Associations of Erythrocyte Membrane Proteins

BINDING OF PURIFIED BANDS 2.1 AND 4.1 TO SPECTRIN*

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Jonathan M. Tyler,‡ Bruce N. Reinhardt, and Daniel Branton

From the Cell and Developmental Biology Department, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Specific associations of spectrin with Bands 2.1 and 4.1 have been examined by measuring the binding of purified 125I-Band 2.1 and 125I-Band 4.1 to [32P]spectrin in solution. Binding of Bands 2.1 and 4.1 to spectrin was measured as 125I radioactivity precipitated by an anti-spectrin-Staphylococcus aureus complex. The association between spectrin and Band 2.1 is characterized by relatively high affinity ($K_d = 10^{-7} M$ at pH 7.6) and saturation of available binding sites at a molar ratio of 1:1 (Band 2.1/spectrin heterodimer). Band 4.1 binding to spectrin is characterized by a similar affinity ($K_d = 10^{-7} M$ at pH 7.6) with saturation of available sites occurring at a stoichiometric ratio of 2:1 (Band 4.1/spectrin heterodimer). Scatchard plots of Band 4.1 binding to spectrin are curvilinear and consistent with a positively cooperative interaction.

Bands 2.1 and 4.1 bind to different sites on the spectrin molecule: unlabeled Band 4.1 does not competitively displace 125I-Band 2.1 from spectrin in solution, and low angle rotary-shadowed platinum-carbon replicas of these polypeptides reveal two discrete binding sites.

Spectrin is the major extrinsic protein of the erythrocyte membrane. It is attached to the membrane by polypeptides that have been termed ankyrin (1) or syndeins (2) and which we refer to as Band 2.1, following the nomenclature of Steck (3). Band 2.1 is in turn anchored to the membrane by its association with the major transmembrane protein, Band 3 (4). Together with actin and Band 4.1, spectrin lines the cytoplasmic surface of the red cell membrane with a meshwork that remains after the membrane is extracted with nonionic detergents such as Triton X-100 (5, 6). Complexes of spectrin and Band 4.1 (7) and of spectrin and actin (8) can be formed, and complexes containing spectrin, actin, and Band 4.1 have been observed (9-11).

To measure the interactions between spectrin and Bands 2.1 and 4.1, we have quantified the association of these polypeptides in solution. By using purified proteins, we avoided the potentially complicating effects of multiple protein-protein interactions in membrane preparations and have determined the stoichiometry and affinity of associations between spectrin and Band 2.1, and between spectrin and Band 4.1.

EXPERIMENTAL PROCEDURES

Materials

Carrier-free (32P)orthophosphoric acid in 0.02 M HCl and (32P)-labeled Bolton-Hunter reagent ([125I]iodinated p-hydroxyphenyl prolionic acid N-hydroxysuccinimide ester) in benzene (approximately 1500 Ci/mmol) were obtained from New England Nuclear. Disopropyl fluorophosphate (DFP), adenosine, dithiothreitol, benzylpenicillin G (sodium salt, 1650 IU/mg), d-biotin, and bovine serum albumin were from Sigma. Friend's adjuvant was purchased from Calbiochem-Behring. Gel filtration media were from Pharmacia and DEAE-cellulose (DE52) was from Whatman. Protein A on inactivated, lyophilized Staphylococcus aureus cells was from Enzyme Center, Ferritin-avidin (10 mg/ml) was purchased from Vector Laboratories. All other chemicals were of at least reagent grade. Freshly drawn human blood was obtained from the Northeast Regional Red Cross Blood Program.

Methods

Purification of Human Spectrin—Erythrocyte ghosts (12) prepared in NaPO₄ (5 mM), EDTA (1 mM) (7), were given a final wash in cold TES (N-[Tris(hydroxymethyl)methyl-2-arnino)ethanesulfonic acid] (1 mM) at pH 7.6, EDTA (0.1 mM), and then incubated in this buffer at 37°C for 30 min. The suspension was then centrifuged (225,000 x g, 15 min) and the supernatant immediately loaded onto a Sepharose 4B column (2.5 x 90 cm). Elution with NaPO₄ (5 mM) at pH 7.6, EDTA (1 mM), KCl (20 mM), dithiothreitol (0.2 mM), and NaNO₂ (2 mM) yielded the heterodimer fraction ($V_d/V_o = 1.7$) containing only Bands 1 and 2 (Fig. 1). Tetrameric spectrin was obtained by extended dialysis of ghosts at 0-4°C against TES (1 mM) at pH 8.0, EDTA (0.1 mM) followed by ultracentrifugation and column chromatography of the supernatant on Sephrose 4B. This spectrin ($V_d/V_o = 1.4$) existed as a tetramer in solution was confirmed by low angle rotary shadowing (7, 13). For quantitative immunoprecipitation assays, spectrin was radiolabeled with [125I]-labeled Bolton-Hunter reagent (7, 14-16) or [32P] (7, 16-18).

Purification of Bands 2.1 and 4.1—Bands 2.1 and 4.1 were purified from human erythrocyte membranes as previously described (7), with some modifications. The crude [125I]-labeled 2.1/4.1 extract was loaded onto a DEAE-cellulose (0.6 x 25 cm) column and eluted with a stepped series of salt concentrations that we formulated knowing the salt-dependent elution characteristics of Bands 2.1 and 4.1 observed with continuous, linear gradients (Fig. 2). After loading, 50 mM KCl was used to elute minor contaminants, 100 mM KCl was used to elute pure, concentrated Band 2.1 and, following a wash in 165 mM KCl, 200 mM KCl was used to elute pure Band 2.1. The purified proteins were dialyzed against phosphate-buffered saline (5 mM NaPO₄ at pH 7.6, 1 mM EDTA, 130 mM KCl, 20 mM NaCl, 0.2 mM dithiothreitol, 2 mM NaNO₂) and used for binding analyses within 18 h.

Preparation of Anti-Spectrin—Antibodies against human erythrocyte spectrin were produced by multiple site injections of 200 to 400 μg of dialyzed spectrin in 0.9% saline emulsified with Freund's complete adjuvant. Initial intramuscular injections were followed by intramuscular and subcutaneous booster injections at 12- to 14-day intervals. Rabbits were bled at 1- to 3-week intervals beginning at the second booster injection, and the antisera were titered by precipitin tests. The γ-globulins were precipitated...
and further purified by passage through a DEAE-cellulose column in affinity chromatography (19,20). Purified human spectrin (1.2 mg/ml) was dialyzed extensively against ice-cold phosphate-buffered saline, was loaded onto the spectrin-Sepharose affinity column, and eluted with a continuous linear gradient of KCl in a phosphate/salt buffer as described under "Methods." The polypeptides were analyzed against phosphate-buffered saline prior to passing it through activated Sepharose 4B (2.0 ml). After thorough washing, the conjugate was packed in a borosilicate glass column (1.0 cm) and equilibrated with isotonic phosphate buffer (10 mM) at pH 8.0.

The eluate fractions were analyzed for radiolabeling. Affinity-purified anti-spectrin is used in binding assays. Affinity-purified anti-spectrin is shown in Lane e. Proteins were prepared as described under "Methods," and 10- to 40-μl aliquots containing 10 to 75 μg of protein were analyzed by electrophoresis in the presence of 0.2% sodium dodecyl sulfate on 5% polyacrylamide gels.

Fig. 2. Salt-dependent elution characteristics of Bands 2.1 and 4.1 from a DEAE-cellulose column (1.6 x 30 cm). Approximately 4 mg of 125I-labeled crude high salt extract derived from 50 ml of packed human erythrocytes was loaded onto the column in a low salt buffer as described under "Methods." The polypeptides were eluted with a continuous linear gradient of KCl in a phosphate/EDTA buffer (—). The eluate fractions were analyzed for 125I activity (——) and by sodium dodecyl sulfate-gel electrophoresis. The DEAE-cellulose-treated IgG fraction was then purified by affinity chromatography (19,20). Purified human spectrin (1.2 mg/ml in 0.1 M NaHCO3 buffer, pH 9.0) was coupled to cyanogen bromide-activated Sepharose 4B (2.0 mg of spectrin/ml of swollen Sepharose). After thorough washing, the conjugate was packed in a borosilicate glass column (1.0 x 12 cm) and equilibrated with isotonic phosphate-buffered saline (5 mM NaPO4 at pH 7.6, 150 mM NaCl). The IgG fraction (10 mg/ml), dialyzed extensively against ice-cold phosphate-buffered saline, was loaded onto the spectrin-Sepharose affinity column and washed with phosphate-buffered saline until the eluate absorbance (280 nm) had returned to base-line. Anti-spectrin was eluted as a sharp peak with glycine/HCl (0.2 M) at pH 2.7, NaCl (2 M), and immediately titrated to neutrality with 1 M Tris (Tris-(hydroxymethyl)aminomethane) buffer. The material was then dialyzed against phosphate-buffered saline prior to passing it through either 2.1- or 4.1-Sepharose affinity columns to remove components directed against these polypeptides. Anti-spectrin so purified was treated with iPr2FP 1 (0.4 M) and stored at 0–4°C in a buffer (5 mM NaPO4 at pH 7.6, 1 mM EDTA, 130 mM KCI, 20 mM NaCl, 0.2 mM dithiothreitol, 2 mM NaN3) suitable for binding assays.

Immunodiffusion and Immunoprecipitation.—The specificity of antibody preparations was determined by double immunodiffusion in agar (21) (Fig. 3). Quantitative precipitin tests were performed by incubating varying concentrations of 125I-labeled antigen and antibody together in phosphate-buffered saline (5 mM NaPO4, 1 mM EDTA, 130 mM KCI, 20 mM NaCl, 0.2 mM dithiothreitol, 2 mM NaN3) for 12 to 18 h at 4°C. The resulting immunoprecipitates were washed twice in this buffer and pelleted by centrifugation at 30,000 x g for 10 min at 4°C. Washed precipitates were assayed for 125I activity by counting in a Beckman model 800 y counter. Equivalence was estimated at an antibody/antigen ratio of 4:1 (w/w). Tests were also performed with Staph A to ensure complete removal of antibody and antigen from solution under conditions used for binding assays.

Assay of Band 2.1 and Band 4.1 Binding to Spectrin.—125I-Labeled Band 2.1 (0.75 to 20 μg, 1200 to 6400 cpm/μg) or Band 4.1 (0.4 to 15 μg, 1800 to 7900 cpm/μg) was incubated at 0°C in glass test tubes (10 x 75 mm) in μg of 18O-water in a 0.2-ml volume containing 10 to 17 μg of 125I-spectrin and various concentrations of NaPO4, EDTA, KCl, dithiothreitol, NaN3, Triton X-100, and bovine serum albumin as described in the figure legends. After a 90-min incubation, affinity-purified anti-spectrin (40 to 70 μg) was added and the incubation continued for 20 min on ice. Staph A (10% suspension in phosphate-buffered saline containing 0.2% Triton X-100 and 1 mg/ml of bovine serum albumin) was then added, and the mixture was centrifuged (8000 rpm, 10 s at speed) after 200 min of 5 to 7 rain. The pellet was resuspended and washed three times in phosphate-buffered saline containing 0.2% Triton X-100 and 1 mg/ml of bovine serum albumin. Spectrin-bound 125I radioactivity was determined in a Beckman model 8000 y counter. Total spectrin pelleted was assayed as 32P radioactivity by counting in 8 ml of Aquasol using a Beckman LS-230 scintillation counter (350 to 1000 window). Corrections for 127I/32P cross-talk were made using standard methods.

Ferritin Labeling of Band 4.1.—Biotinyl-N-hydroxysuccinimide ester, prepared essentially as described by Reitzmann and Richards (22), was dissolved in dimethylformamide at a concentration of 12 mg/ml. This was added to purified Band 4.1 (500 μg/ml in 100 mM borate at pH 8.5, 100 mM KCl, 3 mM NaN3, and 0.4 mM iPr2FP) to achieve a final concentration of 7.5% dimethylformamide. The labeling reaction was allowed to proceed for 3 h at 23°C, and the material was then desalted on a Sephadex G-25 column (1.0 x 5.0 cm) to remove unreacted succinimide ester and equilibrate the protein in phosphate-buffered saline (5 mM NaPO4 at pH 7.6, 130 mM KCl, 20 mM NaCl, 0.2 mM dithiothreitol, 2 mM NaN3) suitable for binding assays. Biotin-labeled Band 4.1 was incubated with dimeric or tetrameric spectrin in a 500-μl volume for 180 min at 4°C. Ferritin-avidin (5 μg) was then added and the material was prepared for low angle rotary-shadowing electron microscopy.
and 4.1 were estimated by the method of Lowry et al. (24) in the determination of \(^{125}\)I-specific activities. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed essentially by the method of Fairbanks et al. (25). Low angle rotary-shadowing electron microscopy was conducted as previously described (26).

RESULTS

Purification of Bands 2.1 and 4.1—The extraction of Bands 2.1 and 4.1 from spectrin-depleted erythrocyte membranes at high ionic strength in the presence of potent protease inhibitors has been noted previously (7). The polypeptides are purified to homogeneity in water-soluble, biochemically active forms by DEAE-cellulose chromatography. Amino acid compositions for purified Bands 2.1 and 4.1 are presented in Table I. A \(I_{50}\) values of 6.5 and 8.0 are estimated for Bands 2.1 and 4.1, respectively.

The purified proteins, stored at 4°C in buffers of moderate ionic strength with IPrFP, are stable for several weeks: sodium dodecyl sulfate-polyacrylamide gels of material stored for 4 weeks under these conditions exhibit no new Coomassie-staining bands indicative of proteolytic degradation, and these preparations retain their spectrin-binding properties. Band 2.1 remains monomeric upon storage (7), but Band 4.1, isolated as a monomer, can give rise to dimeric and tetrameric forms as well. This phenomenon has also been observed by other.
Oligomerization prior to incubating with spectrin. Accordingly, a size distribution of bound Band 2.1 molecules is observed. The use of ferritin-labeled Band 4.1 in d confirms the location of the Band 4.1 binding sites at the extreme ends of the tetrameric molecule.

**Fig. 5.** Inability of purified unlabeled Band 4.1 to displace 125I-labeled Band 2.1 competitively. 125I-Labeled Band 2.1 (5.2 μg/ml, 1850 cpm/μg) was incubated for 90 min at 0°C in a 0.2-ml volume containing NaPO₄ (5 mM) at pH 7.6, EDTA (1 mM), KCl (130 mM), NaCl (20 mM), dithiothreitol (0.2 mM), NaN₃ (2 mM), Triton X-100 (0.1%), bovine serum albumin (1 mg/ml), purified spectrin heterodimer (17 μg), and various concentrations of unlabeled Band 4.1. Spectrin-bound radioactivity was determined as described under “Methods.”

**Fig. 6.** Electron micrographs of low angle rotary-shadowed platinum-carbon replicas of (a) spectrin tetramers alone, (b) spectrin tetramers to which Band 2.1 (arrows) has bound, (c) spectrin tetramers to which Band 4.1 (arrows) has bound, and (d) spectrin tetramers to which Band 4.1 has bound and been labeled with ferritin (arrows). Molecules and molecular complexes were prepared as described under “Methods,” sprayed onto freshly cleaved mica in buffer containing 70% glycerol, and dried under vacuum. Rotary shadowing was performed with platinum-carbon and carbon anodes heated by electron bombardment from tungsten cathodes. Replicas were floated onto distilled water and mounted on 400 mesh copper grids. In c, the Band 4.1 molecules were aged to promote oligomerization prior to incubating with spectrin. Accordingly, a size distribution of bound Band 4.1 molecules is observed. The use of ferritin-labeled Band 4.1 in d confirms the location of the Band 4.1 binding sites at the extreme ends of the tetrameric molecule.

**Fig. 7.** Effect of increasing concentrations of 125I-labeled Band 4.1 on the binding of 125I-labeled Band 4.1 to spectrin heterodimers (C, D) in solution at pH 7.6. Various concentrations of 125I-labeled Band 4.1 (5000 cpm/μg) were incubated for 90 min at 0°C in a 0.2-ml volume containing NaPO₄ (5 mM) at pH 7.6, EDTA (1 mM), KCl (130 mM), NaCl (20 mM), dithiothreitol (0.2 mM), NaN₃ (2 mM), Triton X-100 (0.1%), bovine serum albumin (1 mg/ml), and purified spectrin heterodimer (10 μg). Spectrin-bound radioactivity was determined as described under “Methods.” In B, the data are plotted according to the Scatchard equation, B/F = nK · B, where B is micrograms of Band 4.1 bound/mg of spectrin, F is the concentration of unbound Band 4.1 (μg/ml), n is the total number of binding sites (μg/mg), and K is the equilibrium constant (micrograms per ml). Each point represents the average value for an independent experiment. The first experiment was done in triplicate; the second was done in duplicate. The binding is corrected for nonspecific components (A) by subtracting the values obtained when spectrin, but not anti-spectrin or Staph A, is excluded from the assay (A, inset).

Researchers using gel filtration analysis⁷ and appears to be both time- and concentration-dependent. For this reason, Band 4.1 binding experiments were performed within 18 h of isolation, at which time the majority of molecules existed as monomers (as judged by gel filtration and sedimentation analyses).

**Affinity-purified Anti-Spectrin—** Affinity-purified antispectrin gave single, sharp, confluent precipitin lines when tested by double immunodiffusion against purified spectrin (Fig. 3). Similarly, purified spectrin used in the binding assays gave single, sharp, confluent precipitin lines when tested against anti-spectrin, while neither purified Band 2.1 nor Band 4.1 showed any precipitin bands.

**Band 2.1-Spectrin Binding—** Specific binding of spectrin to Band 2.1 has been demonstrated, both on the erythrocyte membrane (1), and in solution (7). To assay the reassociation of Band 2.1 with spectrin in solution, we separated free and spectrin-bound Band 2.1 rapidly by forming a spectrin-anti-spectrin complex that was precipitated by the F₅-specific antibody-binding agent, Protein A on inactivated S. aureus cells (27). Tests were performed to determine the relative concentrations of anti-spectrin and Staph A required to bring about maximal precipitation of spectrin, while minimizing nonspecific binding of 125I-Band 2.1 to the antibody-Staph A complex in the absence of spectrin. This nonspecific component of binding increased linearly with increasing 125I-Band 2.1 concentration, and was usually less than 35% of the total binding observed.

The association between Band 2.1 and spectrin assayed by this technique is characterized by high affinity (Kₐ = 10⁻⁷ M at pH 7.6) with a maximal binding capacity of approximately 425 μg of Band 2.1/mg of spectrin (Fig. 4). Assuming molecular weights of 210,000 for Band 2.1 and 460,000 for the spectrin heterodimer, saturation of available binding sites occurs at a

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³C. Eliot and G. Ralston, personal communication.
mole ratio of approximately 1:1 (Band 2.1/spectrin heterodimer). This value is consistent with that measured by Bennett and Stenbuk (1) using the trypsin-derived $M_r = 72,000$ binding fragment of Band 2.1.

Band 2.1 binds to the $\alpha_2\beta_2$ tetramer of spectrin with an affinity equivalent to that observed with the $\alpha_2\beta_2$ heterodimer. Saturation occurs at a mole ratio of 2:1 (Band 2.1/spectrin tetramer).

Binding of $^{125}$I-Band 2.1 to spectrin could be abolished effectively by incubating in the presence of a 100-fold weight excess of unlabeled Band 2.1, indicating that the Band 2.1-spectrin interaction observed is specific and unaffected by the $^{125}$I-labeled Bolton-Hunter reagent. In contrast, $^{125}$I-Band 2.1 could not be displaced competitively by incubating in the presence of unlabeled Band 4.1 (Fig. 5), a finding which suggests that these polypeptides bind to different sites on the spectrin molecule. This has been confirmed by low angle rotary shadowing of the molecular complexes (Fig. 6).

Band 4.1-Spectrin Binding—$^{125}$I-Band 4.1 binds to spectrin in a saturable manner. Scatchard plots of data corrected for nonspecific binding of $^{125}$I-Band 4.1 to the anti-spectrin-Staph A complex are indicative of positive cooperativity (Fig. 7). From the linear portion of the curve, the $K_d$ is estimated to be $10^{-7}$ M at pH 7.6. Thus, the Band 4.1-spectrin-binding affinity is on the average equivalent to that observed for the Band 2.1-spectrin interaction. Saturation of available binding sites occurs at a concentration of approximately 340 pg of Band 4.1/mg of spectrin. Assuming a molecular weight of 82,000 for the Band 4.1 monomer, this yields a stoichiometric ratio of 2:1 (Band 4.1/spectrin heterodimer). $^{125}$I-Band 4.1 binds to the $\alpha_2\beta_2$ tetramer of spectrin as if it were two $\alpha_2\beta_2$ heterodimers; no spectrin-spectrin associative effect is apparent.

Competitive displacement of $^{125}$I-Band 4.1 by purified, unlabeled Band 4.1 (not shown) demonstrates that binding is saturable, and the $^{125}$I-labeled Bolton-Hunter reagent is not responsible for the spectrin-Band 4.1 interaction.

Electron micrographs of low angle rotary-shadowed spectrin molecules that had bound Band 4.1 indicated that Band 4.1 binds to the end of the spectrin dimer that is most distant from the end that participates in tetramer formation. As Shotten et al. (13) noted, linear high order oligomers of spectrin are never seen, but they and we occasionally have noted nonlinear hexamers (Fig. 8). Given the reciprocal head-to-head binding of the tetramer (7), the binding of monomer strands in the hexamer is explicable as another example of the same reciprocal head-to-head binding. The postulated arrangement of monomer strands in the hexamer is confirmed by experiments in which Band 4.1 was used to identify the orientation of subunits (Fig. 8).

RESULTS

Band 2.1 has been identified as a link between spectrin and the intrinsic membrane protein, Band 3 (4, 27). It is presumably the reassociation between spectrin and Band 2.1 that is measured in the vesicle-binding assay of Bennett and Branton (17), because the 72,000-dalton fragment of Band 2.1 competes with the vesicles in binding spectrin. But, because the assay involves a complex membrane fraction as one of the binding components, it cannot resolve multiple protein-protein or protein-lipid association states. For example, Band 4.1 retains an association with the spectrin-depleted inside-out vesicles used in the Bennett and Branton (17) assay. This association has not been characterized, but could contribute to the spectrin binding measured in such an assay. Having now measured the binding interactions between purified components in solution, we can be certain that the associations observed are a function of these proteins alone and cannot be attributed to the presence of lipids or other membrane-associated polypeptides.

Results obtained in Band 2.1-spectrin-binding assays are in agreement with predicted (29) and experimental values obtained by alternate methods (4). In whole cells or ghosts, Band 2.1 and the sequence-related polypeptides, Bands 2.2 through 2.6, are present in sufficient copies relative to spectrin to account for the observed stoichiometric ratios. It remains to be determined whether all of these spectrin-binding components are simultaneously accessible on the membrane. Preliminary studies in which $^{32}$P-spectrin was reassociated with membrane-bound $^{125}$I-Band 2.1 suggests that as much as 50% of the membrane-associated Band 2.1 may be unavailable for spectrin binding.3

At least two models may be postulated to account for the stoichiometric ratio of Band 4.1 to spectrin observed in the present study: either two spatially distinct Band 4.1 binding sites exist on the spectrin heterodimer, or only one binding site exists per heterodimer and the second Band 4.1 molecule binds via a dimerization event to the molecule previously bound to spectrin. Either of these models could account for the positive cooperativity observed, the former by a Band 4.1-dependent conformational change in the spectrin molecule which increases the affinity of the remaining Band 4.1 binding site; the latter by a spectrin-dependent conformational change in the Band 4.1 molecule which shifts the thermodynamic equilibrium governing oligomerization of Band 4.1. A resolution of these models awaits a detailed analysis of the monomer/dimer/tetramer equilibrium of Band 4.1.

Evidence has accrued implicating Band 4.1 in the modulation of spectrin-actin interaction (9–11). The binding site for

3 J. M. Tyler, unpublished observation.

**FIG. 8.** Proposed orientation of subunit strands in various equilibrium states of spectrin. **Left panel** electron micrographs of each pair show examples of naked spectrin molecules. **Right panels** show ferritin-labeled Band 4.1 bound to dimeric, tetrameric, and hexameric spectrin. The location of this binding site confirms the reciprocal head-to-head binding of subunits in the spectrin molecule.
G- and F-actin on the spectrin molecule has been ascertained (11) and it maps at a position indistinguishable from the Band 4.1 binding site. We have noted previously (7) that spectrin to which Band 4.1 has bound often has a morphology distinct from that of the naked molecule: the individual strands are sometimes seen lying in a parallel array (Fig. 6c) and occasionally the strands separate at the ends, a condition never observed in tetramers alone. This separation of strands may have a role in the increased viscosity observed in spectrin-actin-Band 4.1 complexes in vitro (10, 11).

The physiological significance of the Band 4.1-spectrin complex remains obscure, although the Band 4.1-spectrin-actin complex is clearly implicated in the formation of the two-dimensional meshwork that comprises the erythrocyte cytoskeleton.

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