Beta Spectrin Bestows Protein 4.1 Sensitivity on Spectrin–Actin Interactions

Thomas R. Coleman,* Alan S. Harris,‡ Sheenah M. Mische,‡ Mark S. Mooseker,* and Jon S. Morrow‡

Departments of *Biology and ‡Pathology, Yale University, New Haven, Connecticut 06511-8112

Abstract. The ability of protein 4.1 to stimulate the binding of spectrin to F-actin has been compared by cosedimentation analysis for three avian (erythrocyte, brain, and brush border) and two mammalian (erythrocyte and brain) spectrin isoforms. Human erythroid protein 4.1 stimulated actin binding of all spectrins except the brush border isoform (TW 260/240). These results suggested that the beta subunit determined the protein 4.1 sensitivity of the heterodimer, since all avian alpha subunits are encoded by a single gene. Tissue-specific posttranslational modification of the alpha subunit was excluded by examining the properties of hybrid spectrins composed of the purified alpha subunit from avian erythrocyte or brush border spectrin and the beta subunit of human erythrocyte spectrin. A hybrid composed of avian brush border alpha and human erythroid beta spectrin ran on nondenaturing gels as a discrete band, migrating near human erythroid spectrin tetramers. The actin-binding activity of this hybrid was stimulated by protein 4.1, while either chain alone was devoid of activity. Therefore, although both subunits were required for actin binding, the sensitivity of the spectrin–actin interaction to protein 4.1 is a property uniquely bestowed on the heterodimer by the beta subunit. The singular insensitivity of brush border spectrin to stimulation by erythroid protein 4.1 was also consistent with the absence of proteins in avian intestinal epithelial cells which were immunoreactive with polyclonal antisera sensitive to all of the known avian and human erythroid 4.1 isoforms.

In the erythrocyte, protein 4.1 promotes spectrin–actin interactions and provides a secondary membrane anchor for the cytoskeleton (Goodman and Zagon, 1984; Bennett, 1985; Lazarides and Nelson, 1985). Proteins immunoreactive with 4.1 have been identified in a diverse range of tissues, including fibroblasts and endothelial cells where they appear to be in association with membranes and actin filaments (Cohen et al., 1982; Goodman et al., 1984; Spiegel et al., 1984). One such immunoreactive analog in brain is synapsin I (Baines and Bennett, 1985), a multiphosphorylated protein associated with synaptic vesicles (Navone et al., 1984). Multiple 4.1 isoforms also exist in embryonic tissue, as demonstrated by the unique 4.1s that appear in a sequential, developmentally regulated fashion in chicken erythroid cells (Granger and Lazarides, 1985).

Corresponding to the protein 4.1 variability is a similar diversity of spectrin isoforms (Goodman and Zagon, 1984; Bennett, 1985; Lazarides and Nelson, 1985). In avians, at least three spectrin isoforms exist: a 240/220-kD or 240/230-kD heterodimer found in erythrocytes, brain, and skeletal muscle; a 240/235-kD heterodimer present in brain and most other tissues; and a 240/260-kD found exclusively in intestinal brush borders (Lazarides and Nelson, 1983; Nelson and Lazarides, 1983; Glenney and Glenney, 1983*a*; Lazarides and Nelson, 1985). All of these spectrins share a common alpha (240 kD) subunit (Glenney et al., 1982*c*) encoded by a single gene (Curtis et al., 1985; Birkenmeier et al., 1985).

In mammalian tissue, the relationship of the spectrin isoforms to each other is more complex, as both subunits of the different isoforms vary. Mammalian spectrins are composed of at least five immunologically distinct subunits: two (240 kD) alphas and three (235, 235E, 220 kD) betas. The predominant form in brain is a 240/235-kD heterodimer, although a different isoform (240/235E) is also present which is immunologically more similar to mammalian erythroid spectrin (240/220 kD; Riederer et al., 1986). Multiple isoforms also exist in mammalian skeletal muscle, including a 240/235-kD form, a 240/235E form (potentially identical to the brain 240/235E), and a 240/220-kD variant which may be very similar to mammalian erythroid spectrin (Shile et al., 1985; Riederer et al., 1986; Bloch and Morrow, manuscript submitted for publication). Despite their diversity, a common property of all spectrins is their ability to bind F-actin, an interaction often simulated by protein 4.1 (Goodman and Zagon, 1984; Bennett, 1985; Lazarides and Nelson, 1985).

The molecular nature of the spectrin/protein 4.1/actin ternary complex remains enigmatic. Ultrastructural studies have detected actin and protein 4.1 binding to the end of the spectrin molecule opposite the oligomerization site (Tyler et al., 1980). In vitro, individual spectrin subunits bind protein 4.1 with low affinity, but the intact heterodimer is required for actin binding (Cohen and Langley, 1984). An 8-kD fragment of protein 4.1 has been identified which promotes spec-

trin–actin interactions with an activity and stoichiometry comparable to that of the entire molecule (Correas et al., 1986). Whether direct associations between protein 4.1 and actin occur and to which spectrin subunit(s) protein 4.1 binds remains unclear.

One approach to identify the spectrin subunit most critical for 4.1 regulation is to compare the effects of subunit replacement within the heterodimer on its ability to undergo 4.1–enhanced actin binding. By selecting spectrins which share a common alpha subunit, such as those found in the chicken, we made use of a spectrin family in which "subunit replacement" naturally occurred. Erythrocyte and brain spectrins from chicken and human tissue co-sedimented with actin in a 4.1–dependent manner, whereas chicken intestinal brush border spectrin (TW 260/240) did not. Furthermore, a chicken brush border alpha/human erythroid beta hybrid spectrin demonstrated both potent protein 4.1–dependent actin binding and ran as discrete bands on nondenaturing gels with migration rates similar to erythroid spectrin tetramer and hexamer. Thus using both avian and hybrid spectrins we have demonstrated that the beta spectrin subunit determined the ability of the heterodimer to interact with protein 4.1. Consistent with the lack of protein 4.1 sensitivity in brush border spectrin, no immunoreactive 4.1 analogs were detected in avian intestinal epithelial cells.

Materials and Methods

Isolation of Chicken Brush Borders and TW 260/240

Brush borders were isolated from chicken small intestines according to the method of Keller and Mooseker (1982). Leupeptin (2.5 μg/ml), chymostatin (0.5 μg/ml), pepstatin (0.5 μg/ml), soybean trypsin inhibitor (30 μg/ml), diisopropylfluorophosphate (1.5 mM), aprotinin (80–120 trypsin inhibitor units [TIU][1]/liter) and phenylmethylsulfonyl fluoride (PMSF) (0.2 mM) were used to control proteolysis during the initial homogenization steps.

TW 260/240 was purified by a modification of the methods of Glenney et al. (1982*a*) as described by Pearl et al. (1984). Fractions enriched in TW 260/240 (containing primarily TW 260/240 with small amounts of brush border myosin) from the gel filtration column were concentrated by dialysis against 20 mM Tris, pH 8.0, 2 mM $MgCl_2$, 0.2 mM EGTA, 1 mM dithiothreitol (DTT), 0.02% NaN_3, and 20 TIU aprotinin per liter containing ammonium sulfate to 65% saturation. The precipitated protein was dissolved in a minimal volume of 150 mM KCl, 10 mM Tris, pH 7.5, 1 mM DTT, with 0.2 mM PMSF and 20 TIU aprotinin per liter, and dialyzed against the same buffer. Precipitated brush border myosin was removed by centrifugation (12,000 *g*, 15 min) leaving purified TW 260/240 at ~1 mg/ml.

Purification of Chicken Erythrocyte Spectrin

Chicken erythrocytes were harvested according to established procedures (Granger and Lazarides, 1984). All steps were performed at 4°C and monitored by phase-contrast light microscopy. Blood was collected and processed according to the methods of Howe et al. (1985). Lysis was accomplished by repeated (7–8 times) resuspension and centrifugation in 30–40 vol of buffer containing 5 mM $MgCl_2$, 5 mM NaN_3, 1 mM EGTA, 1 mM DTT, 5 mM Tris, pH 7.5, with 0.2 mM PMSF and 20 TIU aprotinin per liter. The lysed cells were enucleated by homogenization in either a tight-fitting dounce homogenizer or an Omni mixer (model 17105, Dupont Instruments, Newtown, CT). Rehomogenization of the intact cell layer remaining after centrifugation was repeated 4–5 times. Avian red cell ghosts were stored overnight on ice in lysis buffer. Before spectrin extraction, the membranes were washed twice in 2 mM EDTA, 10 mM Tris, pH 7.4, to remove any residual Mg^{2+}. Spectrin was extracted according to the methods of Howe et al. (1985). The extracted protein was freed from actin and other contaminants by gel filtration and concentrated by ammonium sulfate dialysis exactly as described for TW 260/240.

Preparation of Human Erythrocyte Spectrin and Protein 4.1

Hemoglobin-free human erythrocyte ghosts were prepared according to established procedures (Morrow et al., 1980; Bennett, 1983). Spectrin was extracted at 4°C for 36 h in 0.1 mM EDTA, 0.05 mM sodium phosphate, pH 9.0, and purified by gel filtration on Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) in 130 mM KCl, 20 mM NaCl, 0.1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.05 mM PMSF, pH 7.4. Protein 4.1 was extracted and purified from human red cell ghosts according to the methods of Tyler et al. (1979, 1980).

Isolation of Fodrin, Calmodulin, and Actin

Human fodrin was prepared from cadaver brains removed at autopsy 2–4 h postmortem with permission of the family. The brain tissue was cleaned of extraneous membranes and blood vessels and the cerebral cortex diced and quick-frozen in 2-methylbutane cooled in liquid nitrogen and stored until use. Brain spectrin was extracted by either low salt or high salt procedures, and purified by gel filtration and ion exchange chromatography (Glenney et al., 1982*b*; Davis and Bennett, 1984; Harris et al., 1985). Calmodulin was prepared from bovine testes and purified by ion exchange and gel filtration chromatography (Burgess et al., 1980). Chicken breast muscle actin was prepared from acetone powders according to the methods of Spudich and Watt (1971). Protein concentrations were determined either by the method of Lowry et al. (1951) or by using the following values for $A_{280}^{1\%}$: TW 260/240, 12.0 cm^{-1}; G-actin, 10.9 cm^{-1}.

Spectrin Subunit Separation and Reconstruction

Human erythrocyte beta chain was purified by the method of Yoshini and Marchesi (1984). The subunits of TW 260/240 were separated by the method of Glenney and Weber (1985). This procedure made use of the fact that the avian alpha (240 kD) subunit binds calmodulin in a Ca^{++}-dependent manner (Glenney et al., 1982*a*). Purified TW 260/240 was dialyzed against 2 M KI, 10 mM Tris, pH 7.5, 2 mM DTT, 1 mM $CaCl_2$, and 0.02% NaN_3 with 0.2 mM PMSF and 20 TIU aprotinin per liter. After dialysis, the solution was sonicated (Cell Disruptor 200; Branson Sonic Power Co., Danbury, CT) and incubated 1 h at room temperature. TW 260/240 was loaded onto a 1.5-ml calmodulin affinity column (10 mg calmodulin/ml settled gel; CNBr-activated Sepharose 4B, Pharmacia Fine Chemicals). After washing with ~15 column volumes, the 240-kD alpha-subunit was eluted using the same buffer in which 5 mM EGTA was substituted for 1 mM $CaCl_2$.

Spectrin subunit reconstitution was accomplished by combining the chains of interest and dialyzing out of KI into 65% glycerol, 20 mM Tris, pH 7.5, 20 mM 2-mercaptoethanol. Reconstituted spectrin was dialyzed out of glycerol into binding buffer (see below). This glycerol dialysis step minimized aggregation, especially of the beta-subunit.

Cosedimentation Assay

Unless otherwise indicated, cosedimentation studies were conducted in binding buffer containing 150 mM KCl, 10 mM imidazole, pH 7.5, 2 mM $MgCl_2$, 0.2 mM DTT, with 0.2 mM PMSF and 20 TIU aprotinin per liter. G-actin was added to spectrin- and/or protein 4.1–containing solutions and allowed to polymerize 45 min at room temperature. The samples (200–250 μl) were gently sheared by pipetting several times during the incubation period. Care was taken to maintain the same order of addition and comparable mixing for all assays. Cosedimentation was performed at 100,000 *g* for 1 h at 15°C. Supernates were removed and the pellets resuspended in an identical volume. Both supernate and pellet fractions were analyzed by PAGE in the presence of SDS (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R by the method of Fairbanks et al. (1971). Stained gels were scanned with a model 1650 Scanning Densitometer (BioRad Laboratories, Richmond, CA). The areas under the peaks were measured using an electronic planimeter (model 1224 Electronic Digitizer; Numonics Corp., Lansdale, PA). Using this staining technique, known spectrin standards bound Coomassie Blue in a linear manner over a 12.5–500 μg/ml range (these experiments were performed at 200 μg/ml total spectrin). For each condition, the percent spectrin pelleted was determined by dividing the area of the spectrin in the pellet by the total spectrin area (supernate plus

1. *Abbreviations used in this paper*: R_f, relative mobility. TIU, trypsin inhibitor unit.

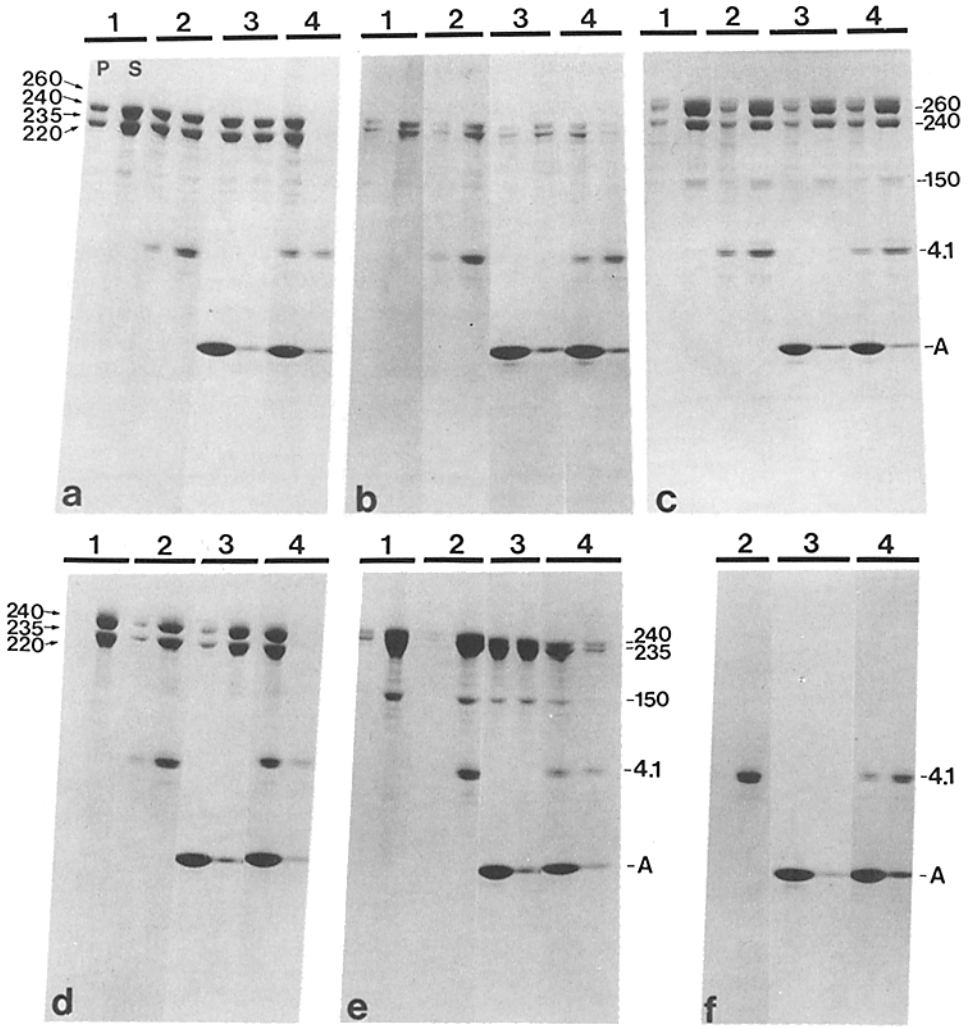

Figure 1. Protein 4.1 stimulated the cosedimentation of all spectrins with actin except the brush border isoform. Chicken spectrin isoforms from erythrocyte (*a*), brain (*b*), and brush border (*c*) were examined. Controls included human erythrocyte (*d*), human brain (*e*), and no added spectrin (*f*). Pellet (*P*, or left side of unmarked lanes) and supernate (*S*, or right side of unmarked lanes) fractions after 100,000 *g* spin were analyzed on 5–15% SDS polyacrylamide gels. Sedimentation was performed with spectrin alone (groups *1*), plus protein 4.1 (groups *2*), plus actin (groups *3*), and plus protein 4.1 and actin (groups *4*). The relative mobilities (R_f's) of the chicken and human alpha spectrin subunits (*240*), and the beta subunits of chicken and human erythrocyte (*220*), brain (*235*), and brush border (*260*) spectrins along with band 4.1 (*4.1*) and actin (*A*) are indicated. When purified in the presence of many protease inhibitors TW 260 was resolved into several subbands (Fig. 1 *c*; Pearl et al., 1984; Howe et al., 1985). The minor components, between 240,000 and 150,000 M_r (*150*) represent spectrin proteolytic fragments frequently observed in these preparations. Protein 4.1 enhanced the cosedimentation of all spectrins with actin except TW 260/240 (Fig. 1 *c*, lanes *4*). Before starting the assays spectrins were prespun at 100,000 *g* for 1 h. Final protein concentrations were as follows: spectrins, 0.2 mg/ml (except 0.1 mg/ml for chicken fodrin, Fig. 1 *b*); protein 4.1, 0.09 mg/ml; and actin 0.25 mg/ml.

pellet) and multiplying by 100. Nondenaturing PAGE was performed in unstabilized 2–4% polyacrylamide gels at 4°C as described by Morrow and Haigh (1983).

Immunoblotting

Polypeptides were transferred (TE Series Transphor Electrophoresis Unit; Hoefer Scientific Instruments, San Francisco, CA) from SDS polyacrylamide 5–16% minigels (Matsudaira and Burgess, 1979) to 0.2-μm pore nitrocellulose paper (Schleicher and Schuell, Inc., Keene, NH) in 25 mM Tris, pH 7.0, 192 mM glycine, 0.1% SDS, 20% methanol at 40 V, stained for total protein with 0.2% ponceau S in 3% TCA, destained with dH_2O, marked, and trimmed. Incubations were performed with gentle shaking. Nonspecific protein binding was blocked with Tris-buffered saline (TBS)/BLOTTO (Johnson et al., 1984; 50 mM Tris, pH 8.0, 150 mM NaCl, 5% nonfat dry milk, Carnation Co., Los Angeles, CA) at 37°C for 1 h. Sheets were then incubated overnight with rabbit sera (anti-avian protein 4.1, Granger and Lazarides, 1984; anti-human brain spectrin alpha chain, Harris et al., 1985; or anti-human protein 4.1, Croall et al., 1986; 1:500 dilution in TBS/BLOTTO) at room temperature and treated sequentially with TBS, peroxidase-conjugated goat anti-rabbit immunoglobulin (Cooper-Biomedical Inc., Malvern, PA; 1:1,000 dilution in TBS/BLOTTO), TBS, and TBS made 0.01% in Tween 20. Blots were developed with 0.05% 3,3′ diaminobenzidine, 0.01% H_2O_2 in PBS (10 mM Na phosphate, pH 7.2, 150 mM NaCl).

Results

Protein 4.1 Does Not Stimulate Actin Binding of Chicken Brush Border Spectrin (TW 260/240)

Using avian spectrins, with their common alpha subunit as an internal control, we investigated the spectrin/protein 4.1/actin ternary complex (Fig. 1). Control experiments demonstrated small and variable sedimentation of the pure spectrin isoforms alone (0–25%; Fig. 1, groups 1). This aggregation occurred despite clarifying (100,000 *g*, 1 h) the spectrin immediately before the assay. Aggregation was most pronounced for the avian spectrins and may be attributed to the greater tendency of these proteins to self associate (Pearl et al., 1984; Howe et al., 1985). The addition of protein 4.1 had variable effects on spectrin sedimentation, causing a slight decrease for that of the fodrins and an increase for the brush border and erythroid isoforms (Fig. 1, groups 2). The addition of actin increased the sedimentation (17–58%; Fig. 1, groups 3) of all spectrins over that seen in its absence. The addition of both protein 4.1 and actin dramatically increased

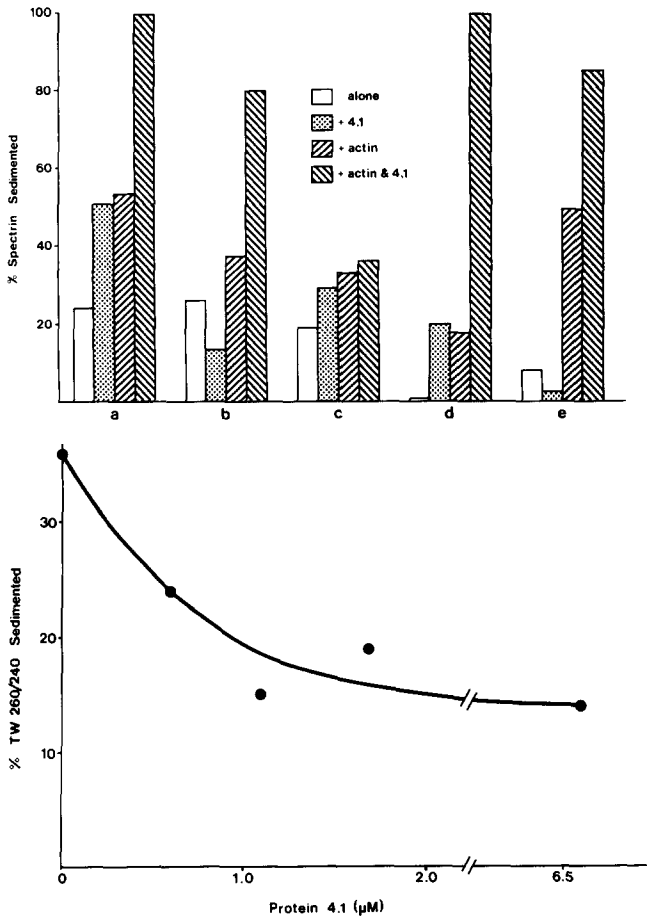

Figure 2. (*Top*) Quantitation of the gels presented in Fig. 1. Histograms are plotted as percent spectrin pelleted as determined by densitometric scanning of the spectrin bands (for details see Materials and Methods). The different spectrins (*a–e*) are as indicated in Fig. 1. Protein 4.1 enhanced the spectrin–actin interaction for all spectrins except the brush border isoform (group *c*). (*Bottom*) Protein 4.1 weakly inhibited the TW 260/240-actin interaction at pH 6.9. Concentration of TW 260/240 was 0.2 mg/ml; buffer conditions were as indicated in Materials and Methods, but at pH 6.9 to maximize the TW 260/240–actin interaction (Pearl et al., 1984; Fishkind et al., 1985). The percent TW 260/240 pelleted in the presence of the various protein 4.1 concentrations (0–17-fold molar excess, relative to TW 260/240; based on a dimer 500,000 M_r) was determined exactly as in the top panel. The plotted data has been corrected for nonspecific TW 260/240 pelleting.

the sedimentation (80–100%; Fig. 1, groups 4) of all spectrins except the brush border isoform (TW 260/240). Control experiments done in the absence of added spectrin demonstrated a weak direct interaction between protein 4.1 and F-actin (Fig. 1 *f*).

Quantitation of this 4.1-dependent spectrin–actin sedimentation is presented in Fig. 2 (*top*). Clearly, protein 4.1 enhanced this spectrin–actin interaction for all spectrins except the brush border isoform (Fig. 2 (*top*, group *c*). In fact, in many TW 260/240 cosedimentation assays, protein 4.1 slightly inhibited the TW 240/260–actin interaction over that in its absence (data not shown). Erythroid protein 4.1 stimulated brain and erythrocyte spectrins to bind actin to varying degrees: the spectrins of erythroid origin (Fig. 2, *top*, groups *a* and *d*) were maximally stimulated; both fodrins (Fig. 2, *top*, groups *b* and *e*) showed significant but lower activity, as noted previously (Burns et al., 1983; Lin et al., 1983).

Additional cosedimentation experiments were performed using conditions known to favor the TW 260/240–actin interaction (reduced ionic strength and lower pH; Pearl et al., 1984; Fishkind et al., 1985). Reducing the KCl concentration to 100 mM (pH 7.5) enhanced the amount of TW 260/240 pelleted by actin, but also revealed a slight inhibitory effect of protein 4.1 on the TW 260/240–actin binding (data not shown). Since lowering the ionic strength interferes with spectrin–protein 4.1 binding (Ohanian et al., 1984), we examined the concentration dependence of protein 4.1 on TW

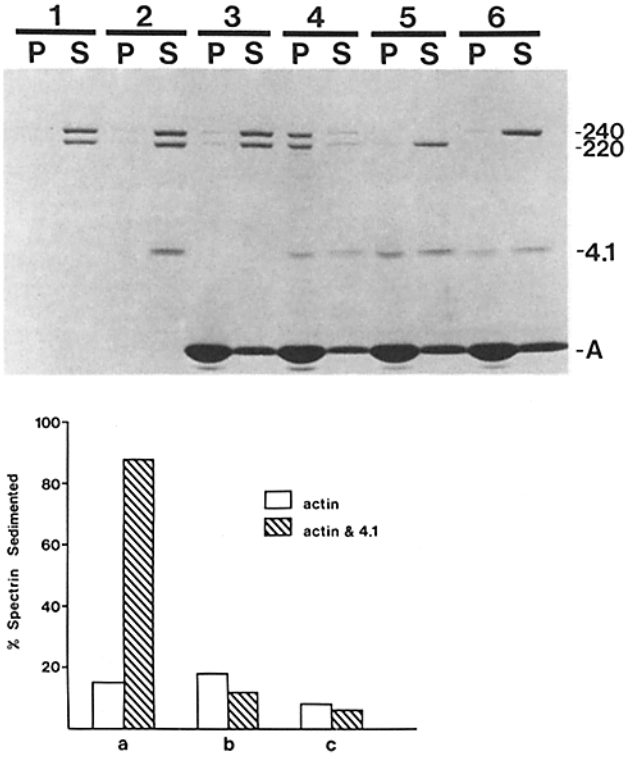

Figure 3. (*Top*) Protein 4.1 enhanced the cosedimentation of hybrid (chicken intestinal TW 260/240 alpha/human erythrocyte beta) spectrin–actin interactions. Sedimentation was performed with hybrid spectrin alone (group *1*), plus protein 4.1 (group *2*), plus actin (group *3*), and plus protein 4.1 and actin (group *4*). Controls include human erythrocyte beta subunit plus protein 4.1 and actin (group *5*) and chicken intestinal TW 240 (alpha) subunit plus protein 4.1 and actin (group *6*). Pellet (*P*) and supernate (*S*) fractions after 100,000 *g* spin were analyzed on a 5–15% SDS polyacrylamide gel. The R_f's of the chicken alpha (*240*) and human beta (*220*) spectrins along with protein 4.1 (*4.1*) and actin (*A*) are indicated. Final protein concentrations were as follows: chicken alpha and human beta spectrin subunits, 0.03 mg/ml; protein 4.1, 0.04 mg/ml; actin, 0.25 mg/ml. (*Bottom*) Quantitation of the gel presented in the top panel. Hybrid (chicken intestinal alpha/human erythroid beta) spectrin (*a*) and controls (chicken intestinal alpha [*b*] and human erythroid beta [*c*]) are shown under the two conditions indicated (plus actin and plus protein 4.1 and actin). Protein 4.1 enhanced the cosedimentation of only viable alpha/beta complexes (*a*) and had little, if any, effect on the individual subunits (*b* and *c*). Histograms are plotted as percent spectrin pelleted as determined by densitometric gel scanning (see Fig. 2 for details).

260/240–actin interaction at 150 mM KCl and a somewhat lower pH (6.9), conditions which also favor the binding of TW 260/240 to actin (Pearl et al., 1984; Fishkind et al., 1985). Under these conditions, protein 4.1 is maximally active (Ohanian et al., 1984). Increasing amounts of protein 4.1, up to a 17-fold molar excess, weakly inhibited the interaction between TW 260/240 and actin (Fig. 2, *bottom*).

Human Beta Spectrin Hybridizes with Chicken Brush Border Alpha and Bestows the Ability To Undergo Protein 4.1–stimulated Actin Binding

Although the avian alpha spectrins have identical iodopeptide maps (Glenney and Glenney, 1984) regardless of tissue origin and are encoded by a single gene (Birkenmeier et al., 1985; Curtis et al., 1985), it is conceivable that minor post-translational modifications might alter the sensitivity of the brush-border alpha (TW 240) subunit to protein 4.1. To exclude this possibility, we formed hybrid spectrin molecules. The avian beta subunits showed a remarkable propensity to self-associate (data not shown; in agreement with other work: Davis and Bennett, 1984*b*; Glenney and Weber, 1985), making sedimentation analysis of avian intestinal alpha/avian erythroid beta hybrid spectrins difficult to interpret. Human erythroid beta, however, did not aggregate in the presence of sufficient sulfhydryl reductants, and despite species differences, complexed with avian intestinal alpha. Like the erythroid spectrin, this hybrid cosedimented with actin in a protein 4.1–dependent fashion (Fig. 3, *top*). Control experiments demonstrated minimal sedimentation of hybrid spectrin alone (3–10%; Fig. 3, *top*, group *1*) or in the presence of either protein 4.1 (3–11%; Fig. 3, *top*, group *2*) or actin (7–15%; Fig. 3, *top*, group *3*). The addition of both protein 4.1 and actin dramatically increased the sedimentation (82–88%, group *4*) of hybrid spectrin. Control experiments using the isolated subunits themselves showed no significant sedimentation in the presence of both protein 4.1 and actin (6 and 12%, groups *5* and *6*, respectively). Nearly identical results to those presented in Fig. 3 were obtained using an avian erythroid alpha/human erythroid beta hybrid spectrin (data not shown), underscoring the similarities between avian alpha subunits. Quantitative analysis of the gels presented in Fig. 3 confirmed that only the hybrid spectrin bound actin in a 4.1–dependent manner (Fig. 3, *bottom*); the individual subunits remained in the supernate under all conditions (Fig. 3, *bottom*, groups *b* and *c*).

The fidelity of reassociation of the hybrid spectrin was examined using nondenaturing PAGE (Fig. 4; Morrow and Haigh, 1983). In these gels, isolated spectrin subunits ran as diffuse bands. Most of the isolated chicken brush border alpha (TW 240) remained as a very large aggregate at the top of the gel; that which entered the gel migrated just ahead of human erythroid spectrin tetramer (Fig. 4, lane *1*). Isolated human erythroid beta ran just behind human erythroid spectrin dimer (Fig. 4, lane *2*; in agreement with earlier work, Yoshino and Marchesi, 1984). The chicken brush border alpha/human erythroid beta hybrid spectrin ran as two sharply focused bands near the human erythroid spectrin tetramer and hexamer bands (Fig. 4, lane *3*). Control lanes included human erythrocyte spectrin which migrated as discrete dimer, tetramer, and oligomer bands (Fig. 4, lane *4*) and TW 260/240 which migrated primarily as a diffuse band just above human erythroid spectrin hexamer, presumably representing tetrameric TW 260/240 and as a very large aggregate which failed to enter the gel (Fig. 4, lane *5*). In addition, several less abundant bands were observed in the TW 260/240 preparations which presumably represent a dimeric form and proteolytic fragments (Howe et al., 1985).

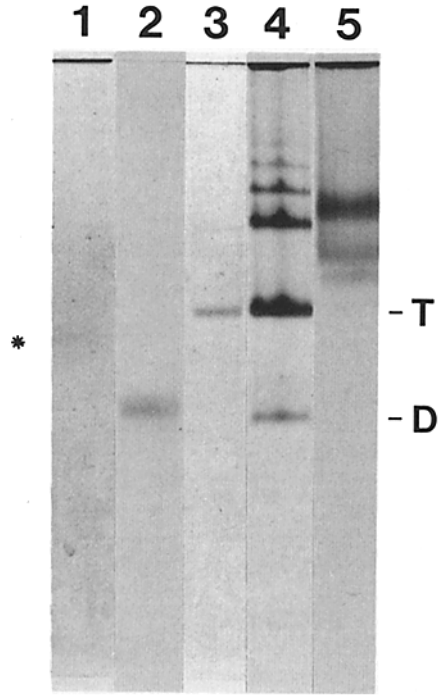

Figure 4. Analysis of spectrin subunits and avian intestinal alpha (TW 240)/human erythrocyte beta hybrid spectrin by nondenaturing gel electrophoresis. Lanes *1* and *2* show migration patterns of isolated subunits alone: chicken intestinal alpha (60 μg: * indicates diffuse band of that which entered the gel) and human erythroid beta (60 μg), respectively. Chicken brush border alpha/human erythroid beta hybrid spectrin (120 μg) ran as discrete bands (lane *3*), migrating near the native human erythrocyte tetramer and hexamer bands (lane *4*). Native TW 260/240 migrated as several bands above human erythrocyte tetramer and as an aggregate which failed to enter the gel (lane *5*). The R_f's of the native human erythrocyte spectrin dimers (*D*) and tetramers (*T*) are indicated.

An Immunoreactive Avian Protein 4.1 Isoform Is Not Present in Chicken Intestinal Epithelial Cells

The results presented above have demonstrated differences in the interactions of various chicken spectrins with human erythrocyte protein 4.1 and indicated that these interactions were mediated through the beta spectrin subunit. To examine the possibility that, unlike avian erythroid and brain spectrins, TW 260/240 requires an avian protein 4.1 isoform to interact with actin, we searched for protein 4.1 isoforms in chicken intestinal cells by immunoanalysis (Fig. 5). We used a polyclonal antiserum (kindly provided by E. Lazarides) specific for the six avian protein 4.1 isoforms (for characterization of this sera see Granger and Lazarides, 1984), all of which are derived from a single primary transcript (Ngai, J., R. T. Moon, and E. Lazarides, manuscript in preparation).

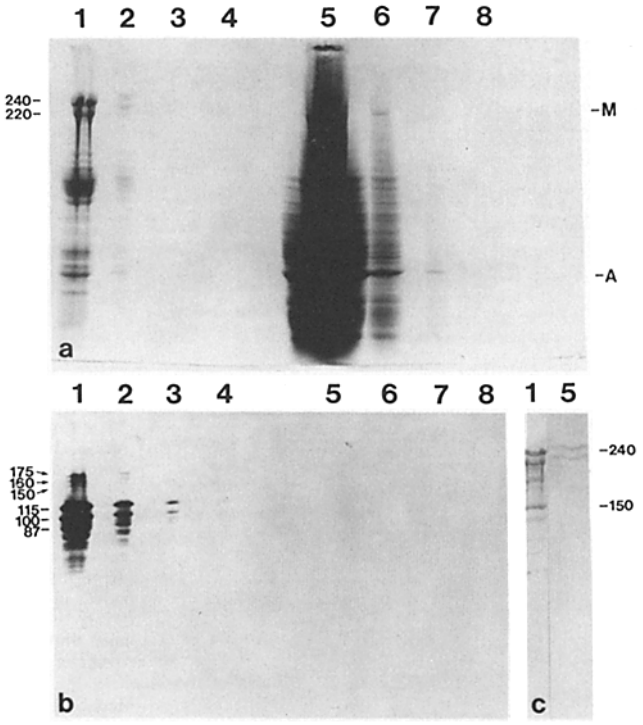

Figure 5. Immunoblot analysis showed no avian protein 4.1 is present in whole epithelial cells. Identical samples were run on identical SDS polyacrylamide gels; one was stained with Coomassie Blue (*a*) and the other two were electrophoretically transferred to nitrocellulose and probed with either an avian protein 4.1 polyclonal antibody and peroxidase-conjugated goat anti-rabbit immunoglobulin (*b*) or an anti-human brain alpha spectrin polyclonal antibody and peroxidase-conjugated goat anti-rabbit immunoglobulin (*c*). Lanes *1–6* are serial dilutions of a one-tenth suspension of chicken red cell ghosts (lane *1* has 3.2 μg total protein); each lane is an order of magnitude less protein than the one preceeding it. Lanes *5–8* are a similar serial dilution of whole intestinal epithelial cells (lane *5* has 40 μg total protein). Protein 4.1 was detected in a 1:1,000 (lane *4*) dilution of avian ghosts (3.2 ng total protein), but was not present in intestinal epithelial tissue (lanes *5–8*). The R_f's of the major bands erythroid spectrin alpha (*240*) and beta (*220*), myosin (*M*), actin (*A*), and the six major protein 4.1 bands (*175, 160, 150, 115, 100, 87*) are indicated.

To standardize the amount of protein 4.1 one would expect to detect if it was present in a ratio to spectrin similar to that found in the red blood cell, we have also probed these blots with an anti-alpha spectrin antibody. Gels were deliberately overloaded with whole epithelial cell sample (Fig. 5, lanes *5*) and a serial dilution thereof (Fig. 5, lanes *6–8*) to maximize the chance of detecting immunoreactive protein 4.1 isoforms. As a positive control, similar serial dilutions of chicken erythrocyte membranes were run and blotted in parallel (Fig. 5, lanes *1–4*). Even in the extremely overloaded lane, no immunoreactive protein 4.1 was detected in whole epithelial cells (Fig. 5 *b*, lane *5*) where there is at least one half as much TW 240 (and its proteolytic fragments) as erythroid alpha spectrin (Fig. 5 *c*). Conversely, immunoreactive protein 4.1 was detected in all dilutions of erythrocyte membranes (Fig. 5 *b*, lanes *1–4*). Similar studies using a mammalian erythrocyte protein 4.1 polyclonal antisera reactive with chicken protein 4.1 also showed no reactivity with intestinal epithelial cells.

Discussion

Spectrin, an extended molecule composed of two parallel chains, contains multiple linearly aligned structural domains (Morrow et al., 1980). This study focuses on those domains (presumably alpha-V and beta-IV) which contain the actin- and protein 4.1–binding site(s). Although both subunits weakly bind protein 4.1, only the intact heterodimer binds actin (Cohen and Langley, 1984). The results presented here clearly demonstrate that the beta subunit is the critical determinant of protein 4.1 specificity and sensitivity in this spectrin–actin interaction. Protein 4.1 does not stimulate actin binding of brush border spectrin (Figs. 1 and 2) but does stimulate actin binding of brush border alpha/human erythroid beta hybrid spectrin (Fig. 3). Furthermore, protein 4.1 only enhances the cosedimentation of viable alpha/beta hybrid complexes and has no effect on the individual subunits.

Several mechanisms may account for the ability of the beta subunit to alter the interaction of spectrin with protein 4.1 and/or actin. The simplest mechanism would postulate that erythrocyte (220 kD) and brain (235 kD) beta spectrins contain a specific protein 4.1–binding site, which although of low affinity in the absence of actin, is nevertheless important for initiating the formation of the tertiary complex. Independent cross-linking experiments using radiolabeled protein 4.1 support this hypothesis (Becker and Lux, 1984). Presumably, neither the brush border (TW 260) beta nor the alpha subunit from any spectrin contain such a regulatory protein 4.1 binding site. An alternative mechanism would involve the alpha subunit more directly in the protein 4.1 interaction, but still require the participation of the beta subunit for its expression, in a fashion analogous to the stimulation of spectrin oligomerization by ankyrin and band 3 (Morrow and Giorgi, manuscript submitted for publication).

Although a distinction cannot at present be made between these mechanisms, it is clear that the beta subunit is the critical subunit determining spectrin–protein 4.1 interactions. It is also clear that the ability of erythrocyte protein 4.1 to stimulate spectrin–actin binding varies quantitatively with the tissue of origin. For example, the strongest stimulation was observed in the erythroid spectrins; intermediate stimulation was detected for the brain spectrins, and no stimulation occurred for the brush border spectrin. This result is analogous to that seen with the interaction between the various beta spectrins and ankyrin. Although most spectrins bind ankyrin (Bennett et al., 1982; Burridge et al., 1982), the strength of the interaction varies widely depending on the tissue of origin of both the spectrin and the ankyrin (Davis and Bennett, 1984*a*; Howe et al., 1985; Harris et al., 1986), and in fact such binding is not detected in avian brush border spectrin. These results lend quantitative support to the notion that specific spectrin isoforms may be matched to unique isoforms of protein 4.1 or ankyrin, and that it is through such matched interactions that distinct membrane skeletal domains are established (Glenney and Glenney, 1983*a*; Lazarides and Nelson, 1985).

Our search for chicken intestinal protein 4.1 isoforms (potentially specific TW 260/240–actin stimulators) revealed no immunoreactive analogs (Fig. 5). This result is in agreement with the lack of specific binding of this antibody on frozen sections of chicken small intestines (Granger and Lazarides, 1984). While this finding is consistent with the insensitivity of TW 260/240 to stimulation by erythroid pro-

tein 4.1, it does not exclude the existence of other TW 260/240-actin cofactors. For example, mammalian synapsin I has many structural properties in common with erythroid protein 4.1, including binding spectrin at potentially identical sites (Baines and Bennett, 1985). In chicken, synapsin I has recently been purified from brain homogenates (Bixby and Reichardt, 1985). Chicken synapsin I does not cross-react with the anti-avian protein 4.1 antiserum (Lazarides, E., personal communication) and, consequently, it is a potential candidate for a TW 260/240-actin effector.

It is also noteworthy that the avian alpha subunits can form active hybrid complexes with human erythroid beta subunits (Figs. 3 and 4). Most of the chicken alpha subunit (TW 240) failed to enter nondenaturing gels (Fig. 4, lane *1*). In contrast, the human erythrocyte beta subunit migrated as a single diffuse band near that of native erythrocyte spectrin dimers (Fig. 4, lane *2*). When combined, the human beta spectrin induces a spontaneous disaggregation of chicken alpha spectrin, causing it to not only enter the gel but also to form discrete bands aligned with those of human erythrocyte spectrin tetramers and hexamers (Fig. 4, lane *3*). Such an interspecies, cross-tissue hybrid demonstrates a remarkable conservation of spectrin chain–chain binding sites. Newly synthesized avian alpha spectrin adopts one of two conformations, heterodimer or homo-oligomer (Woods and Lazarides, 1986), suggesting that chain–chain interactions are important even among identical subunits. Although it has been postulated that subunit–subunit associations may involve interactions along a hydrophobic face of the 106-residue spectrin repetitive unit (Speicher and Marchesi, 1984; Woods and Lazarides, 1986), it seems likely that these interactions occur at discrete sites (Morrow et al., 1980; Sears et al., 1986) since some, but not all, spectrin peptide fragments are competent for reassembly. Although other functions have yet to be identified specifically for the avian alpha subunit, one factor acting to preserve its structure through evolution may be the requirement that it combine faithfully with several diverse beta subunits.

We wish to thank Drs. James M. Anderson, Joan M. Carboni, Carol Cianci, Karen Conzelman, Sarah Fashena, Toshiko Shibayama, and Bruce R. Stevenson for their friendship and help during the course of this work; Drs. Bruce L. Granger and Elias Lazarides for graciously providing their anti-avian protein 4.1 antiserum; Dr. Richard A. Anderson for occasionally supplementing our erythroid protein 4.1 supply; Michael Boyd and Ann Golgia for their invaluable technical assistance; and Kris Hall Mooseker for her help preparing the manuscript.

This material is based upon work supported under a National Science Foundation Graduate Fellowship (T. R. Coleman), a Muscular Dystrophy postdoctoral fellowship (A. S. Harris), grants AM25387 (to M. S. Mooseker) and HL28560 (to J. S. Morrow) from the National Institutes of Health, and March of Dimes grants 1-924 (M. S. Mooseker) and 5-377 (J. S. Morrow).

Received for publication 30 June 1986, and in revised form 1 November 1986.

References

Baines, A. J., and V. Bennett. 1985. Synapsin I is a spectrin-binding protein immunologically related to erythrocyte protein 4.1. *Nature (Lond.).* 315:410–413.

Becker, P. S., and S. E. Lux. 1984. Protein 4.1 binding peptides of human erythrocyte spectrin identified by use of radiolabel-transfer cross-linker. *J. Cell Biol.* 99(4, Pt. 2):113a. (Abstr.)

Bennett, V. 1983. Proteins involved in membrane-cytoskeleton association in human erythrocytes: spectrin, ankyrin, and band 3. *Methods Enzymol.* 96:313–324.

Bennett, V. 1985. The membrane skeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* 54:273–304.

Bennett, V., J. Davis, and W. Fowler. 1982. Brain spectrin: a membrane-associated protein related in structure and function to erythrocyte spectrin. *Nature (Lond.).* 299:126–131.

Birkenmeier, C. S., D. M. Bodine, E. A. Repasky, D. M. Helfman, S. H. Hughes, and J. E. Barker. 1985. Remarkable homology among the internal repeats of erythroid and nonerythroid spectrin. *Proc. Natl. Acad. Sci. USA.* 82:5671–5675.

Bixby, J. L., and L. F. Reichardt. 1985. The expression and localization of synaptic vesicle antigens at neuromuscular junctions in vitro. *J. Neurosci.* 5:3070–3080.

Burgess, W. H., D. K. Jemiolo, and R. H. Kretsinger. 1980. Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate. *Biochim. Biophys. Acta.* 623:257–270.

Burns, N. R., V. Ohanian, and W. B. Gratzer. 1983. Properties of brain spectrin (fodrin). *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 153:165–168.

Burridge, K., T. Kelley, and P. Mangeat. 1982. Nonerythrocyte spectrins: actin–membrane attachment proteins occurring in many cell types. *J. Cell Biol.* 95:478–486.

Cohen, C. M., and R. C. Langley, Jr. 1984. Functional characterization of human erythrocyte spectrin alpha and beta chains: association with actin and erythrocyte protein 4.1. *Biochemistry.* 23:4488–4495.

Cohen, C. M., S. F. Foley, and C. Korsgren. 1982. A protein immunologically related to erythrocyte band 4.1 is found on stress fibers of non-erythroid cells. *Nature (Lond.).* 299:648–650.

Correas, I., T. L. Leto, D. W. Speicher, and V. T. Marchesi. 1986. Identification of the functional site of erythrocyte protein 4.1 involved in spectrin–actin associations. *J. Biol. Chem.* 261:3310–3315.

Croall, D. E., J. S. Morrow, and G. N. DeMartino. 1986. Limited proteolysis of the erythroid membrane skeleton by calcium dependent proteinases. *Biochim. Biophys. Acta.* 882:287–296.

Curtis, P. J., A. Palumbo, J. Ming, P. Fraser, L. Cloe, P. Meo, S. Shane, and G. Rovera. 1985. Sequence comparison of human and murine erythrocyte alpha-spectrin cDNA. *Gene.* 36:357–362.

Davis, J. Q., and V. Bennett. 1984*a*. Brain ankyrin: a membrane associated protein with binding sites for spectrin, tubulin, and the cytoplasmic domain of the erythrocyte anion channel. *J. Biol. Chem.* 259:13550–13559.

Davis, J. Q., and V. Bennett. 1984*b*. Brain spectrin isolation of subunits and formation of hybrid with erythrocyte spectrin subunits. *J. Biol. Chem.* 258: 7757–7766.

Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.

Fishkind, D. J., M. S. Mooseker, and E. M. Bonder. 1985. Actin assembly and filament cross-linking in the presence of TW 260/240, the tissue specific spectrin of the chicken intestinal brush border. *Cell Motil.* 5:311–322.

Glenney, J. R., Jr., and P. Glenney. 1983. Fodrin is the general spectrin-like protein found in most cells whereas spectrin and the TW protein have restricted distribution. *Cell.* 34:503–512.

Glenney, J. R., Jr., and P. Glenney. 1984. Comparison of spectrin isolated from erythroid and non-erythroid sources. *Eur. J. Biochem.* 144:529–539.

Glenney, J. R., Jr., and K. Weber. 1985. Separation of fodrin subunits by affinity chromatography on calmodulin–Sepharose. *Anal. Biochem.* 150:364–368.

Glenney, J. R., Jr., P. Glenney, M. Osborn, and K. Weber. 1982a. An F-actin- and calmodulin-binding protein from isolated intestinal brush borders has a morphology related to spectrin. *Cell.* 28:843–854.

Glenney, J. R., Jr., P. Glenney, and K. Weber. 1982*b*. F-actin-binding and cross-linking properties of porcine brain fodrin, a spectrin-related molecule. *J. Biol. Chem.* 257:9781–9787.

Glenney, J. R., Jr., P. Glenney, and K. Weber. 1982*c*. Erythroid spectrin, brain fodrin, and intestinal brush border proteins (TW 260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type–specific subunit. *Proc. Natl. Acad. Sci. USA.* 79:4002–4005.

Goodman, S. R., and I. S. Zagon. 1984. Brain spectrin: a review. *Brain Res. Bull.* 13:813–832.

Goodman, S. R., L. A. Cusorin, D. B. Coleman, and I. S. Zagon. 1984. Identification and location of brain protein 4.1. *Science (Wash. DC).* 224: 1433–1436.

Granger, B. L., and G. Lazarides. 1984. Membrane skeletal protein 4.1 of avian erythrocytes is composed of multiple variants that exhibit tissue-specific expression. *Cell.* 37:595–607.

Granger, B. L., and E. Lazarides. 1985. Appearance of new variants of membrane skeletal protein 4.1 during terminal differentiation of avian erythroid and lenticular cells. *Nature (Lond.).* 313:238–241.

Harris, A. S., J. P. Anderson, P. D. Yurchenco, L. A. D. Green, K. J. Ainger, and J. S. Morrow. 1985. Mechanism of cytoskeletal regulation: functional and antigenic diversity in human erythrocyte and brain beta spectrin. *Biochim. Biophy. Acta.* 817:147–158.

Harris, A. S., J. P. Anderson, P. D. Yurchenco, L. A. D. Green, K. J. Ainger, and J. S. Morrow. 1986. Mechanisms of cytoskeletal regulation: functional and antigenic diversity in human erythrocyte and brain beta spectrin. *J. Cell. Biochem.* 30:51–69.

Howe, C. L., L. M. Sacramone, M. S. Mooseker, and J. S. Morrow. 1985. Mechanisms of cytoskeletal regulation: modulation of membrane affinity in

avian brush border and erythrocyte spectrins. *J. Cell Biol.* 101:1379–1385.

Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Technique.* 1:3–8.

Keller, T. C. S., and M. S. Mooseker. 1982. Ca^{++}-calmodulin-dependent phosphorylation of myosin and its role in the brush border contraction in vitro. *J. Cell Biol.* 95:943–959.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lazarides, E., and W. J. Nelson. 1983. Erythrocyte and brain forms of spectrin in cerebellum distinct membrane-cytoskeletal domains in neurons. *Science (Wash. DC).* 220:1295–1296.

Lazarides, E., and W. J. Nelson. 1985. Expression and assembly of the erythroid membrane-skeletal proteins ankyrin (globin) and spectrin in the morphogenesis of chicken neurons. *J. Cell. Biochem.* 27:423–441.

Lin, D. C., M. D. Flannagan, and S. Lin. 1983. Complexes containing actin and spectrin from erythrocyte and brain. *Cell Motil.* 3:375–382.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Matsudaira, P. T., and D. R. Burgess. 1979. Identification and organization of the components in the isolated microvillus cytoskeleton. *J. Cell Biol.* 83:667–673.

Morrow, J. S., and W. B. Haigh, Jr. 1983. Erythrocyte membrane proteins: detection of spectrin oligomers by gel electrophoresis. *Methods Enzymol.* 96:298–304.

Morrow, J. S., D. W. Speicher, W. J. Knowles, C. J. Usu, and V. T. Marchesi. 1980. Identification of functional domains of human erythrocyte spectrin. *Proc. Natl. Acad. Sci. USA.* 77:6592–6596.

Navone, F., P. Greengard, and P. DeCamilli. 1984. Synapsin I in nerve terminals: selective association with small synaptic vesicles. *Science (Wash. DC).* 226:1209–1211.

Nelson, W. J., and E. Lazarides. 1983. Switching of subunit composition of muscle spectrin during myogenesis in vitro. *Nature (Lond.).* 304:364–368.

Ohanian, V., L. C. Wolfe, K. M. John, J. C. Pinder, S. E. Lux, and W. B. Gratzer. 1984. Analysis of the ternary interaction of the red cell membrane skeletal proteins spectrin, actin and 4.1. *Biochemistry.* 23:4416–4420.

Pearl, M., D. Fishkind, M. Mooseker, D. Keene, and T. Keller III. 1984. Studies on the spectrin-like protein from the intestinal brush border, TW 260/240, and characterization of its interaction with the cytoskeleton and actin. *J. Cell Biol.* 98:66–78.

Riederer, B. M., I. S. Zagon, and S. R. Goodman. 1986. Brain spectrin (240/235) and brain spectrin (240/235E): two distinct spectrin subtypes with different locations within mammalian neural cells. *J. Cell Biol.* 120:2088–2097.

Sears, D. E., V. T. Marchesi, and J. S. Morrow. 1986. A calmodulin and alpha subunit binding domain in human erythrocyte spectrin. *Biochim. Biophys. Acta.* 870:432–442.

Shile, P., A. Harris, and J. S. Morrow. 1985. Human skeletal muscle contains a unique tissue-specific spectrin isoform. *Fed. Proc.* 44:742a.

Speicher, D. W., and V. T. Marchesi. 1984. Erythrocyte spectrin is comprised of many homologous triple helical segments. *Nature (Lond.).* 311:177–180.

Spiegel, J. E., D. S. Beardsley, F. S. Southwick, and S. E. Lux. 1984. An analogue of the erythroid membrane protein 4.1 in nonerythroid cells. *J. Cell Biol.* 99:886–893.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. 1. Biochemical studies of interaction of the tropomyosin–troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

Tyler, J. M., W. R. Hargreaves, and D. Branton. 1979. Purification of two spectrin-binding proteins: biochemical and electron microscopic evidence for site-specific reassociation between spectrin and bands 2.1 and 4.1. *Proc. Natl. Acad. Sci. USA.* 76:5192–5196.

Tyler, J. M., B. N. Reinhardt, and D. Branton. 1980. Associations of erythrocyte-membrane proteins. *J. Biol. Chem.* 255:7034–7039.

Woods, C. M., and E. Lazarides. 1986. Spectrin assembly in avian erythroid development is determined by competing reactions of subunit homo- and hetero-oligomerization. *Nature (Lond.).* 321:85–91.

Yoshino, H., and V. T. Marchesi. 1984. Isolation of spectrin subunits and reassociation *in vitro*. *J. Biol. Chem.* 259:4496–4500.