9 and lower molecular weight band 4.9 fragments.
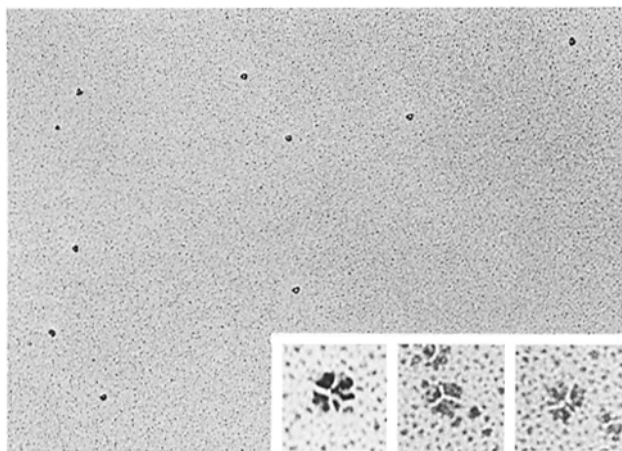
FIGURE 3 Low-angle, rotary-shadowed images of purified band 4.9. Specimens were sprayed onto mica in 30% glycerol according to the method of Tyler and Branton (96). (Band 4.9 trimers dissociated into monomeric and dimeric species in the higher glycerol concentrations recommended by Tyler and Branton.) × 56,000 (inset, × 550,000).

complexing actin monomers, as does profilin (12) or DNAse I (45). The additional actin in the supernatant could also be accounted for if band 4.9 helped to nucleate actin polymerization, producing many short, slowly sedimenting filaments, as does villin (8, 20, 35, 64) or accumentin (82, 83).

To distinguish among these possibilities, we measured the equilibrium viscosities of actin filaments with and without band 4.9 (Fig. 5). Band 4.9 did not alter the critical actin monomer concentration. Furthermore, no interaction between monomeric actin and band 4.9 was detected by sucrose density gradient centrifugation, gel filtration, or cross-linking studies (data not shown). Short actin filaments were in fact found when negatively stained supernatants containing the extra nonsedimented actin were examined in the electron microscope. No filaments were observed in the supernatants of the controls (data not shown). Finally, when the sedimentation assay of Fig. 4 was repeated using longer centrifugation or greater centrifugal force, the supernatant in the band 4.9–containing tubes was depleted of the extra actin and contained only an amount of actin identical to that in the control without band 4.9 (data not shown). These results indicated that the extra actin was in the form of short filaments that were generated by some interaction with band 4.9.

Light and Electron Microscopy of Actin/Band 4.9 Structures

Large fibers, up to 0.8 μm in diameter, formed when G-actin was polymerized in the presence of band 4.9 (Fig. 6a). Each of these fibers was a bundle of many actin filaments (Fig. 6b), reminiscent of actin bundles generated by vinculin (51, 52), fimbrin (8), or high concentrations of magnesium chloride (41). In fibers of actin/band 4.9 preparations aged
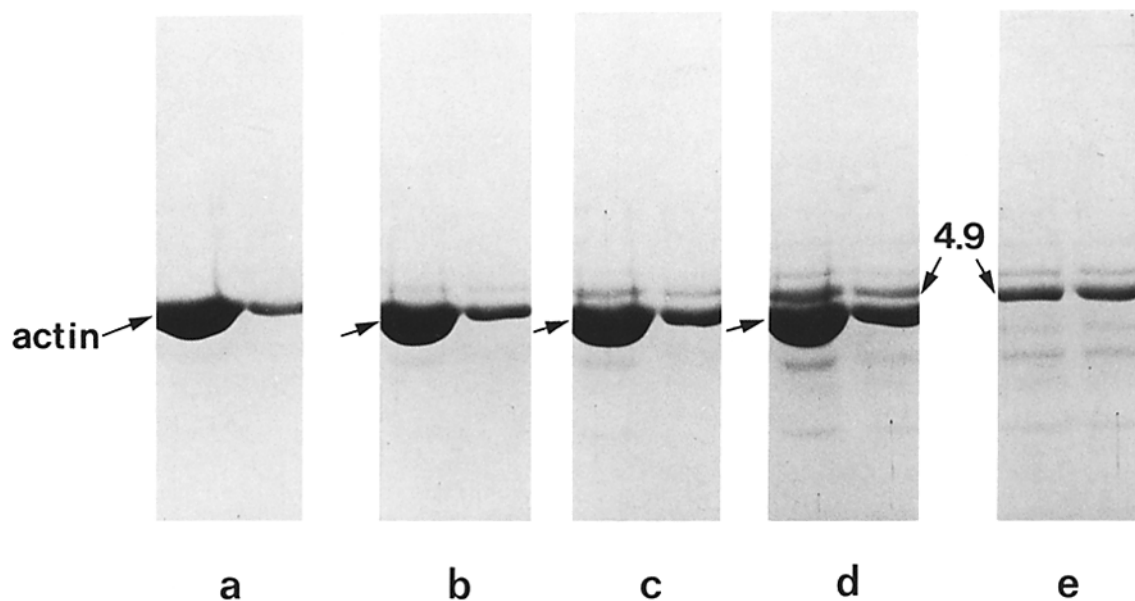
FIGURE 4 Effect of band 4.9 on the high-speed sedimentation of F-actin. Gel-filtered G-actin (a) or band 4.9 (e) or both (b–d) were incubated under polymerization conditions for 2 h at 25°C and centrifuged for 20 min at 95,000 g (average) in a Beckman airfuge (Beckman Instruments, Palo Alto, CA). The SDS polyacrylamide gel shows the composition of the initial total sample mixtures and of the supernatants after centrifugation (left and right lanes within each panel, respectively). All samples contained 200 μg/ml actin, 50 mM KCl, 28 mM Tris, 1 mM ATP, 2 mM MgCl$_2$, 0.02 mM CaCl$_2$, 0.8 mM EGTA, 1 mM dithiothreitol, 0.02% NaN$_3$ (pH 7.6) but the molar ratios of band 4.9 to actin were (b) 1:50, (c) 1:20, and (d) 1:12. Note that as the amount of band 4.9 increases (b–d), more actin remains in the supernatant than in the control (a). Approximately 20–30% of band 4.9 molecules pellet in the presence (b–d) but not the absence (e) of actin (determined by densitometry of pyridine–eluted Coomassie Blue stain from gel bands [27] and by measurement of the distribution of ^{32}P-labeled band 4.9 by scintillation counting of supernatants and pellets).

for 1 wk at 4°C, the filaments were more tightly packed, and the banding pattern typical of in-register filaments was noticeable at intervals of ~36 nm (arrow heads, Fig. 6c). Because short actin filaments broke off where the fibers bent, the bundles appeared brittle (Fig. 6b). These short filaments released from the brittle fibers were most likely the source of the extra nonsedimented actin observed in the sedimentation studies (Fig. 4).

Steady-state Low Shear Viscosity of Actin in the Presence of Band 4.9

The steady-state low shear viscosity of actin was measured in a falling-ball viscometer. In the presence of increasing amounts of band 4.9, the viscosity of the actin/band 4.9 preparations decreased as the concentration of band 4.9 approached a band 4.9/actin molar ratio of about 1:30 (Fig. 7). At higher concentrations of band 4.9, the ball displayed a saltatory movement, falling freely between patches of flocculent material that retarded its movement. At stoichiometric concentrations of band 4.9, the patches of flocculent material became so large that the ball's movement was totally obstructed (Fig. 7, last data point and inset). Microscopic analysis of the flocculus showed that it comprised actin bundles whose diameter increased with increasing concentration of band 4.9. Therefore, at low band 4.9 to actin molar ratios (1:30), band 4.9 lowered the apparent steady-state low shear viscosity by sequestering filaments into thin bundles; at higher ratios, the bundles became thicker and obstructed the ball's movement leading to an apparent increase in steady-state viscosity.

Polymerization Kinetics of Actin in the Presence of Band 4.9

To further characterize the actin/band 4.9 interaction, we followed the kinetics of the polymerization process in the presence of band 4.9 with high shear Ostwald viscometry or with the increase in the fluorescence of pyrene-labeled actin. The advantage of the latter technique is that the fluorescence signal emitted by the pyrene-labeled actin as it polymerizes is proportional only to the polymer mass and is unaffected by filament length (19). Ostwald viscometry showed that band 4.9 increased the length of the lag phase (Fig. 8) and both techniques showed that band 4.9 decreased the rate of elongation during actin polymerization (Figs. 8 and 9a). Furthermore, the final level of viscosity was lower than that of the control (Fig. 8), as would be expected if many small filaments of actin were released from the brittle actin bundles as they were sheared by the viscometer.

When polymerization was nucleated by the addition of F-actin seeds to eliminate the lag phase, elongation was still attenuated (Fig. 9b). This showed that band 4.9 affects the elongation phase of polymerization independent of any possible effect on nucleation. Of particular interest was the observation that the final level of fluorescence was greater in the presence of band 4.9 than in the control. A simple interpretation of this result is that more polymer formed. In theory, this could result from the binding of band 4.9 molecules to the pointed ends of actin filaments which would shift the critical concentration to the lower value that characterizes the barbed end. But in practice this change would be very small (less than a 2% increase in the amount of polymerized actin) and would probably not be detected by our technique (82).

Alternatively, the bundling of actin fibers might sufficiently alter the environment of the pyrene probe and increase its quantum yield. To distinguish among these two possibilities, we measured the fluorescence emission during the polymerization of pyrene-labeled actin into magnesium-induced actin paracrystals (Fig. 10). The kinetics of assembly was qualitatively similar to polymerization in the presence of band 4.9. In subsequent experiments, band 4.9 to actin ratios were found that produced fluorescence curves quantitatively identical to those for the magnesium-induced bundles (data not shown). These experiments suggest that whatever mechanisms are responsible for the band 4.9–induced inhibition of actin filament growth and the higher steady-state fluorescence lev-
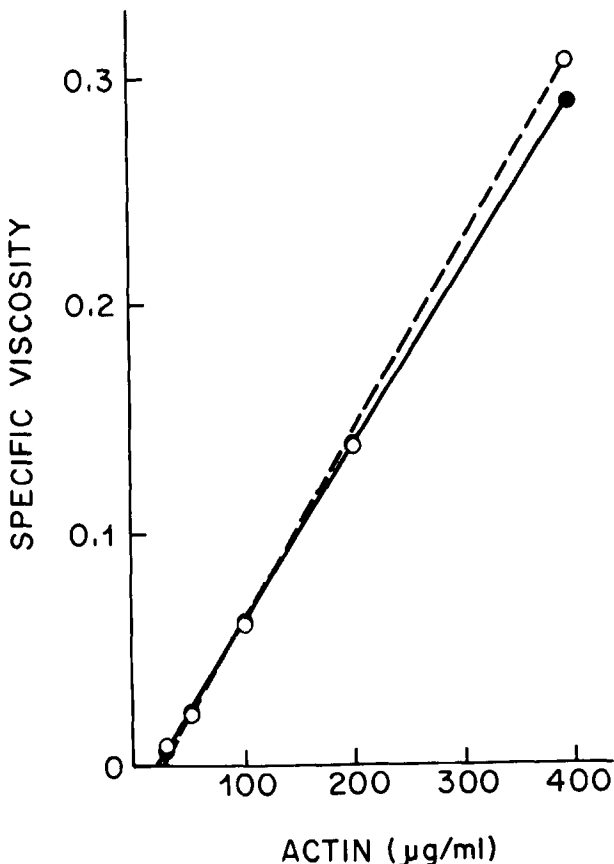

FIGURE 5 Effect of band 4.9 on the equilibrium viscosity of actin filaments. Gel-filtered G-actin at concentrations between 25 and 400 μg/ml was polymerized for 16 h at 25°C in 100 mM KCl, 20 mM imidazole, 1 mM dithiothreitol, 1 mM ATP, 15 mM Tris, 0.03 mM $CaCl_2$, 0.73 mM EGTA, 0.02% NaN_3 (pH 7.0) with a fixed molar ratio of band 4.9 (1:20, band 4.9 to actin) (○) or without (●). Equilibrium viscosities were measured in an Ostwald viscometer (18). Critical monomer concentration was defined as the intercept on the x axis of the line representing specific viscosity as a function of actin concentration (18). Correlation coefficients from linear regression analyses of data points with and without band 4.9 were 0.9996 and 0.9999, respectively.

els, they may be a consequence of the rapid aggregation of growing filaments into tightly packed bundles.

The effects of band 4.9 on actin sedimentation, steady-state viscosity, and polymerization kinetics were independent of the presence of either free calcium or magnesium concentrations between 0.3 nm and 2.0 mM.

DISCUSSION

Comparison of Band 4.9 to Other Actin-bundling Proteins

Our results show that band 4.9 polypeptides are the subunits of a trimeric actin-bundling protein. Actin filaments polymerized in the presence of band 4.9 (Fig. 6) form bundles that are similar in appearance to those induced by other actin-bundling proteins (9, 34, 51, 97). We compared band 4.9 with four other known actin-bundling proteins: fimbrin (34), fascin (10, 67, 85), villin (8, 20, 36, 64), and "vinculin" (51, 52). ("Vinculin" is in quotation marks because recent experiments [24] indicate that the polypeptides that bind to F-actin and that are responsible for the diminished viscosity and the bundling of actin are probably in the mixture of unnamed contaminants with various molecular weights that are found in the usual preparations of vinculin.) Although all five proteins appear to differ from one another by at least one criterion, some of their similarities are striking. With the exception of fascin-induced actin bundles which show a marked 11-nm banding pattern, the negatively stained images of bundles generated by the other four proteins are virtually indistinguishable from one another. For fimbrin, Bretscher reported that the bundles frequently had kinks and breaks in them which suggests that they are rather rigid and brittle (9). In addition, the polymerization kinetics for actin/"vinculin" solutions measured by high shear viscometry (9, 51, 52, 97) are qualitatively identical to the kinetics of actin polymerization in the presence of band 4.9 (Fig. 8). The saltatory movement of the steel ball observed at high band 4.9 concentrations during low shear viscometry experiments (Fig. 7) has been observed for other actin/actin-bundling protein interactions (Dr. J. Cooper, personal communication).

Because the 55,000-mol-wt polypeptide that co-purifies with band 4.9 does not bind to actin, we are confident that band 4.9 and the closely related 52,000-mol-wt polypeptide are the components responsible for the actin bundling activity and viscosity alterations we have observed. But the precise reason why our band 4.9 preparations reduce the rate of actin polymerization remains an open question. While band 4.9 does not alter the critical actin monomer concentration (Fig. 5), both high shear viscometry (Fig. 8) and fluorescence measurements (Fig. 9) show that band 4.9 increases the length of the lag phase and decreases the rate of elongation during actin polymerization. Similar changes in the kinetics of polymerization are noted when actin is polymerized in magnesium (Fig. 10) or with "vinculin" (52, 97). It is possible that the binding of a band 4.9 trimer to the side of an actin

FIGURE 6 Microscopy of actin/band 4.9 complexes. Preparations of actin (0.4 mg/ml) polymerized in the presence of band 4.9 (band 4.9 to actin molar ratio, 1:10) were viewed at low magnification by phase-contrast (a) (× 1,900) and at higher magnifications by negative staining (b and c) (× 100,000 and × 250,000, respectively). d shows the control specimen of actin alone (× 100,000). The preparations in a and b were viewed immediately after polymerization; that in c was aged for 1 wk at 4°C. Buffer conditions were identical to those in Fig. 5.
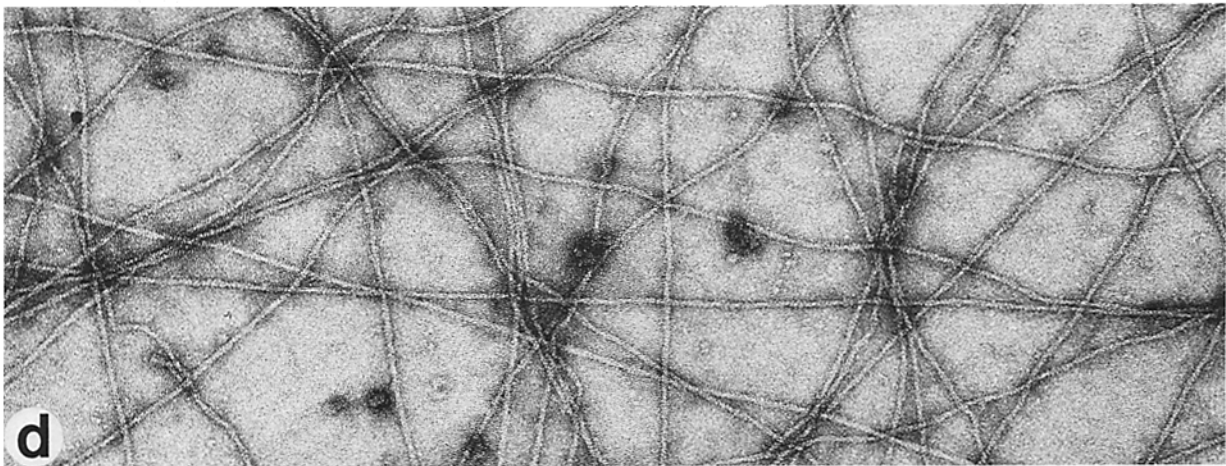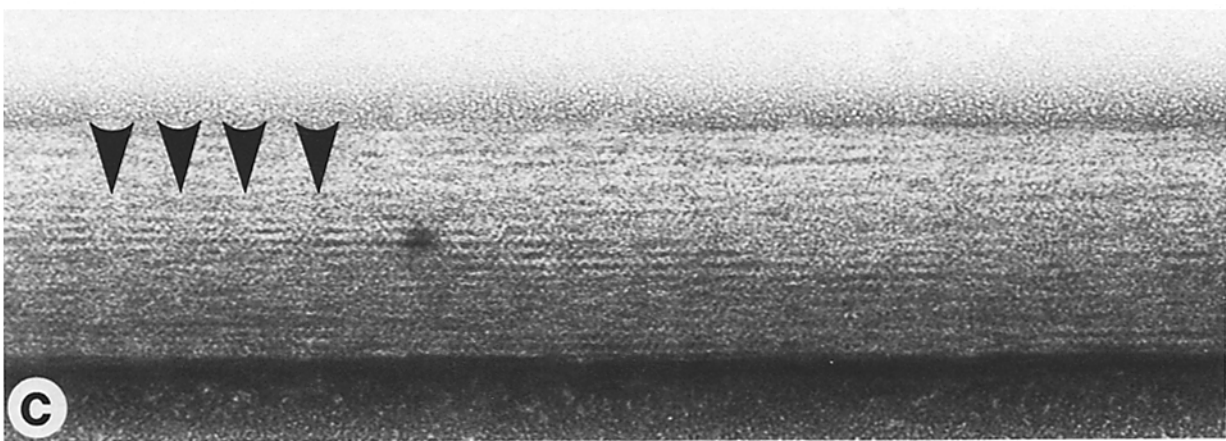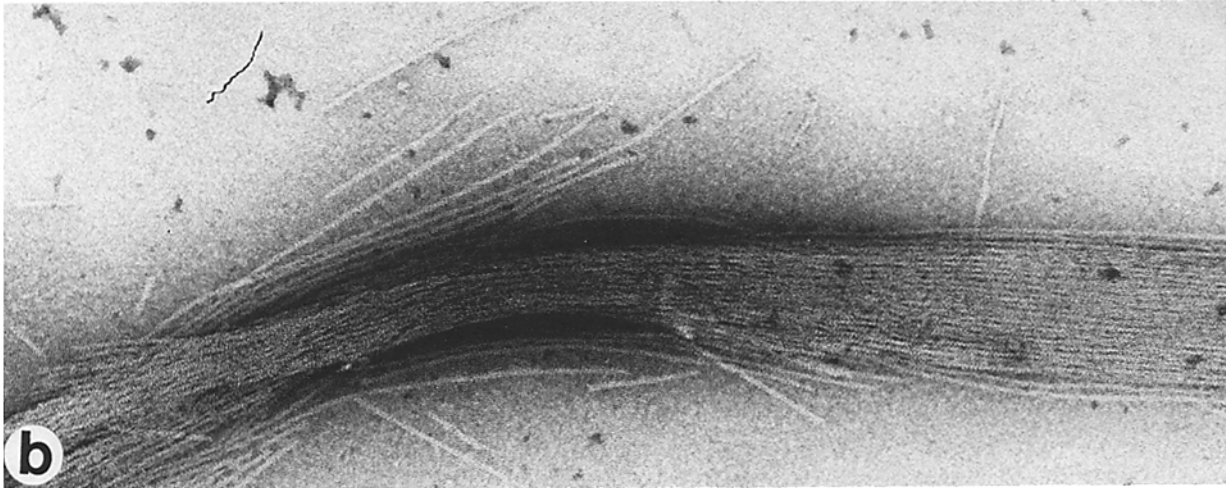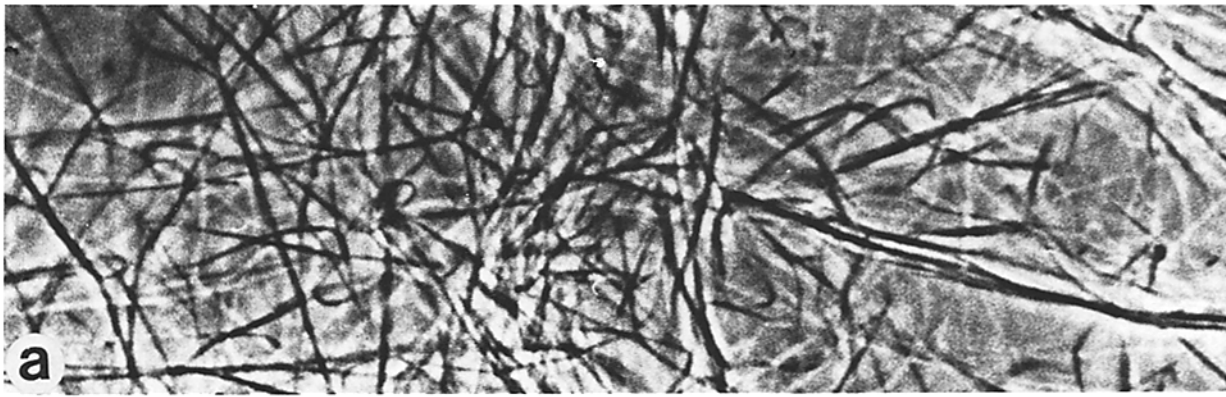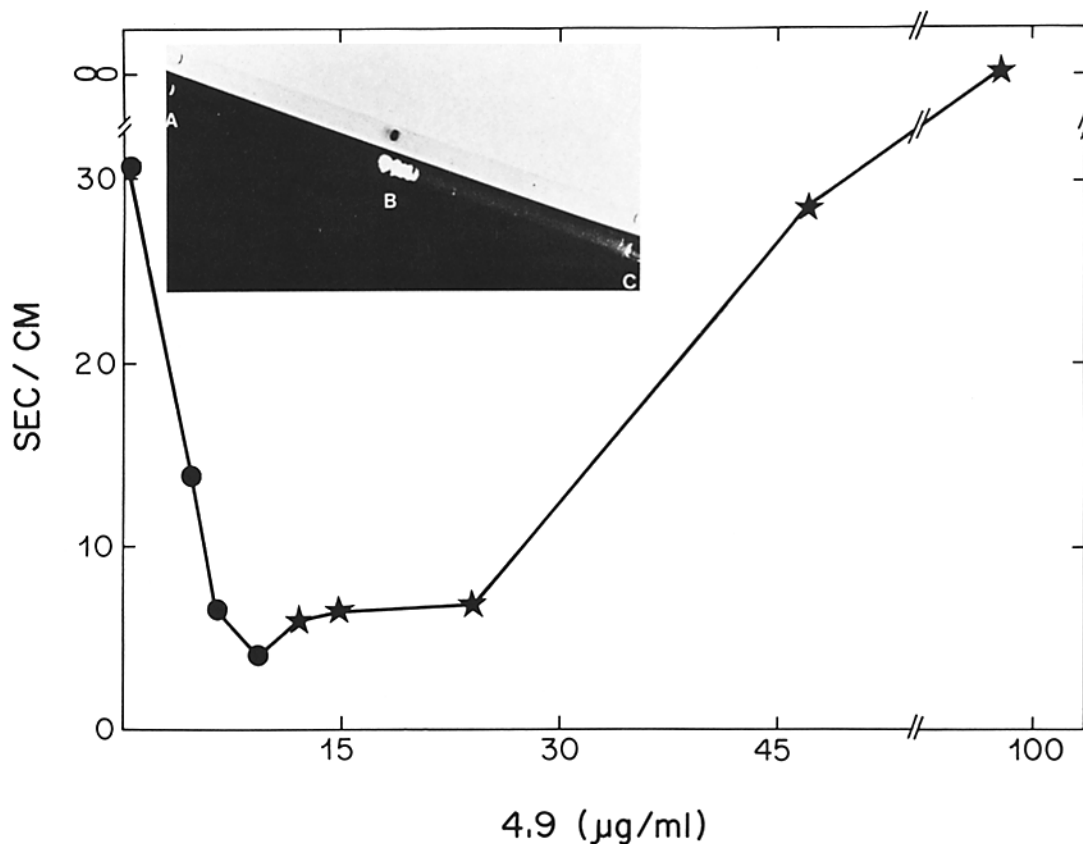
FIGURE 7 The effect of band 4.9 concentration on actin's steady-state low shear viscosity. Gel-filtered G-actin (final concentration 400 μg/ml) was mixed with various concentrations of band 4.9. Polymerization was induced by addition of salt and samples were immediately drawn into 100-μl capillary pipettes and allowed to incubate at 25°C for 6 h. Buffer conditions were identical to those in Fig. 5. Each point represents the mean of three measurements. Above 10 μg/ml band 4.9 (data points indicated by stars) the ball fell in a saltatory fashion (see text for details). *(Inset)* A microcapillary pipette containing pyrene-labeled actin incubated with the maximum band 4.9 concentration plotted on the graph was illuminated by white (a) or ultraviolet (b) light. The actin-containing flocculus accumulated around the stainless steel ball as it fell from the meniscus (point A) to its final position (point B). Note the absence of fluorescent material in the region through which the ball passed, and its presence in the region below the ball's resting place (points B to C). The high degree of fluorescence visible to the unaided eye is a consequence of the bundling of the actin fibers by band 4.9, since the fluorescence in pipettes containing only pyrene-labeled actin filaments is not visible in these conditions (data not shown).

filament alters the actin subunit's conformation in a way that lowers its affinity for other monomers (cf. reference 3).

Band 4.9 Capping

Since band 4.9 does not raise the critical actin monomer concentration, it is unlikely that it caps the barbed end of actin filaments. This is further supported by the fact that the barbed end of the actin contained within the spectrin/actin/band 4.1/band 4.9 complexes isolated from ghost extracts is free to nucleate actin growth (13, 59–61) and that in vivo the barbed ends (72) and perhaps both ends (94) of actin are free.

Function of Band 4.9 in the Erythrocyte

The membrane skeleton has been viewed as a two-dimensional network constructed of spectrin tetramers cross-linked by actin and band 4.1–containing centers and attached to the membrane via ankyrin and band 3 (see Fig. 5) (5). But the in situ organization of the membrane skeleton components has not been extensively studied and the precise organization of actin at the junction points is not clear. It is believed that the actin is in the form of short "protofilaments" (6, 7, 13, 72). To understand how band 4.9 may interact with actin in vivo,

it is useful to consider the roles actin-bundling proteins are thought to play in other cell types. For example, "vinculin" will pack actin into long bundles in vitro like band 4.9 (51, 52), but in vivo it is believed to be located at the tips of long actin filaments holding them in a bunch so that they can connect with spectrin-like cross-linking proteins (11, 29, 32, 33, 92). It is reasonable to suppose that, before enucleation, developing erythrocytes also possessed actin bundles as well as microtubules, organelles, and other common cellular components. If so, then during development, the transcellular actin filaments might depolymerize up to the membrane where the spectrin molecules insert. This would leave short stubs of actin clustered together by band 4.9. The essential feature of this model is the suggestion that an actin-containing center could consist of more than one protofilament.

Although the existence of such truncated bundles in erythrocytes has yet to be demonstrated, the 15–100-nm diameter globular complexes cross-linked by spectrin seen in thin-sectioned, negatively stained, or rotary-shadowed images of cytoplasmic red cell membrane surfaces (66, 91, 93) appear too large to comprise a single actin protofilament even if one adds on the size of several band 4.1 molecules. In other cell types, spectrin-like proteins cross-link bundles of actin fila-
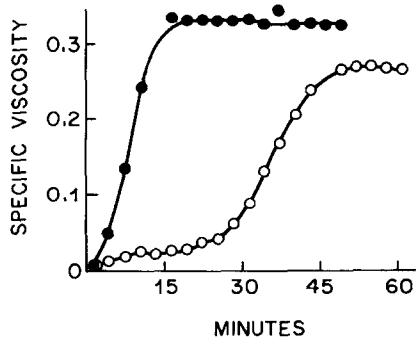
FIGURE 8 Effect of band 4.9 on the time course of actin polymerization as measured by high-shear viscometry. Polymerization of gel-filtered actin in the presence and absence of band 4.9 was followed with an Ostwald viscometer at 25°C after the addition of salt at time zero. Conditions: actin (0.3 mg/ml) alone (●) or with band 4.9 (○), molar ratio band 4.9 to actin, 1:14; 50 mM KCl, 0.23 mM ATP, 20 mM imidazole, 6.7 mM Tris, 0.06 mM CaCl₂, 1.2 mM EGTA, 1.5 mM dithiothreitol, 0.02% NaN₃ (pH 7.0).
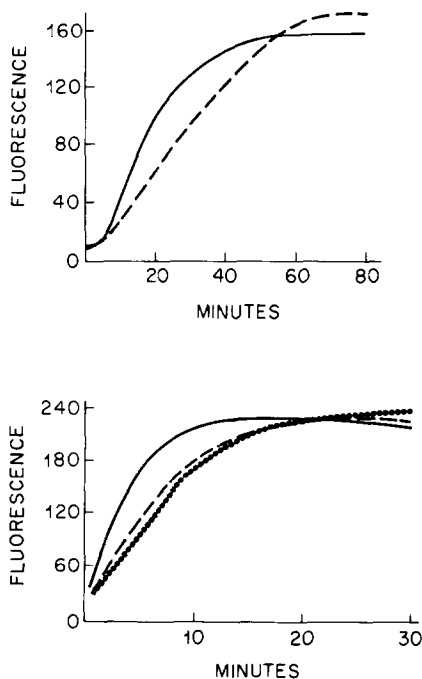

FIGURE 9 Effect of band 4.9 on the polymerization kinetics of pyrene-labeled actin. (a) The polymerization of gel-filtered pyrene-labeled actin in the presence and absence of band 4.9 was followed by its increase in fluorescence at 25°C after the addition of salt at time zero. Conditions: actin (0.2 mg/ml) alone (solid line) or with band 4.9 (dashed line); molar ratio of band 4.9 to actin, 1:10; 50 mM KCl, 0.5 mM ATP, 20 mM imidazole, 8 mM Tris, 1.5 mM dithiothreitol, 0.36 mM CaCl₂, 0.8 mM EGTA, 0.02% NaN₃ (pH 7.0). (b) Same as a except polymerization was nucleated by the addition of F-actin seeds at time zero. Conditions: actin (0.2 mg/ml G-actin plus 20 μg/ml sheared F-actin filaments) alone (solid line) or with band 4.9 to actin ratio of 1:45 (broken line) or 1:20 (dotted line); 55 mM KCl, 0.6 mM ATP, 22 mM imidazole, 7 mM Tris, 1.5 mM dithiothreitol, 0.04 mM CaCl₂, 0.88 mM EGTA, 0.02% NaN₃ (pH 7.0).

ments, not individual filaments (37, 43, 44, 58), and band 4.1 is located on stress fibers, i.e., actin bundles (16). Thus, band 4.9, together with actin, erythrocyte tropomyosin (band 7) (31) and the spectrin/band 4.1 complex, may form structures
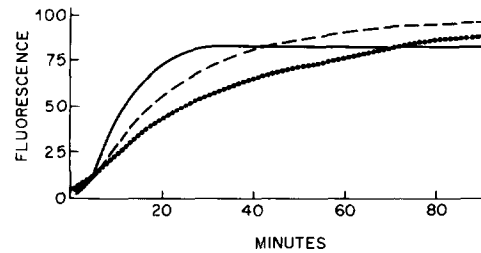

FIGURE 10 Polymerization kinetics of magnesium-induced actin paracrystal formation measured with pyrene-labeled actin. The polymerization of gel-filtered pyrene-labeled actin in the presence of high magnesium chloride concentrations that induce the formation of actin paracrystals was followed by the increase in fluorescence at 25°C after the addition of salt at time zero. For comparison, actin was also polymerized in the presence of band 4.9. Conditions: actin (0.4 mg/ml) polymerized in the presence (dotted line) or absence (solid line) of 50 mM MgCl₂ or in the presence of band 4.9 (broken line), molar ratio of band 4.9 to actin, 1:3. Buffer conditions were 100 mM KCl, 1 mM ATP, 20 mM imidazole, 15 mM Tris, 0.5 mM dithiothreitol, 0.03 mM CaCl₂, 0.74 mM EGTA, 0.02% NaN₃ (pH 7.0), except (dotted line) which contained 50 mM MgCl₂.

in erythroid precursor cells analogous to those formed by fimbrin, actin filaments, tropomyosin, and TW 260/240 in intestinal epithelial brush borders.

Does Band 4.9 Connect Actin to the Membrane?

The fact that the bulk of band 4.9 remains bound to the membrane when spectrin and actin are removed by low salt extraction (86) and the fact that band 4.9 binds to actin in solution (see Results) raises the possibility that band 4.9 might bind to a specific site on the membrane and serve as an additional link between actin and the membrane. An analogous role in non-erythroid cells has been suggested for "vinculin" (11, 29, 32, 33, 92). Although the binding of preformed F-actin to spectrin/actin–depleted inside-out vesicles is stimulated by the prior reconstitution of the vesicles with purified spectrin (14, 15, 30), a significant amount of F-actin binds to unreconstituted vesicles via some trypsin-sensitive site (14). It has been suggested that band 4.1 may be responsible for this association (14), but a role for band 4.9 in this interaction cannot be ruled out at this time.

Does Phosphorylation Modulate Band 4.9's Activity?

The phosphorylation of red cell membrane components has long been thought to play a role in determining cell shape (69). Although initially this regulation was ascribed to the phosphorylation state of spectrin (2), more recent work has not supported this proposal (1, 71). The fact that band 4.9's phosphorylation is controlled by a membrane-bound, cAMP-dependent kinase, unlike the other major membrane skeletal components (25, 40, 76, 89), and the observations that band 4.9 is more highly phosphorylated in sickle cells (23) and in calcium-treated normal cells (53) (which also have an altered shape) raise the possibility that band 4.9 phosphorylation modulates cell morphology. We have observed that when spectrin- and actin-depleted inside-out membrane vesicles were prepared from ghosts derived from ³²P-labeled erythrocytes, the band 4.9 molecules present on the vesicles had a significantly lower specific activity than those that eluted with spectrin, actin, and band 4.1 in the low ionic strength extract

(see Results). Now that the characterization of band 4.9 has been initiated, it will be possible to determine whether its phosphorylation alters its actin- or membrane-binding affinities.

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