Modulation of Actin Polymerization by the Spectrin–Band 4.1 Complex†

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ABSTRACT: The effect of human erythrocyte spectrin dimer and band 4.1 on the polymerization of actin was studied by two independent methods: by following the increase in fluorescence of actin covalently conjugated to *N*-pyrenyliodoacetamide (pyrenylactin) and by following the increase in light scattered by actin polymers. Both techniques indicated that the complex of spectrin dimer and band 4.1, but neither spectrin nor band 4.1 alone, stimulates the rate of nucleation (decreases the lag phase of polymerization) and stabilizes oligomers of F-actin. While the band 4.1–spectrin complex, but not spectrin alone, had no immediate effect on the rate of polymerization after the lag phase, it eventually decreases the rate of actin filament growth when the molecular ratio of actin–spectrin–band 4.1 approaches the physiological range. The complex has no detectable effect on the critical actin concentration and does not significantly alter the apparent order of the nucleation reaction.

Spectrin, actin, and band 4.1 are major protein constituents of the mammalian erythrocyte cytoskeleton. Studies of the purified cytoskeletal proteins in solution have helped to elucidate the structure of the erythrocyte cytoskeleton and predict some of its functions [reviewed in Branton et al. (1981) and Lux (1979)]. Recent studies (Ungewickell et al., 1979; Cohen et al., 1980) have demonstrated that in solution actin together with spectrin tetramer and band 4.1 forms an extensively cross-linked network of actin filaments. While spectrin tetramers alone induce viscosity increases in solutions containing F-actin (Brenner & Korn, 1979), Fowler & Taylor (1980) and Ungewickell et al. (1979) observed that band 4.1 stimulates gelation of F-actin–spectrin tetramer mixtures. Cohen & Korsgren (1980) reported that spectrin tetramer and actin form gels which will recover their viscosity after shearing only when band 4.1 is present. Taken together, these findings suggest that band 4.1 contributes to the structural stability of the cytoskeleton by enhancing the interaction between spectrin and actin.

Band 4.1 binds with high affinity to the tail of the spectrin molecule which is also the site of spectrin association with F-actin (Tyler et al., 1980). Furthermore, the binding affinity of band 4.1 to spectrin is increased significantly in the presence of F-actin (unpublished results).

Since the band 4.1–spectrin complex interacts with F-actin, we investigated whether this complex could modulate the self-assembly of actin. This study shows that the spectrin–band 4.1 complex stimulates the rate of actin nucleation and stabilizes F-actin oligomers.

Materials and Methods

Materials

Gel filtration media and DEAE-Sephacel were from Pharmacia. Ammonium sulfate, sucrose, and guanidinium chloride were Schwarz/Mann products. *N*-(1-Pyrenyl)iodoacetamide was from Molecular Probes. One- to two-day-old blood was obtained from the Northeast Regional Red Cross Blood Program.

Methods

Preparation of Erythrocyte Ghosts. One unit of human blood was centrifuged (5 min at 16000*g*), and the red cells were suspended in phosphate-buffered saline [5 mM sodium phosphate, 155 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA),[1] and 0.02% NaN_3, pH 8.0]. Following three washes in this solution, the erythrocytes were lysed in 20–30 volumes of lysis buffer (5 mM sodium phosphate, 1 mM EDTA, and 20 μg/mL PMSF, pH 8.0) at 4 °C. Lysed cells were washed free of hemoglobin by passage through a Millipore Pellicon cassette system according to the manufacturer's recommendations. Six to eight liters of lysis buffer was required to produce white ghosts which were brought to a final volume of 500 mL.

Purification of Band 4.1. Band 4.1 was purified by slight modifications of previous methods (Tyler et al., 1980). The salt-extracted supernatant containing band 4.1, band 2.1, band 4.9, and residual spectrin was dialyzed against 7.5 mM sodium phosphate, 1 mM EDTA, 0.2 mM DTT, and 20 mM NaCl, pH 8.0, and then loaded on a DEAE-Sephacel column (2.5 × 28 cm) equilibrated in the same buffer. Proteins were eluted from the column with a NaCl gradient from 50 to 250 mM. Fractions containing purified band 4.1 were pooled, dialyzed, and concentrated on a 1-mL DEAE-Sephacel column.

Preparation of Spectrin Heterodimer. Spectrin was prepared by well-established methods (Tyler et al., 1980; Ralston, 1976): low ionic strength extraction of dimer followed by concentration using $(NH_4)_2SO_4$ and column chromatography on Sepharose CL-4B (5 × 90 cm) equilibrated in 5 mM sodium phosphate, 0.5 mM DTT, 20 mM KCl, and 1 mM EDTA, pH 7.6. Small amounts of contaminating actin were separated from this spectrin by centrifugation of the spectrin on 5–20% sucrose gradients in phosphate-buffered saline (18 h, SW40 rotor, at 40000 rpm) (Bennett & Branton, 1977).

Actin Purification. G-Actin was prepared from acetone powder of rabbit back and leg muscles by the method of Spudich & Watt (1971). G-Actin was gel filtered on a

† From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received January 25, 1984.* This work was supported by Grant HL 17411 from the National Institutes of Health (to D.B.).

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[1] Abbreviations: DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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Sephadex G-150 column (2.5 × 50 cm) equilibrated in buffer A (2.0 mM Tris-HCl, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$, and 0.02% NaN_3, pH 8.0). G-Actin concentration was measured by the absorbance at 290 nm, assuming an absorptivity of 0.62 mL/mg (Eisenberg & Moos, 1967; Gordon et al., 1976).

Preparation of Fluorescently Labeled Actin. The protocol of Kouyama & Mihashi (1981) was used to obtain pyrenyliodoacetamide-labeled F-actin (pyrenylactin). Unpolymerized pyrenylactin was chromatographed on a Sephadex G-150 column to remove actin binding proteins and actin oligomers. To avoid photobleaching of the fluorophore, the pyrenylactin was stored at 0 °C in the dark.

Measurements of Fluorescence and Light Scattering. An SLM Instruments spectrofluorometer was used to monitor the fluorescence of actin. The excitation and emission wavelengths were 368 and 408 nm, respectively. Polymerization was initiated by adding a small aliquot of 5× polymerization buffer (250 mM KCl, 100 mM PIPES, pH 7.4, 100 mM EGTA, 0.5 mM $CaCl_2$, and 5.0 mM ATP) to protein samples prewarmed to 23 °C. The same instrument was used for light-scattering measurements. The instrument was set to 546 nm for excitation and emission wavelengths. The turbidity was determined at a 90° angle with respect to the incident light.

Viscosity Measurements. Relative viscosity was measured by using a low-shear rolling-ball viscometer similar to that described by Griffith & Pollard (1978). To a G-actin-containing solution (total concentration 0.084 mg/mL) were added spectrin dimer (0.168 mg/mL final concentration) and band 4.1 (0.03 mg/mL final concentration) or a mixture of both (same final concentrations as when added individually); 5× polymerization buffer was added to initiate actin polymerization, and samples were drawn up into 100-μL micropipets and incubated at 23 °C for 90 min. Final buffer conditions were identical with those described in Figure 1.

Results

The effect of spectrin dimer, band 4.1, or a mixture of the two on the polymerization of actin was studied by two independent methods: by following the increase in fluorescence during polymerization of pyrenylactin or by following the increase in light scattering of polymerizing unlabeled actin solutions.

Pyrenylactin has been shown by a number of investigators to be a very sensitive probe of actin polymerization (Kouyama & Mihashi, 1981; Tobacman & Korn, 1983). The label reacts specifically with sulfhydryl groups on the surface of the actin molecule and reports local conformational changes induced by formation of actin filaments. A greater than 25-fold enhancement of fluorescence intensity is observed as a result of actin polymerization.

A representative experiment (more than 30 runs were performed) is plotted in Figure 1 and demonstrates that in polymerization buffer (20 mM PIPES, pH 7.4, 50 mM KCl, 2 mM EGTA, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$) dimeric spectrin mixed with band 4.1 at a molar ratio of 1:1 (henceforth referred to as the "spectrin–band 4.1 complex") consistently enhanced the rate of actin nucleation (decreased the lag phase) and had little effect on the rate of elongation in the first ca. 15 min, but it decreased the rate of actin elongation after this initial period. After 75 min, no further changes in fluorescence were observed for solutions of pyrenylactin or pyrenylactin containing spectrin–band 4.1 complex. The final fluorescence values for both samples were, within the error of method, identical. In addition, band 4.1 alone at the physiological molar ratio of actin to band 4.1 (5:1) decreased

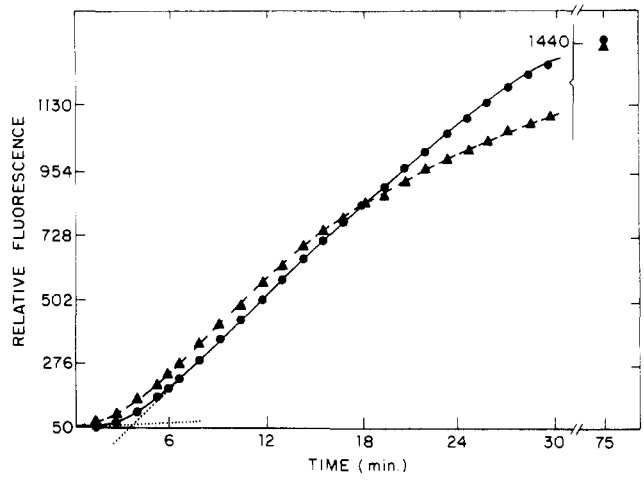

FIGURE 1: Kinetics of G-actin polymerization in the presence (▲) and absence (●) of spectrin dimer–band 4.1 complex shown as a plot of relative fluorescence vs. time. Spectrin dimer–band 4.1 complex is a solution containing a 1:1 molar mixture of the two purified proteins and is added to G-actin prior to initiation of polymerization. To initiate polymerization (t = 0), 5× polymerization buffer was added to make the final buffer condition 20 mM PIPES, pH 7.4, 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM $CaCl_2$, and 2 mM EGTA at 24 °C. Total actin concentration was 0.085 mg/mL, and the molar ratio of actin to spectrin to band 4.1 was 8.5:1:1. The dotted lines show an example of the intersecting lines used to derive the data plotted in Figure 2.

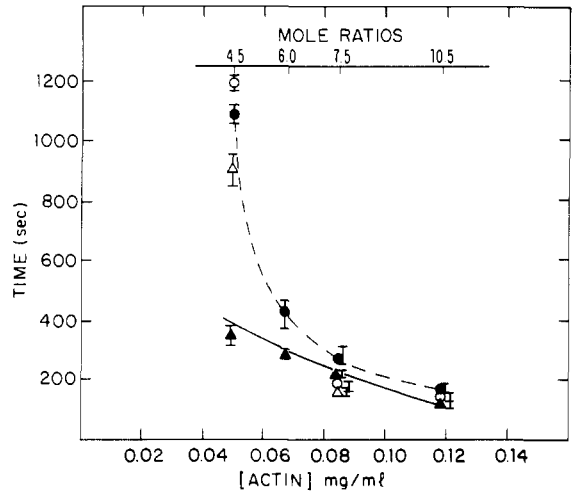

FIGURE 2: Effect of spectrin dimer–band 4.1 complex on the lag phase of actin polymerization: (●) actin alone; (▲) actin and spectrin dimer–band 4.1 complex; (○) actin and band 4.1; (△) actin and spectrin dimer. Polymerization buffer conditions were identical with those described in Figure 1. The spectrin concentration (0.12 mg/mL) and the band 4.1 concentration (0.02 mg/mL) were constant, but the actin concentration was varied as shown. The consequent mole ratios of actin–spectrin–band 4.1 complex in the complete mixture containing actin and the spectrin dimer–band 4.1 complex as well as the mole ratios of actin–spectrin alone or actin–band 4.1 alone are shown on the inserted scale. Lag time was determined from plots as in Figure 1 and is the intersection of a line tangent to the curve at t = 0 and another line tangent to the elongation phase portion of the curve as shown in Figure 1.

the rate of elongation but did not affect either the lag phase or the final extent of actin polymerization (not shown).

The increase in the rate of nucleation and the eventual decrease in the rate of elongation were only observed when the molar ratio of actin to spectrin–band 4.1 complex was close to the physiological range (between 4.5 to 1 and 7.5 to 1). At higher ratios of actin to complex, the effect was greatly diminished or nil. Figure 2 summarizes several experiments designed to measure the effect of varying the ratio of the

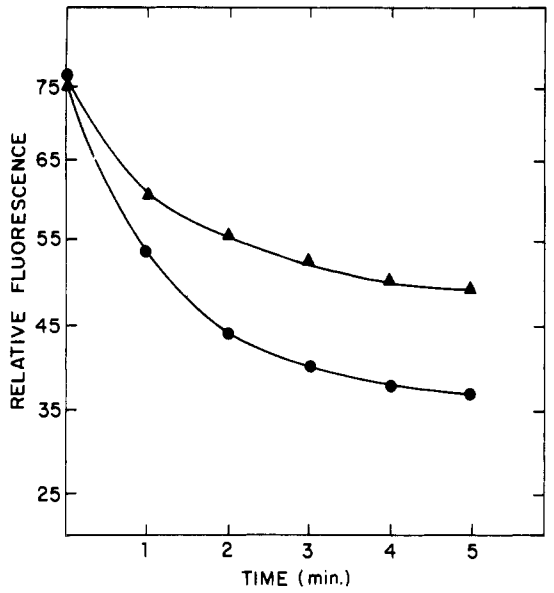

FIGURE 3: Depolymerization of F-actin by 0.4 M guanidinium chloride in the presence (▲) and absence (●) of spectrin dimer–band 4.1 complex. An aliquot of 5 M guanidinium chloride was added to the actin-containing solutions after polymerization was completed. Mixing was accomplished by inverting the fluorescence cuvette 2 times. [Actin] = 0.085 mg/mL. The molar ratio of actin to the spectrin–band 4.1 complex was 4.5:1. Other experimental conditions were identical with those in Figure 1.

complex, spectrin alone, or band 4.1 alone on the length of the lag phase at different actin concentrations. When the stoichiometry of actin, spectrin, and band 4.1 in solution approaches the physiological range of 5:1:1, the spectrin–band 4.1 complex is a more potent effector of nucleation than either spectrin or band 4.1 alone (Figure 2). In addition to the ability of the spectrin–band 4.1 complex to increase the rate of actin nucleation and decrease the rate of elongation (Figures 1 and 2), the complex stabilizes actin filaments. Stabilization was judged by measuring F-actin depolymerization when in 0.4 M guanidinium chloride (Figure 3). The complex decreased both the rate and the extent of depolymerization (not shown). Guanidinium chloride at 0.4 M is known to dissociate but not denature F-actin (Blikstad et al., 1978). Our observation that spectrin–band 4.1 stabilizes actin filaments confirms and extends the results of Pinder & Gratzer (1983), who found that the ability to dissociate erythrocyte-associated F-actin into monomers was increased after trypsin degradation of band 4.1 and spectrin.

We assume that the nucleation step can be described by

$$v = k_{app}[Ac]^n$$

where v is the velocity of the reaction, k_{app} is the apparent rate constant of the reaction, [Ac] is the total actin concentration, and n is the apparent order of the reaction. Using two independent techniques (Figure 4), we found that the spectrin–band 4.1 complex did not significantly alter the apparent order of the reaction. Linear regression analysis (Figure 4, legend) of our data showed that plots of log [Ac] vs. log v did not differ consistently with and without the spectrin–band 4.1 complex. Although the particular experiment shown in Figure 4 shows a slightly lower slope in the presence of spectrin–band 4.1, other experiments, on different days or with different batches of protein, showed that this was not a consistent difference. On the other hand, slopes of log [Ac] vs. log v were consistently lower when measured by fluorescence than when measured by light scattering. [Compare also the data of Tobacman & Korn (1983) with those of Kasai et al. (1962).] It is probable

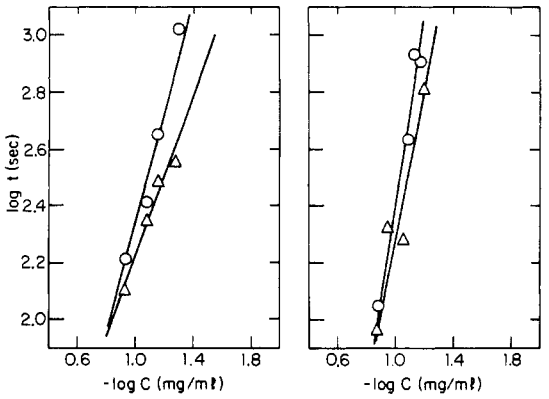

FIGURE 4: Effect of spectrin dimer–band 4.1 complex on the apparent order (n) of actin nucleation in the absence (△) and presence of the complex. Left panel; fluorescence of pyrenylactin; right panel, light scattering. Experimental conditions are the same as in those in Figure 1, except that light-scattering measurements were determined at 30 °C. The slope from these plots, n, represents the order of nucleation reactions described by the equations in the text. The concentration of the spectrin–band 4.1 complex was kept constant while the actin concentration was varied. Linear regression analysis of the fluorescence data shown here (left panel) indicated slopes of 2.2 (with a correlation coefficient $r = 0.98$) and 1.3 ($r = 0.98$) in the absence and presence of the complex, respectively. The light-scattering data (right panel) generated values for the slope of 3.2 ($r = 0.97$) and 2.4 ($r = 0.94$) in the absence and presence of the complex, respectively.

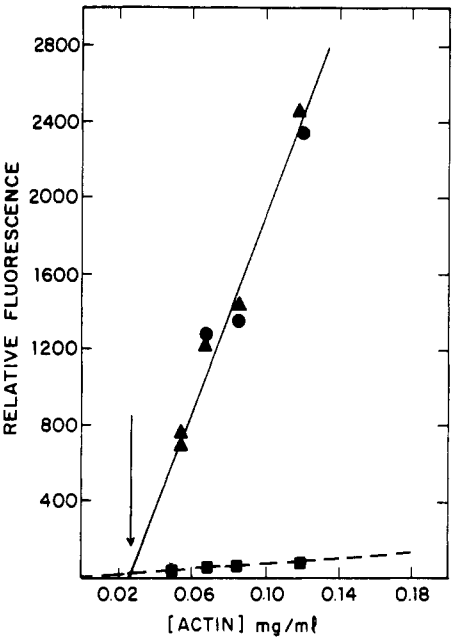

FIGURE 5: Effect of spectrin dimer–band 4.1 complex on the critical actin concentration in the absence (●) and presence (▲) of the complex. Experimental conditions are the same as in those in Figure 1. Relative fluorescence is the final fluorescence of the polymerized actin solutions at equilibrium. The concentration of spectrin–band 4.1 complex was held constant so that [spectrin] = 0.11 mg/mL and [band 4.1] = 0.02 mg/mL. The arrow indicates the critical actin concentration determined as the intersection of a line derived from fluorescence values prior to (---) and at the completion of (—) polymerization. The closed square (■) represents the relative fluorescence of actin solution under nonpolymerizing conditions.

that fluorescence and light scattering measure somewhat different aspects of the polymerization process.

The spectrin–band 4.1 complex had no significant effect on the critical actin concentration measured either by fluorometric methods (Figure 5) or by light-scattering techniques using unlabeled actin (not shown). These results are consistent with the assumption that pyrene-labeled actin behaves indistin-

guishably from the unlabeled protein.

The low-shear viscosity of actin-containing solutions is not appreciably changed by spectrin dimer or band 4.1 alone but is markedly increased by the presence of mixtures of the spectrin–band 4.1 complex at actin:spectrin dimer:band 4.1 molar ratios of 5:1:1. The apparent viscosities (in centipoise) for actin, actin with spectrin dimer, actin with band 4.1, and actin with spectrin dimer and band 4.1 were 19.6 ± 2, 22.8 ± 2, 19.4 ± 2, and more than 600, respectively. While we observed large viscosity increases when spectrin dimer is incubated at 23 °C with band 4.1 and actin, Fowler & Taylor (1980) observed large viscosity increases with spectrin dimer only after incubation at 32 °C, a temperature which favors the association of spectrin dimers into tetramers. Although small amounts of tetramer may form at 23 °C, the discrepancy between these results is probably due to our use of gel-filtered actin, which is known to form longer filaments than the actin used by Fowler & Taylor (1980), which was not subjected to gel chromatography. Thus, the high viscosity that we observed with spectrin dimer is probably not due to cross-linking actin filaments but due to the decoration and stabilization of long F-actin filaments. Negative staining of the spectrin–band 4.1–F-actin mixture (not shown) reveals long actin filaments heavily decorated with elongated spectrin molecules consistent with the results of Cohen et al. (1980) and Cohen & Foley (1983). The filament length cannot be judged accurately because individual fibers often extend beyond the viewing area even at low magnification and because the density of filaments makes the identification of an entire single filament difficult.

Discussion

Although tetrameric spectrin is necessary to form a cross-linked cytoskeletal network, our results suggest that a complex of dimeric spectrin and band 4.1 is capable of regulating the rate of actin polymerization. The spectrin–band 4.1 complex affects the self-assembly of actin by shortening the lag phase and by stabilizing the filaments of actin. The complex may stabilize actin filaments in part by decreasing spontaneous actin filament fragmentation. However, the observed modulation of actin behavior apparently takes place without altering the basic mechanism of actin polymerization: the apparent order of the reaction, the critical concentration, and the initial rate of polymerization of actin are not affected.

Because the critical actin concentration is not affected by the spectrin-band 4.1 complex, we interpret the shortening of the lag phase as due to the stabilization of short actin protomers by the spectrin–band 4.1 complex. This would lead to earlier accumulation of nucleation centers and is consistent with the possibility that the average length of the actin filaments formed in the presence of the complex is shorter than the length of actin filaments formed in the absence of spectrin and band 4.1 (Cohen et al., 1980; Husain et al., 1983).

Although we do not understand why the spectrin–band 4.1 complex decreases the late phases of actin filament growth, our results do show that dimeric spectrin together with band 4.1 markedly increases the viscosity of long actin filaments. It is possible that the restrictions on the motion of actin filaments that are detected by the viscosity measurement are in fact the basis for the observed late decreases in the rate of actin growth.

Our discussion focuses only on the effect of spectrin and band 4.1 on actin polymerization. This is but one aspect of the reaction or reactions leading to the formation of the cytoskeletal network. Other factors, such as divalent cation concentration, the presence of ATP, membrane binding, or the presence of other proteins, i.e., band 4.9 or band 2.1, also may play a major role in the formation and properties of the erythrocyte cytoskeleton (Palek & Liu, 1979; Branton et al., 1981; Wiedenmann & Elbaum, 1983).

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