

# Mechanisms of Cytoskeletal Regulation: Modulation of Membrane Affinity in Avian Brush Border and Erythrocyte Spectrins

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**ABSTRACT** The spectrins isolated from chicken erythrocytes and chicken intestinal brush border, TW260/240, share a common alpha subunit and a tissue-specific beta subunit. The ability of these related proteins to bind human erythrocyte inside out vesicles (IOVs) and human erythrocyte ankyrin *in vitro* have been quantitatively compared with human erythrocyte spectrin. Chicken erythrocyte spectrin binds human IOVs and human ankyrin with affinities nearly identical to that for human erythrocyte spectrin. TW260/240 does not significantly bind to either IOVs or ankyrin. These results demonstrate a remarkable tissue preservation of ankyrin-binding capacity, even between diverse species, and confirm the role of the avian beta-spectrins in modulating this functionality. Avian brush border spectrin may represent a unique spectrin which serves primarily as a filament cross-linker and which does not interact strongly with membrane-associated proteins.

The cortical cytoplasm of eukaryotic cells is characterized by a dense fibrillar array of interacting filaments and associated linking proteins. Two well-characterized examples of such arrays occur in the human erythrocyte (for review see reference 1) and the avian intestinal brush border (for review see reference 2). In each of these cells spectrin is a prominent component of the cortical cytoskeleton. In the erythrocyte, spectrin is essential for maintenance of membrane integrity (3). Over 70% of the mass of the erythrocyte skeleton is spectrin, which together with lesser amounts of F-actin and protein 4.1 forms an elaborate structure required for support of the plasma membrane. This spectrin-actin lattice is most strongly anchored to the membrane through high affinity binding between the beta-spectrin subunit (220 kD) and ankyrin (4, 5). Ankyrin in turn binds to the cytoplasmic domain of band 3, the anion channel protein. A secondary cytoskeletal membrane attachment exists at the site of spectrin-4.1-actin binding, since 4.1 binds to the cytoplasmic domain of glycoporphin in the presence of polyphosphatidyl inositol (6). The ankyrin binding site on erythrocyte spectrin lies near the site of tetramer and oligomer formation on the beta subunit (4). Conversely, the actin and 4.1 binding site on spectrin appears to involve the end remote from the oligomer-forming domain (for review see reference 1).

Avian brush border spectrin, TW260/240 (7, 8), may play quite a different role from erythrocyte spectrin. Immunolo-

calization (9) and selective extraction studies (8) suggest that the predominate task of TW260/240 is to cross-link actin filaments rather than couple membrane receptors to the cytoskeleton. TW260/240 forms strands that cross-link the basal ends of adjacent microvillar actin bundles in the terminal web region, and most of the spectrin in this region appears to be unassociated with the plasma membrane.

Because of these apparent differences in spectrin function, it is of interest to understand their molecular basis and to quantitate the strength of membrane affinity for each type of spectrin. Earlier work in the avian system (for reviews see references 10 and 11) has indicated that only the beta-spectrin subunit is tissue specific in this species, suggesting that any differences in spectrin function between tissues must be due to variations in this subunit. To explore these hypotheses quantitatively, we have compared the ability of spectrin from chicken brush border (TW260/240) and chicken erythrocytes to bind human erythrocyte inside out vesicles (IOVs)<sup>1</sup> and human erythrocyte ankyrin *in vitro*. The results indicate a remarkable preservation of binding function for the erythrocyte forms of spectrin, even between such diverse species as human and chicken, but almost no membrane-binding affin-

<sup>1</sup> *Abbreviations used in this paper:* DTT, dithiothreitol; IOVs, human erythrocyte inside out vesicles; PMSF, phenylmethylsulfonyl fluoride; RSG, resealed ghost; TIU, trypsin inhibitor units.

ity and undetectable human ankyrin-binding affinity for TW260/240. While enterocyte-specific forms of ankyrin or other membrane binding sites cannot be rigorously excluded, no immunoreactive forms of ankyrin were found in avian brush borders. These results indicate that the paradigm of spectrin as a membrane-filament linker requires extension to include strictly filament-filament interactions.

## MATERIALS AND METHODS

**Isolation of Chicken Brush Borders and TW260/240:** Brush borders were isolated from the small intestines of chickens by the method of Mooseker and Howe (12), as modified by Keller and Mooseker (13).

TW260/240 was isolated by the method of Glenney et al. (7), as modified by Pearl et al. (8). Briefly, brush borders were extracted for 30 min on ice with 0.6 M KCl, 2 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.2 mM dithiothreitol (DTT), 10 mM Tris, pH 8.0, with phenylmethylsulfonyl fluoride (PMSF) and 10–20 trypsin inhibitor units (TIU) aprotinin per liter. The extract was precipitated in 35% (saturated) ammonium sulfate, and gel filtered in 0.6 M KCl, 0.5 mM EDTA, 0.2 mM DTT, 10 mM Tris, pH 8.0, with aprotinin and PMSF using Bio-Rad A-15 M, in a 50-cm column. Fractions containing TW260/240 were chromatographed on a 5-ml hydroxyapatite column (Bio-Rad Laboratories, Richmond, CA) equilibrated with the A-15 column elution buffer containing 5 mM potassium phosphate at pH 7.2. The column was developed with a linear gradient of 5–300 mM phosphate. Peak fractions containing TW260/240 eluted ~110 mM phosphate.

**Purification of Chicken Erythrocyte Spectrin:** Chicken erythrocytes were harvested and processed following established procedures (14). All steps were conducted at 4°C. Blood was collected in 14 mM sodium citrate, 155 mM choline chloride, and 5 mM HEPES, pH 7.4. Erythrocytes were washed 3–5 times in the same buffer without citrate, with careful removal of the overlying leukocyte layer after each centrifugation. Lysis was accomplished in 40 vol of buffer containing 5 mM MgCl<sub>2</sub>, 5 mM NaN<sub>3</sub>, 1 mM EGTA, 1 mM DTT, 5 mM Tris, pH 7.5, with 0.5 mM PMSF and 10–20 TIU aprotinin per liter. This lysis procedure was repeated 3–4 times. The lysed cells were enucleated with a tight fitting Dounce homogenizer. Rehomogenization of the intact cell layer remaining after centrifugation was repeated 3–4 times. Lysed and enucleated membranes were stored on ice in lysis buffer. Prior to spectrin extraction, the membranes were twice washed in 2 mM EDTA, 10 mM Tris, pH 7.4, to remove residual Mg<sup>2+</sup>. Spectrin was extracted in 0.6 M KI, 0.5 mM EDTA, 0.2 mM DTT, 10 mM Tris-HCl buffer at pH 8.0 for 20 min on ice, and the extracted proteins were precipitated by addition of ammonium sulfate to 35% saturation. The extracted protein was freed of actin and other contaminants by gel filtration on Bio-Gel A-15M, exactly as described above for TW260/240 (8). Fractions from the gel filtration column were precipitated by dialysis into 60% saturated ammonium sulfate in 10 mM imidazole, 1 mM MgCl<sub>2</sub>, 0.2 mM DTT, 1 mM EGTA, 0.1 mM PMSF, and 10 TIU aprotinin per liter. The precipitated protein was dissolved and dialyzed against the IOV binding assay buffers. Samples were clarified by centrifugation at 100,000 g for 1 h prior to use.

**Preparation of IOVs, Stripped IOVs, and Human Erythrocyte Spectrin:** Hemoglobin-free human erythrocyte ghosts were prepared from freshly drawn blood by lysis and repeated washing in 5 mM sodium phosphate following established methods (4, 15). Spectrin was prepared by extraction at 0°C for 36 h in 0.1 mM EDTA, 0.05 mM sodium phosphate, pH 9.0, and purified by gel filtration on Sepharose C14-B (Pharmacia Fine Chemicals, Piscataway, NJ) in 130 mM KCl, 20 mM NaCl, 0.1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.05 μM PMSF, pH 7.4 at 4°C (4, 15). The spectrin- and actin-free vesicles remaining after spectrin extraction were harvested for binding studies as before (4, 15). Resealed ghosts (RSGs) used for nonbinding control membranes were prepared from freshly made ghosts by incubation for 30 min in isotonic phosphate-buffered saline (PBS) containing 0.02% NaN<sub>3</sub> (4, 15). IOVs stripped of associated cytoplasmic proteins were prepared by incubation in a 10-fold vol of 1 M KCl, 2.5 M urea, 1.0 mM EDTA, 100 mM glycine, 5 mM NaPO<sub>4</sub>, 0.4 mM diisopropylfluorophosphate, pH 7.4, for 30 min at 37°C, following established methods (16) with modifications.

**IOV Binding Measurements:** The binding of spectrins to human erythrocyte IOVs and RSGs was assayed at room temperature in 75 mM NaCl, 40 mM sodium phosphate, pH 7.2, with 0.1% bovine serum albumin in the final assay mixture. <sup>125</sup>I-labeled spectrin was used at a specific activity of ~50,000 cpm/μg protein. 40 μg of IOV or RSG protein was used per assay, in a total volume of 150 μl. The mixture of protein and vesicles (or RSGs) was incubated for 1 h; the unbound spectrin was separated from the vesicles by

sedimentation at 16,000 g for 30 min through a 150-μl cushion of 10% sucrose (4, 15).

**Protein Iodination and Other Procedures:** Proteins were iodinated at pH 8.0 in 1:2 PBS by either solid-phase lactoperoxidase–glucose oxidase (Enzymobeads, Bio-Rad Laboratories) catalyzed incorporation of <sup>125</sup>I (Amersham Corp., Arlington Heights, IL) onto tyrosine residues (17) or by the Bolton-Hunter reagent (ICN Radiochemicals, Irvine, CA) with incorporation into amino groups (18). Either method gave indistinguishable results in these binding assays. All protein determinations were by the method of Lowry (19). SDS PAGE was performed by the method of Laemmli (20). Nondenaturing PAGE was performed in unstabilized 2–4% polyacrylamide gels at 4°C as described by Morrow and Haigh (21).

## RESULTS

### Multiple Isoforms of Chicken and Human Spectrin Exist

Spectrins isolated from different tissues and different species are often characterized by subunits of variable molecular weight and composition, a fact recognized by several laboratories (10, 11). The spectrins isolated for this study also illustrate this diversity (Fig. 1). Human erythrocyte spectrin is composed of two subunits, an alpha subunit of 240 kD and a beta subunit of 220 kD as determined by PAGE. Chicken erythrocyte spectrin is composed of similar molecular mass subunits, although there appears to be little similarity by <sup>125</sup>I peptide mapping between these subunits and those of the human erythrocyte protein (10) (data not shown). The chicken brush border spectrin, TW260/240, is composed of a 260-kD beta subunit and a 240-kD alpha subunit. It is likely that the 240-kD alpha subunits of chicken erythrocyte and chicken brush border spectrin are identical, as judged by their <sup>125</sup>I peptide maps (10) (data not shown). Also shown in Fig. 1 is human brain spectrin, the predominate non-erythroid form

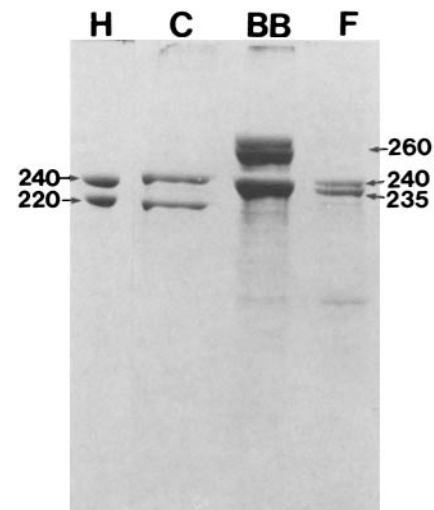


FIGURE 1 All spectrins contain an alpha subunit of 240-kD by SDS PAGE. This Coomassie Blue-stained gel also shows the diversity of the subunit associated with alpha-spectrin in the purified proteins used for this study. In human (H) and chicken (C) erythrocyte spectrin, the beta subunit is 220 kD. In the avian brush border (BB), the associated subunit is a tight doublet centered at 260 kD, leading to the alternative designation of this protein as TW260/240 (7). Shown for comparative purposes in the gel is purified human brain spectrin or fodrin (F), which is a 240- and 235-kD heterodimer (25). The minor component at 150 kD in lanes BB and F represents a proteolytic degradation product of alpha-spectrin frequently observed in these preparations.

of spectrin in mammals (for reviews see reference 11, 22, 23). This spectrin is composed of a 240-kD alpha subunit and a 235-kD beta (or gamma) subunit. Unlike the avian spectrins, the mammalian 240 kD subunits are not identical between erythroid and non-erythroid tissues (10, 24, 25).

### Only Erythroid Spectrins Bind IOVs with High Affinity

Human erythrocyte ghosts may be stripped of their spectrin by low ionic strength extraction, yielding the IOVs (Fig. 2). Also shown are the ankyrin- and 4.1-depleted vesicles obtained after both high salt and urea extraction. Control experiments (Fig. 3) confirmed that human erythrocyte spectrin rebinds to spectrin-depleted erythrocyte IOVs in a saturable fashion (26). The IOVs used in these experiments bound  $^{125}\text{I}$ -labeled human erythrocyte spectrin with an apparent  $K_a$  of  $2.0 \pm 0.3 \times 10^7 \text{ M}^{-1}$  and a limiting stoichiometry of  $\sim 140\text{--}150 \mu\text{g}$  spectrin per mg of IOV protein. This corresponds to  $\sim 100,000$  spectrin molecules (dimer) per erythrocyte, very close to estimates for the number of ankyrin molecules in the erythrocyte (27, 28). Several different preparations of spectrin  $^{125}\text{I}$ -labeled by either lactoperoxidase, Iodobeads (Pierce Chemical Co., Rockford, IL), or Bolton-Hunter Reagent, or  $^{32}\text{P}$ -labeled with  $(^{32}\text{P})\text{-}\gamma\text{-ATP}$  all gave indistinguishable results (data not shown).

Chicken erythrocyte spectrin ( $^{125}\text{I}$ -labeled) also rebound to human IOVs. As shown in Fig. 3, this binding was indistinguishable from the control binding of human erythrocyte spectrin, both in terms of its apparent affinity and stoichiometry. This similarity is most clearly apparent in Fig. 4, where the data in Fig. 3 is presented as a Scatchard analysis

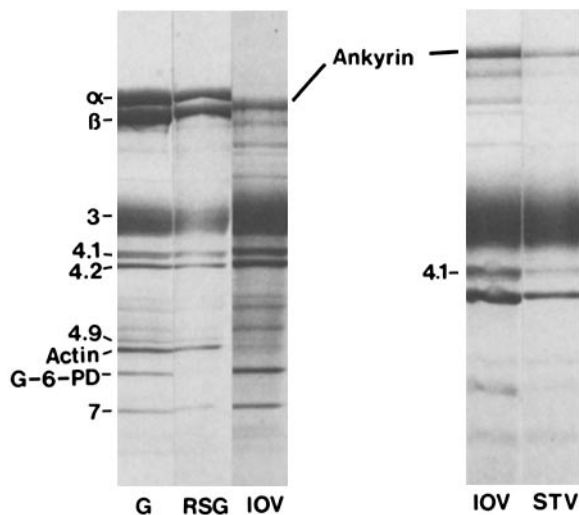


FIGURE 2 The membrane preparations used were all derived from human erythrocyte ghosts (G), shown here after SDS PAGE and Coomassie Blue staining. The major erythrocyte membrane proteins are indicated, using standard nomenclature (for review see reference 1). Treatment of erythrocyte ghosts with PBS removes the glyceraldehyde-6-phosphate dehydrogenase, and renders them impermeable to large proteins (15). These are called resealed ghosts (RSG). Removal of spectrin and actin from the ghosts (see Materials and Methods) produces inverted membrane (inside out) vesicles (IOV). Ankyrin and protein 4.1 are removed from the IOVs by extraction with 1 M KCl and 2.5 M urea, yielding stripped vesicles (STV).

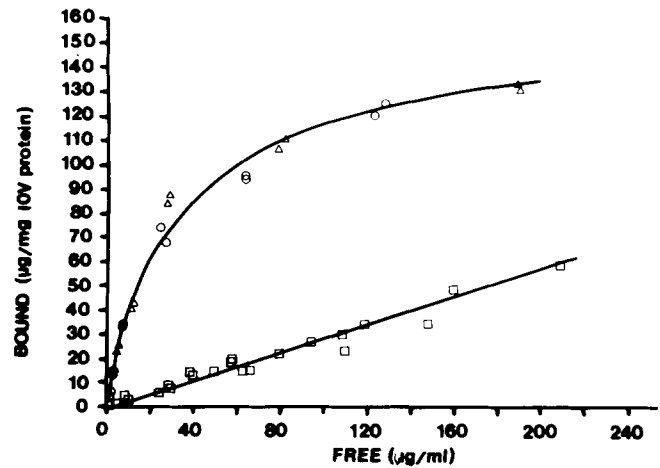


FIGURE 3 Erythroid spectrins from human (O) and chicken ( $\Delta$ ) bind human IOVs almost identically. TW260/240 binds the same vesicles weakly ( $\square$ ). As described in Materials and Methods, increasing amounts of radiolabeled spectrin from human erythrocytes, chicken erythrocytes, or chicken intestinal brush border were incubated with 30–50  $\mu\text{g}$  of IOV protein, and the bound and free components separated by sedimentation through 10% sucrose. Saturation of the TW260/240 spectrin binding could not be observed at the concentrations of protein experimentally obtainable, since above  $\sim 300 \mu\text{g/ml}$  TW260/240 began to aggregate, leading to sedimentation of the protein even in the absence of added vesicles (8).

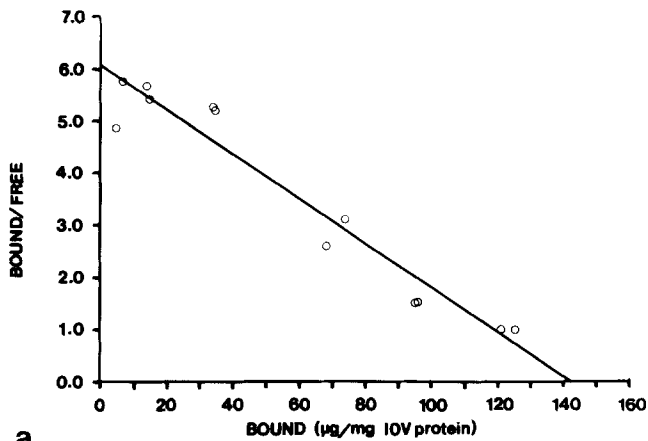
(29). The apparent  $K_a$  of chicken erythrocyte spectrin-binding to IOVs is  $1.0 \pm 0.3 \times 10^7 \text{ M}^{-1}$ . The stoichiometry of binding for both chicken and human erythroid spectrins is also nearly identical. Thus despite the considerable species difference between these two erythroid spectrins, both bind to human IOVs, and presumably to human ankyrin, with similar high affinities.

Chicken brush border spectrin binds weakly to human IOVs. The binding behavior of  $^{125}\text{I}$ -labeled TW260/240 is also shown in Figs. 3 and 4. Unlike its erythrocyte counterpart, this spectrin displayed a very weak, almost linear binding isotherm. Saturation of binding could not be demonstrated, probably due to experimental limitations. At higher concentrations of free TW260/240, the protein began to aggregate as judged from increased precipitation in the absence of added membranes. This phenomenon has been previously documented for this class of spectrins (8). Extrapolations based on Scatchard analysis (Fig. 4) suggest a saturation point near that for the other spectrins, although this determination is obviously less precise than that for the more strongly binding erythrocyte spectrins. The apparent  $K_a$  for TW260/240 binding to human erythrocyte IOVs was  $\sim 1 \times 10^6 \text{ M}^{-1}$ .

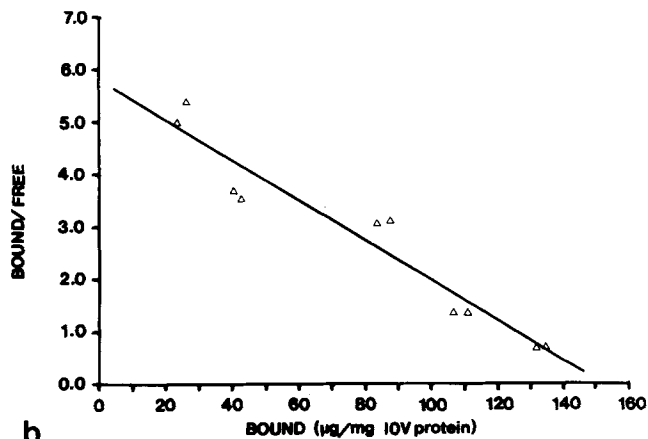
### All Spectrins Bind Erythrocyte Membranes in a Side-specific Fashion

The weak binding of chicken brush border spectrin to IOVs suggested a nonspecific interaction. However, none of the various spectrins bound to resealed human erythrocyte ghosts, a measure of binding to the external membrane surface (Fig. 5). Other experiments with proteolytically treated IOVs also demonstrated no binding of any of the spectrins (data not shown). Thus, even the weak binding displayed by the brush border spectrin appears to involve a protein receptor which is cytoplasmic directed and sensitive to proteolysis.

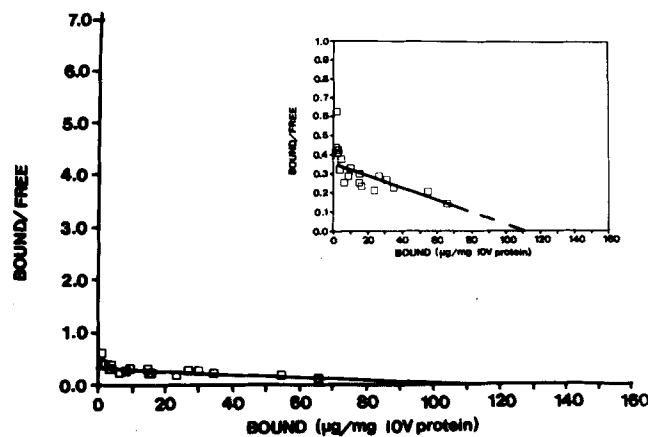
To identify the nature of the protein receptor on the IOVs



a



b



c

FIGURE 4 Scatchard analysis of the data shown in Fig. 3 using a least squares regression analysis confirms the similarity of binding for the two erythroid spectrins. (a) Human erythroid spectrin; the apparent  $K_a = 2.0 \pm 0.3 \times 10^7 M^{-1}$  and the limiting stoichiometry is  $142 \mu g$  spectrin bound per mg IOV protein. (b) Chicken erythroid spectrin; the apparent  $K_a = 1.0 \pm 0.3 \times 10^7 M^{-1}$  and the limiting stoichiometry is  $152 \mu g/mg$ . (c) Chicken brush border spectrin; the weakness of the binding precludes a precise determination of the association constant and stoichiometry. A weighted regression analysis of this data yields an apparent  $K_a$  of  $\sim 1 \times 10^6 M^{-1}$  and a predicted stoichiometry of  $\sim 110 \mu g/mg$ .

for the various spectrins, binding studies were done using the stripped IOVs shown in Fig. 2, followed by selective reconstitution with either ankyrin or protein 4.1. Similar experiments

done with human erythrocyte spectrin have previously demonstrated that ankyrin is required for high affinity membrane binding (16, 27). The results summarized in Fig. 6 confirm this requirement in the case of the human erythrocyte spectrin. For the brush border protein there is no significant effect of ankyrin or protein 4.1 on its binding to the IOVs (Fig. 6B). Chicken erythrocyte spectrin was not examined in this assay.

The ability of human erythrocyte spectrin to competitively inhibit the binding of TW260/240 to IOVs was also examined. In these assays, a paradoxical effect was observed (Fig. 7B). Low concentrations of unlabeled human erythrocyte spectrin stimulated TW260/240 binding, while higher levels subsequently led to competitive inhibition. The  $K_i$  of this inhibition ( $6 \times 10^6 M^{-1}$ ) was identical to that measured in a similar control experiment of human erythrocyte spectrin binding (Fig. 7A). These results suggested that in the presence of erythrocyte spectrin the brush border was as difficult to compete from the membrane as was the erythrocyte protein, despite its much lower affinity in direct binding assays. This unusual effect was traced to the formation of mixed hybrids between brush border and human erythrocyte spectrin (Fig. 7B, inset; and by native gel electrophoresis, data not shown), a phenomenon also noted for other spectrins (30). Thus when radiolabeled brush border spectrin and a small amount of its 150-kD proteolytic product joined noncovalently to the unlabeled erythroid spectrin, the brush border spectrin in effect gained a high affinity membrane binding site. This "new" binding site accounted for the enhanced binding observed in the competition studies at the low initial concentrations of inhibitory spectrin. Subsequent additions of unlabeled erythroid spectrin then competed with the hybrid TW260/240 and erythroid spectrin complex. Independent experiments in which brush border spectrin and human erythroid spectrin were mixed in 6 M urea, and in which the urea was subsequently removed, also demonstrated a several-fold enhancement of membrane binding by the brush border due to hybrid formation between erythroid and brush border spectrin (Fig. 7B, inset).

Finally, the ability of TW260/240 to competitively inhibit the binding of erythroid spectrin to the IOVs was measured (Fig. 7C). In this assay, no inhibition was observed, further confirming the lack of interaction of this protein with ankyrin.

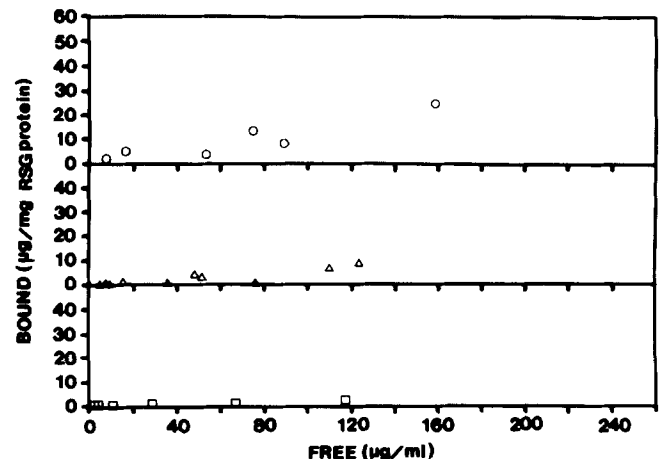
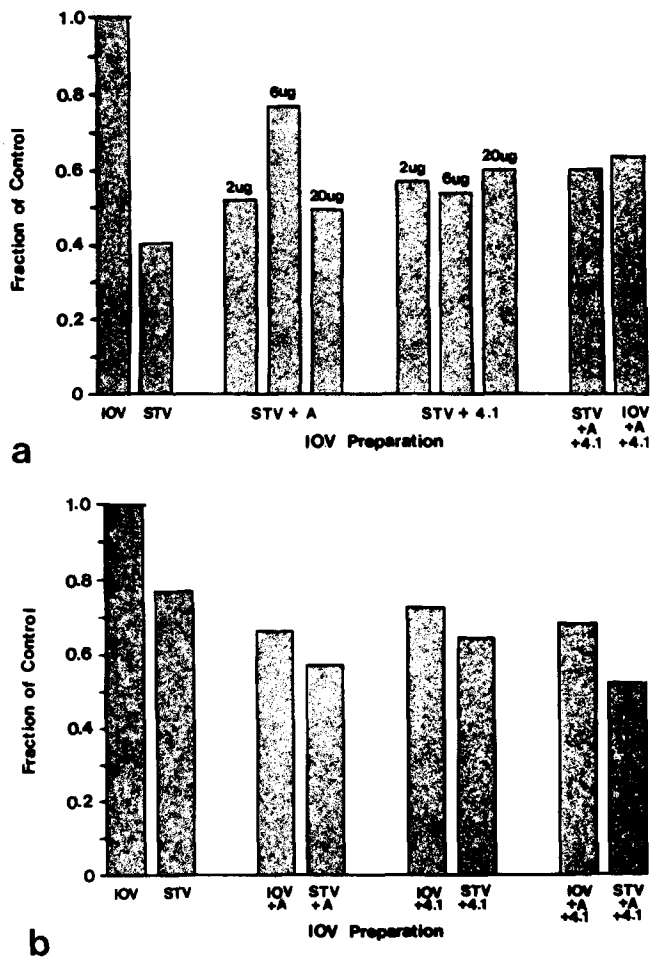


FIGURE 5 The binding of all spectrins is negligible to RSGs. Thus each spectrin binds erythrocyte membranes in a side-specific fashion. Conditions were the same as in the experiments in Fig. 3. O, human erythrocyte spectrin;  $\Delta$ , chicken erythrocyte spectrin;  $\square$ , chicken TW260/240.



**FIGURE 6** Removal of ankyrin and protein 4.1 does not alter the binding of TW260/240 to erythrocyte membranes. (a) Control experiment demonstrating the suppression of human erythrocyte spectrin binding by removal of ankyrin and protein 4.1 from IOVs (cf. Fig. 2). 40  $\mu\text{g}$  of IOV or stripped vesicles (STV) were incubated with 16  $\mu\text{g}$  of radiolabeled spectrin, and various amounts of purified ankyrin or protein 4.1. Binding is expressed as fraction of the initial binding to the IOVs. Conditions were otherwise the same as in Fig. 3. The binding of STV may be largely restored by the addition of increasing amounts of ankyrin (STV + A), although at high levels of ankyrin (20  $\mu\text{g}$ ) inhibition by the free ankyrin overcomes the initial stimulation (16). Protein 4.1 alone does not restore the binding (STV + 4.1). The complex action of ankyrin and 4.1 is illustrated in the last two lanes (STV or IOV + A + 4.1). The addition of 20  $\mu\text{g}$  each of ankyrin and 4.1 stimulates binding to stripped vesicles, but inhibits the binding to IOVs. The inhibition observed in these experiments is presumably due to competition of soluble ankyrin with that bound to the membrane for the available spectrin. (b) The binding of TW260/240 does not appear to require either ankyrin or protein 4.1. The conditions were identical to those described above, except that now the binding protein was chicken brush border spectrin. There is minimal reduction in binding realized when the vesicles are depleted of ankyrin and protein 4.1 (STV). This binding is not restored by the addition of 3–4  $\mu\text{g}$  of either ankyrin or 4.1 (+A or +4.1), and in fact a slight inhibition is even observed. The origin of this mild inhibitory effect is unknown.

### Only the Erythrocyte Spectrins Bind Ankyrin with High Affinity *In Vitro*

Although the stripped vesicle experiments described above indicated that ankyrin was not responsible for the weak

membrane binding of TW260/240, this question was further explored by measuring the direct binding between the purified proteins *in vitro* using nondenaturing PAGE (21) and  $^{125}\text{I}$  Bolton-Hunter-labeled ankyrin (Fig. 8). Native TW260/240 migrated in these gels as a doublet presumably representing the tetrameric form, and as a very large aggregate which failed to enter the gel (Fig. 8A). In addition, several less abundant, more slowly migrating bands were observed, suggestive of intermediate states of association. These results are consistent with earlier ultrastructural and sedimentation velocity measurements indicating that this protein aggregates at low ionic strength (8). When this protein was incubated with ankyrin, no binding of the two was detected (Fig. 8, B and E). The small amount of ankyrin trapped at the top of the gel evident in Fig. 8B was comparable to amounts present in control experiments for this preparation of radiolabeled ankyrin in the absence of spectrin or TW260/240.

The chicken erythrocyte spectrin migrated predominantly as a sharply focused band near that of human erythrocyte spectrin tetramer (Fig. 8C). Lesser amounts of more slowly migrating bands were also present. Radiolabeled ankyrin bound strongly to this avian spectrin (Fig. 8, D and E). By this assay, both human and chicken erythrocyte spectrins demonstrated nearly equivalent binding ( $K_a = 1.2 \times 10^7$  and  $0.9 \times 10^7 \text{ M}^{-1}$ , respectively). These values are comparable to the binding affinities found for the IOVs and confirm that ankyrin is the principal high affinity membrane receptor for these two spectrins.

### DISCUSSION

The rationale for the studies presented here is to quantitatively investigate functional differences among spectrin isoforms, taking advantage of the avian system where any such differences must be contributed by the variable beta subunit. We have focused on spectrin-membrane interaction because this interaction is thought to be fundamental to spectrin function in both erythroid and nonerythroid cells. The identification of both ankyrin (31, 32) and protein 4.1 (33–36) analogs in nonerythroid tissues has provided strong support for the notion that the molecular basis for spectrin-membrane interaction, first defined in the erythrocyte, is universal. The results presented here demonstrate that avian erythrocyte spectrin is virtually indistinguishable from human erythrocyte spectrin with respect to membrane interaction via ankyrin. In fact, avian erythrocyte spectrin binds to human IOVs and ankyrin with much higher affinity than does the human nonerythrocyte spectrin, fodrin (Harris, A. S., J. P. Anderson, P. D. Yurchenco, L. A. D. Green, K. A. Ainger, and J. S. Morrow, manuscript submitted for publication). Thus despite wide species difference, the beta subunit of erythrocyte spectrins are highly conserved with respect to membrane binding.

In contrast to the erythrocyte spectrins, brush border spectrin binds IOVs very weakly. This binding does not appear to be ankyrin mediated, since TW260/240 fails to inhibit the ankyrin-mediated binding of human erythrocyte spectrin. In addition, TW260/240 does not bind ankyrin when measured by native gel electrophoresis. This assay can detect association constants greater than  $1 \times 10^5 \text{ M}^{-1}$ . Consequently, if TW260/240 does bind to ankyrin, it does so with at least a 100-fold lower affinity than either human or avian erythrocyte spectrin. Of course one cannot rigorously exclude a membrane-binding role for avian brush border spectrin, since isoforms of ankyrin

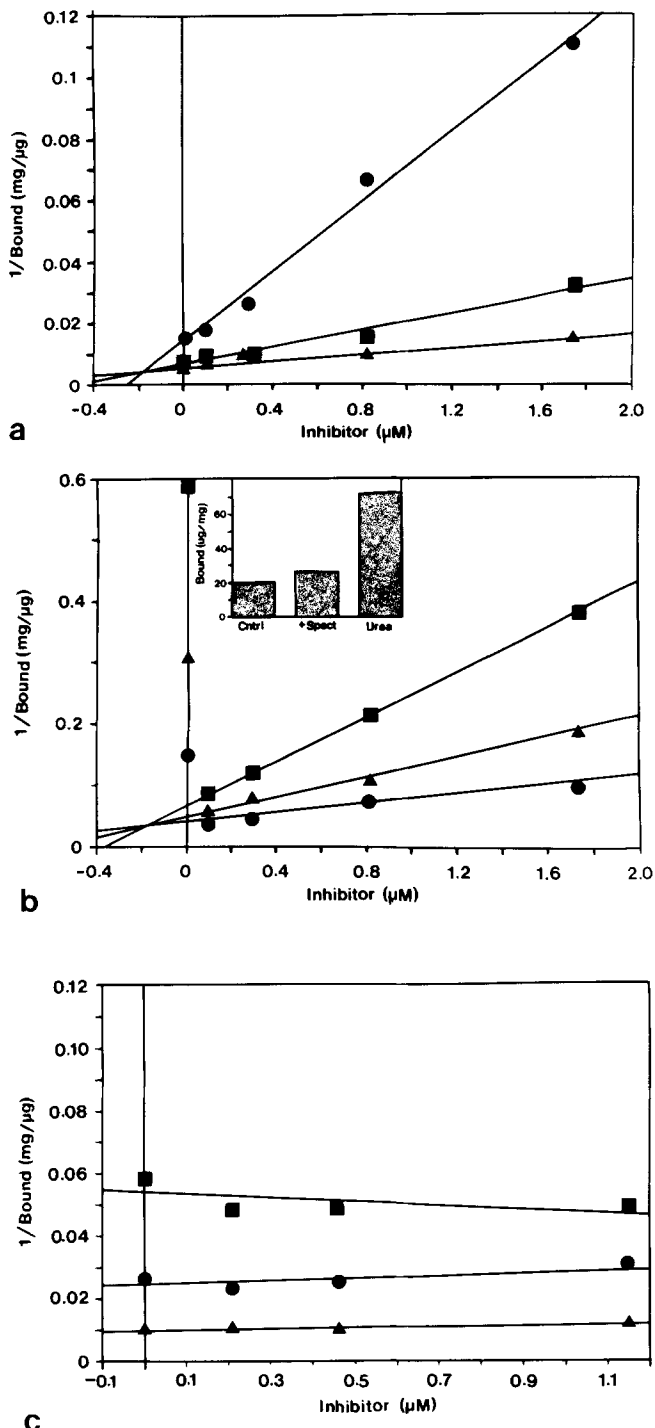


FIGURE 7 Dixon inhibition plots of spectrin binding to IOVs. (a) Control plot of erythroid spectrin inhibition by unlabeled spectrin. 70  $\mu\text{g}$  of IOVs were incubated with 80 ( $\blacktriangle$ ), 40 ( $\blacksquare$ ), or 9 ( $\bullet$ )  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled spectrin and variable amounts of inhibitor as indicated. The  $K_i$  of erythroid spectrin inhibition by erythroid spectrin in this study was  $5\text{E}06$  (M)-1. (b) Inhibition of  $^{125}\text{I}$ -labeled TW260/240 binding by human erythroid spectrin. Conditions were same as above. IOVs were incubated with 44 ( $\bullet$ ), 22 ( $\blacktriangle$ ), or 9 ( $\blacksquare$ )  $\mu\text{g}$  of TW260/240, and increasing concentrations of inhibitory erythroid spectrin as indicated. Note that initially the added unlabeled erythroid spectrin stimulates binding of TW260/240 (cf. points at zero added inhibitor). (Inset) Stimulation of IOV binding of 0.15  $\mu\text{M}$  TW260/240 by 0.2  $\mu\text{M}$  erythroid spectrin, both before (center lane) and after cycling through 6 M urea (right lane). The urea treatment facilitates the formation of brush border and erythrocyte spectrin

might bind TW260/240 with greater affinity. However, immunoreactive analogs of ankyrin are not detected in the brush border by immunoblotting with anti human ankyrin antibodies (data not shown). Thus the most likely interpretation of this data is that avian brush border spectrin cross-links tightly the rootlet microvillar core bundles but does not link these filaments to membranes through an ankyrin-mediated pathway.

At this point it is important to note the studies of Hirokawa et al. (37) on the immunolocalization of spectrin in the terminal web region of the mouse intestinal brush border. These workers have provided evidence for the involvement of spectrin in the cross-linkage of microvillar cores and in the linkage of these cores to the plasma membrane and to vesicles within the terminal web. These results seem inconsistent with the membrane binding studies presented here. However, based on peptide-map analysis of the spectrin isoform present in the human intestinal brush border (data not shown), it appears that the mammalian brush border contains protein probably identical to fodrin rather than a tissue-specific form of brush border spectrin.

In erythroid spectrin a secondary membrane binding site also exists via a protein 4.1-actin-glycophorin complex (6). Protein 4.1 binds mammalian erythrocyte spectrin strongly only in the presence of F-actin, and thereby stimulates the binding of spectrin to F-actin (1). It is possible that TW260/240 uses this alternative mode of membrane attachment. Other evidence tends to exclude this possibility, since TW260/240 does not interact with erythrocyte 4.1, whereas avian erythroid spectrin does (Coleman, T., A. Harris, M. S. Mooseker, and J. S. Morrow, manuscript in preparation).

The significance of the weak IOV binding observed for the TW260/240 in these experiments is thus difficult to interpret. Its genesis remains uncertain. The stimulation of binding observed in the presence of erythroid spectrin, due to mixed hybrid formation between the spectrins, suggests that residual contamination of the vesicles with spectrin may paradoxically offer one form of binding site. Conversely, interactions with other unidentified anchoring proteins cannot be excluded.

The authors wish to thank Dr. Mauro Giorgi for providing a sample of radioiodinated ankyrin, and Ms. Janet Wicklow for expert secretarial assistance.

This work was supported in part by the following grants: HL 28560 and AM 25387 from the National Institutes of Health to J. S. Morrow and M. S. Mooseker, respectively; a Basil O'Connor Starter Grant (No. 5-377) to J. S. Morrow; and a Basic Research Grant (1-924) from the March of Dimes Birth Defects Foundation to M. S. Mooseker.

Received for publication 28 May 1985

hybrids. (c) Absence of inhibition of erythroid spectrin binding to IOVs by added unlabeled TW260/240. In this experiment 27  $\mu\text{g}$  of IOVs were used, and the buffer included 10 mM imidazole and 1 mM  $\text{MgCl}_2$ ; otherwise the conditions were analogous to those above. No inhibition of human erythroid spectrin binding by the indicated amounts of added TW260/240 was observed. The amounts of erythroid spectrin present in each assay was 4.5 ( $\blacktriangle$ ), 1.8 ( $\bullet$ ), or 0.9 ( $\blacksquare$ )  $\mu\text{g}$ .

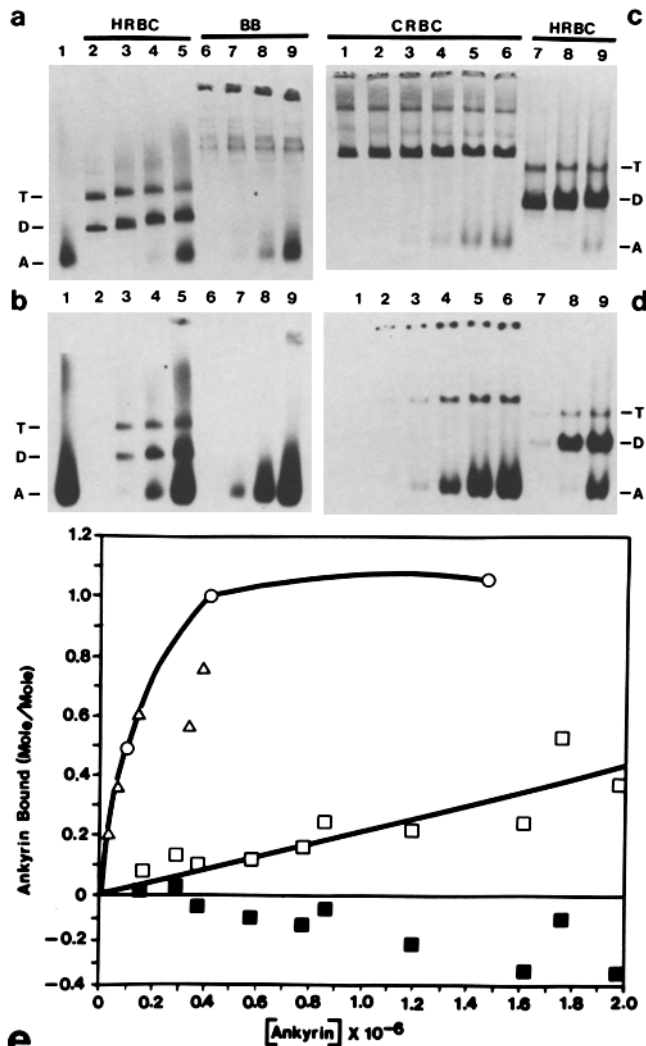


FIGURE 8 Ankyrin binds only to the erythroid spectrins *in vitro* as detected by native PAGE. Various spectrins were incubated with <sup>125</sup>I-labeled ankyrin under isotonic conditions as described in Materials and Methods. Aliquots of the incubation mixture for each ankyrin concentration were electrophoresed at 4°C by native PAGE (21). (a) Coomassie Blue-stained gel of human erythrocyte spectrin (0.3 mg/ml) (lanes 2–5) with <sup>125</sup>I-labeled ankyrin (20,570 cpm/μg). The ankyrin alone is shown in lane 1. The total concentration of ankyrin in the incubation mixtures ranged from 0.5 to 2.4 μM. The migration positions of free erythrocyte spectrin tetramer (T) and dimer (D), as well as free ankyrin (A) are indicated. Chicken brush border spectrin (0.3 mg/ml) (lanes 6–9) under comparable conditions migrates as a multicomponent band near erythrocyte tetramer, and as an aggregate which fails to enter the gel. Note the shift in mobility of the erythrocyte protein induced by binding to ankyrin. (b) Autoradiogram of the gel shown in a. The binding of ankyrin to erythrocyte spectrin is apparent. No binding to the brush border spectrin is apparent (see text). (c) Similar experiment as shown in a, but with chicken erythrocyte spectrin (lanes 1–6) at 0.16 mg/ml. The predominant species migrates in the gel with a mobility similar to tetrameric human erythrocyte spectrin (T). Also shown (lanes 7–9) are control lanes loaded with human erythrocyte spectrin, similar to those in gel a. (d) Autoradiogram of the gel shown in c. The ankyrin used in this experiment (22,835 cpm/μg) was a different preparation from that shown in gel a. This ankyrin contained no aggregated material (cf. lanes b5 and d9). Strong binding of ankyrin to chicken erythrocyte spectrin is observed. (e) Quantitation of ankyrin binding by gamma counting of the native gels. The bound and free ankyrin counts were measured after cutting the bands from the gels shown in a and b. O, human erythrocyte spectrin

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binding; Δ, chicken erythrocyte spectrin binding; □, chicken brush border spectrin binding, uncorrected for nonspecifically aggregated ankyrin which is present at the gel top in the absence of added TW260/240; ■, TW260/240 binding corrected by subtraction of the aggregated ankyrin counts.