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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

(membrane/microfilament/calcium/axonal transport/intestinal epithelium)

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ABSTRACT **Spectrin, fodrin, and TW-260/240 form a group of structurally and functionally similar but not identical high molecular weight actin-binding proteins from chicken erythrocytes, brain tissue, or intestinal epithelial brush borders. Immunological data and one-dimensional peptide maps of the separated subunits suggest that a common (M_r 240,000) and a variant (M_r 220,000, 235,000, or 260,000) subunit account for the three different heterodimers. These results are in line with the related but distinct morphology of the three proteins observed in micrographs of rotary-shadowed molecules and the finding that the common (M_r 240,000) subunit seems to account for the calcium-dependent calmodulin-binding activity displayed by the three proteins. The possible functions of spectrin-like molecules in nonerythroid cells are discussed.**

The cytoskeletal system of the cortical cytoplasm is composed, in part, of actin and associated proteins. These actin-binding proteins can regulate the organization of microfilaments and exert control over such microfilament-related functions as cell shape and deformability, cell movement, and the distribution of membrane proteins. Identification of the actin-binding proteins that are restricted to the submembranous microfilament system should allow better understanding of the way these activities are controlled.

Fractionation of brain tissue (1) and the microfilament organization of intestinal epithelial brush borders (2) has led to the recent identification of two novel, but closely related, high molecular weight actin-binding proteins, which have been described as brain fodrin (1, 2) and intestinal TW-260/240 (2), respectively.* The two proteins share several biochemical properties such as calmodulin-binding and actin-crosslinking activities and are immunologically related (2). They differ in subunit composition, as shown by $NaDodSO_4$/polyacrylamide gel electrophoresis, which indicates that they are composed of dissimilar polypeptides, one that has nearly the same apparent molecular weight (≈240,000) in both proteins and the other that has a higher (260,000 in TW-260) or a lower (235,000 in fodrin) molecular weight. Rotary-shadowing electron microscopy showed a striking morphology (2). Both proteins are long flexible molecules of a distinct double-stranded morphology, raising the possibility of a structural relationship with spectrin (3), which accounts for the submembranous sheath of the erythrocyte (4, 5) and is known to be made up of two nonidentical high molecular weight subunits. Because antibodies against fodrin or TW-260/240 also indicate submembranous localization of the corresponding antigens in many different cell types (1, 2) and a recent study has raised the possibility of spectrin-related molecules in some nonerythroid cells (6), we have concentrated on an immunological and protein-chemical characterization of spectrin, fodrin, and TW-260/240 from chicken cells. Here we show that the three proteins have a similar molecular organization. They contain a highly related subunit (M_r 240,000) that is responsible for calcium-dependent calmodulin binding and a distinct subunit that has a M_r of 260,000, 235,000, or 220,000, depending on the molecular species.

MATERIALS AND METHODS

Purification of Proteins. Chicken TW-260/240 and chicken or rabbit fodrin were prepared from brush borders and brain, respectively, as described (2). Chicken erythrocytes and crude plasma membranes were obtained as described (7). Chicken spectrin was extracted at 37°C with 20 vol of 0.1 mM EDTA, and membranes were removed by centrifugation at 200,000 × g for 1 hr. The low-salt extract was used directly for electrophoresis or spectrin was further purified by chromatography on Sepharose 4B as described for chicken TW-260/240 (2).

Isolation of Polypeptides. The subunits of TW-260/240, fodrin, and spectrin were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis on 4% preparative gels (3 mm thick for TW-260/240 and spectrin; 1.5 mm thick for fodrin). After electrophoresis, separated subunits were visualized by precipitation with 0.3 M KCl at 0°C, observed under dark-field illumination, and carefully excised. The bands were electroeluted for 36 hr at 125 V into dialysis tubing, dialyzed against 0.1% $NaDodSO_4$, and lyophilized. Purity of the isolated peptides was determined by reelectrophoresis.

Antibody Preparation and Characterization. Antisera to the separated TW-260 and TW-240 proteins were elicited in rabbits as described (2). Antisera to electrophoretically purified (but unfractionated) fodrin were prepared as described for the TW proteins. Antiserum to chicken gizzard filamin was as described (2). To test for polypeptide crossreactivity of the various antisera and the isolated polypeptides, a modification of the immunoblotting technique was used. Briefly, 1.5-μl portions of solutions of native proteins or separated denatured polypeptides (≈100 μg/ml) were spotted 1 cm apart onto strips of nitrocel-

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Abbreviation: P_i/NaCl, phosphate-buffered saline.

* Throughout this report, we use the terms fodrin (a complex with subunit polypeptides of M_r 240,000 and 235,000 isolated from brain) and TW-260/240 (a complex with subunits of M_r 260,000 and 240,000 isolated from the terminal web of intestinal epithelial cells). We have avoided obvious names such as brain or intestinal spectrin or nonerythroid spectrin to maintain currently useful distinctions between these molecules.

lulose. Then the strips were air dried, soaked in phosphate-buffered saline (P_i/NaCl)/5% bovine serum albumin for 60 min, sealed in plastic bags containing 4 ml of 1:50 dilution of antiserum in P_i/NaCl/albumin, and incubated at 37°C overnight. Next the strips were washed four times for 5 min each with P_i/NaCl and soaked in P_i/NaCl/albumin containing 10^5 cpm of ^{125}I-labeled protein A per ml for 2 hr at 37°C. Unbound protein A was removed with P_i/NaCl/0.1% Triton X-100 using several washes over 1 to 2 hr. Strips were dried at 80°C and autoradiographed at −70°C for 1–12 hr.

Other Procedures. Low-angle rotary shadowing was carried out by the method of Tyler and Branton (8) as modified (2). Digestion of separated polypeptides with *Staphylococcus aureus* V8 protease was carried out by the procedure of Cleveland *et al.* (9). ^{125}I-Labeled calmodulin binding of proteins in polyacrylamide gels was carried out by the gel-overlay assay (2, 10). ^{125}I-Labeled protein A was prepared by the IODO-GEN procedure (11).

RESULTS

The purity of the three proteins was assessed both by gel electrophoresis (Fig. 1) and electron microscopy using rotary shadowing. Examples of the typical long flexible rod morphology of these molecules, as previously analyzed in detail for human spectrin (3) and chicken TW-260/240 and fodrin (2), are shown in Fig. 2. (For discussion of the similarities and differences, see ref. 2.)

The three chicken proteins are each made up of two dissimilar subunits. In addition to a "common" polypeptide of apparent M_r 240,000, they have additional polypeptides of either higher (260,000 for TW-260/240) or lower (235,000 for fodrin; 220,000 for spectrin) apparent M_r (2). The subunits of TW-260/240 and spectrin could be separated easily by preparative electrophoresis. This was more difficult to achieve in the case of the closely spaced doublet of fodrin; however, by using 4% gels 1.5 mm (rather than 3 mm) thick, good separation with no crosscontamination detectable on reelectrophoresis of the isolated subunits was achieved (Fig. 1).

Immunological Crossreactivity. All four antisera raised against TW-260/240, as well as the two antisera elicited against fodrin, react strongly with native TW protein, fodrin, and spectrin (Fig. 3). Antisera to either of the isolated TW peptides reacted to some extent with the other TW peptide; however, this may be due to crosscontamination of the original antigens used for injection or, alternatively, it may reflect common antigenic determinants on TW-260 and TW-240. The two antisera raised against isolated TW-240 react not only with the original antigen but also with the isolated M_r 240,000 subunit of either fodrin or spectrin but fail to react in our assay with the M_r 235,000 subunit of fodrin or the M_r 220,000 subunit of spectrin. Thus, the three M_r 240,000 subunits of spectrin, fodrin, and TW-260/240 share common antigenic sites. This point is further illustrated by two antisera raised against fodrin. Although reactive with both fodrin subunits, these antisera detect only the M_r 240,000 component of both TW-260/240 and spectrin. It is interesting that all of these sera show a strong species preference and react only weakly with rabbit fodrin or spectrin (Fig. 3). This may explain our previous observation (2) that such sera failed to recognize human spectrin.

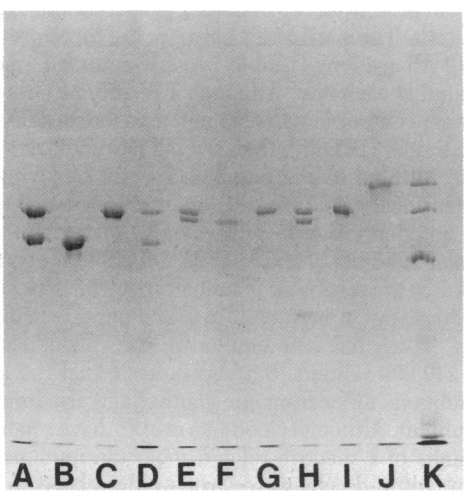

FIG. 1. NaDodSO$_4$/4% polyacrylamide gel electrophoresis of spectrin, fodrin, and TW-260/240. Lanes: K, brush border cytoskeletal protein; J, TW-260; I, TW-240; H, chicken fodrin; G, M_r 240,000 subunit of fodrin; F, M_r 235,000 subunit of fodrin; E, rabbit fodrin; D, low-salt extract of chicken erythrocyte membranes; C, M_r 240,000 subunit of chicken spectrin; B, M_r 220,000 subunit of spectrin; A, rabbit spectrin.

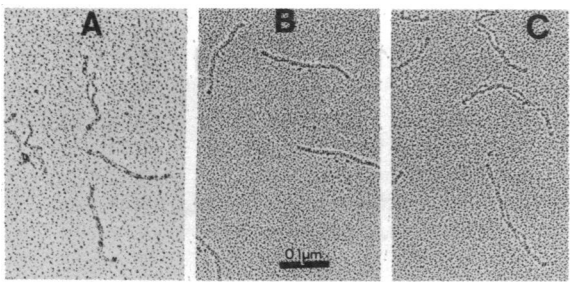

FIG. 2. Chicken spectrin (*A*), fodrin (*B*), and TW-260/240 (*C*) as visualized by low-angle rotary shadowing. (×35,600; bar = 0.1 μm.)

One-Dimensional Peptide Maps of Isolated Subunits. Since the immunological results suggested a high degree of relatedness between the three M_r 240,000 subunits, we carried out a protein-chemical study using limited proteolysis of all six isolated subunits. The results obtained with staphylococcal V8 protease are summarized in Fig. 4, and similar results were obtained with trypsin, chymotrypsin, and thermolysin (not

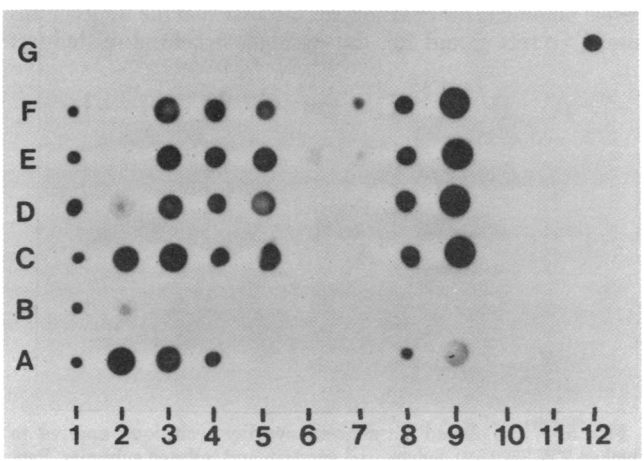

FIG. 3. Immunological reactivity of antisera to TW-260/240, fodrin, and filamin with native TW-260/240, fodrin, spectrin, and filamin or isolated subunits of these proteins. Rabbits were immunized with various antigens: A and B, TW-260; C and D, TW-240; E and F, fodrin; G, filamin. These were tested for reactivity against various peptides: 1, native chicken TW-260/240; 2, separated TW-260; 3, TW-240; 4, native chicken fodrin; 5, M_r 240,000 subunit of fodrin; 6, M_r 235,000 subunit of fodrin; 7, native rabbit fodrin; 8, native chicken spectrin; 9, M_r 240,000 subunit of chicken spectrin; 10, M_r 220,000 subunit of chicken spectrin; 11, rabbit spectrin; 12, chicken gizzard filamin.

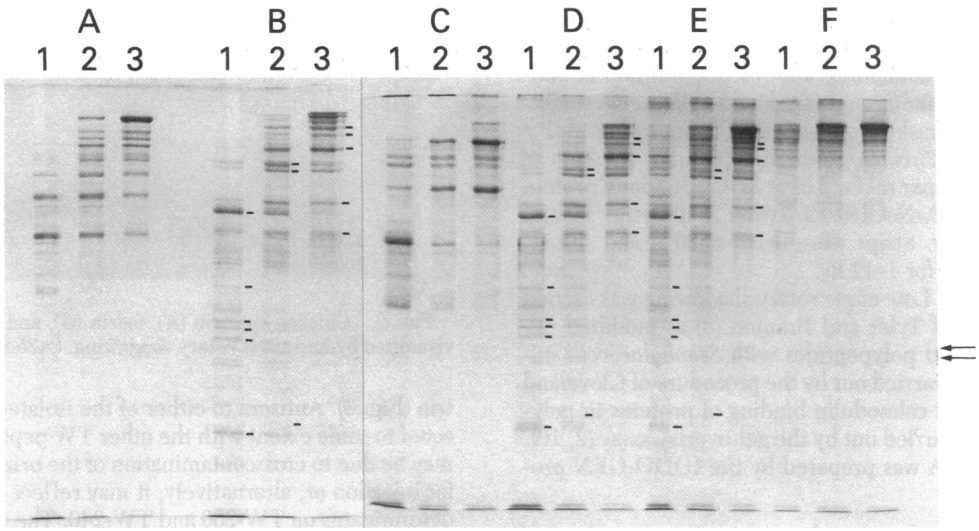

FIG. 4. Partial proteolysis of separated subunits of chicken spectrin, fodrin and TW-260/240 by staphylococcal V8 protease. (*A*) M_r 220,000 subunit of spectrin. (*B*) M_r 240,000 subunit of spectrin. (*C*) M_r 235,000 subunit of fodrin. (*D*) M_r 240,000 subunit of fodrin. (*E*) TW-240. (*F*) TW-260. Subunits were treated with V8 protease for 5 min (lanes 3), 12.5 min (lanes 2), or 30 min (lanes 1) at room temperature, boiled in $NaDodSO_4$ sample buffer, and subjected to $NaDodSO_4$/polyacrylamide (10% acrylamide) gel electrophoresis. For *A* and *B*, a separate gel was used. Staining was with Coomassie blue. –, Similar-sized peptides derived from the three M_r 240,000 subunits; ←, V8 protease alone.

shown). The M_r 240,000 subunits of all three proteins showed strikingly similar cleavage patterns (indicated by symbols in Fig. 4) not recognizable in the three other subunits. These seemed to differ not only from each other but also very strictly from the pattern of the M_r 240,000 subunit.

The M_r 240,000 Polypeptides Bind Calmodulin. The immunological and peptide mapping data together suggest a high degree of structural relatedness of the three M_r 240,000 subunits. Since we had previously identified TW-240 and total fodrin as calcium-dependent calmodulin-binding proteins (2), we used the ^{125}I-labeled calmodulin gel-overlay assay on all isolated subunits (Fig. 5). As expected, all three M_r 240,000 subunits are recognized as calcium-dependent calmodulin-binding proteins whereas the three variant subunits do not show this binding. Although we cannot rule out the possibility that they may reveal binding in other assays (for discussion of the limits of this assay, see refs. 2 and 10), the common structural relatedness

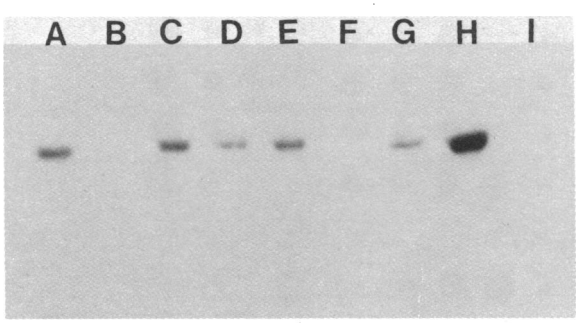

FIG. 5. ^{125}I-Labeled calmodulin overlay technique applied to chicken TW-260/240, fodrin, and spectrin and isolated subunits. Peptides were subjected to electrophoresis in a 5% gel. After fixation of the protein, the gel was soaked in ^{125}I-labeled calmodulin in a calcium-containing buffer. Unbound calmodulin was removed by washing, and the calmodulin-binding proteins were visualized by autoradiography. Lanes: A, unfractionated TW-260/240; B, TW-260; C, TW-240; D, unfractionated fodrin; E, M_r 240,000 subunit of fodrin; F, M_r 235,000 subunit of fodrin; G, unfractionated spectrin; H, M_r 240,000 subunit of spectrin; I, M_r 220,000 subunit of spectrin. Only the relevant portion of the autoradiogram is shown. (For details of procedure, see ref. 10.) Note the strong decoration of the M_r 240,000 subunits in all three molecules.

of the M_r 240,000 subunits is clearly reflected by this property whose functional importance is not yet known. In addition, this result seems to map the calmodulin binding site proposed to exist on spectrin (12). We do not yet know why we have failed to detect this binding in preliminary experiments on human spectrin (2), but our gel-overlay result on chicken spectrin is in agreement with the previous report that spectrin may be a calmodulin-binding protein (12).

DISCUSSION

The present results lead us to the conclusion that spectrin-like molecules are not restricted to erythroid cells but are a component of many other cell types. Concentrating on one animal species, we have found morphological, structural, immunological, and functional relationships among chicken erythrocyte spectrin, brain fodrin, and the microfilament-associated protein TW-260/240 present in brush border preparations of intestinal epithelial cells. The similarities between the three proteins are striking. (*i*) All are long flexible "double-stranded" molecules tightly bound at each end. Although TW-260/240 has a significantly longer contour length (263 nm) than fodrin (200 nm) and spectrin tetramer (195 nm), their overall morphologies are similar (2). (*ii*) All bind to and crosslink F-actin (2) giving rise to three-dimensional gels. (*iii*) All three proteins are constructed from two nonidentical high molecular weight subunits. They share similar subunits of apparent M_r 240,000 that correspond to subunit 1 or the α subunit of human spectrin. This similarity is clearly borne out in immunological and peptide mapping experiments, which not only emphasize the relatedness of the three M_r 240,000 subunits but also indicate that the three associated subunits differ from one another and also from the M_r 240,000 subunit. Although Goodman *et al.* (6) have reported that both subunits of a spectrin-related molecule immunoprecipitated from cultured cells were structurally related to the two spectrin polypeptides, our data suggest that the β chains of the three related molecules are distinct. Currently, the reason for this discrepancy is not known. (4) The calcium-dependent calmodulin binding of spectrin (12), fodrin (2), and TW-260/240 (2) has now been mapped by a gel-overlay assay and shown to be mediated by the M_r 240,000 subunit in all cases. The com-

bined evidence indicates a model in which three cell-type-specific spectrin-like proteins share a related—and perhaps identical—common subunit but differ in the properties of the second and variant subunit to form morphologically distinct high molecular weight actin-binding proteins. The present results do not, however, rule out minor differences or modifications in the M_r 240,000 unit among different tissues that may have functional importance.

It should be noted that, although three distinct variant subunits have been found in the three cell types so far examined—i.e., M_r 220,000 (spectrin) in erythrocytes, M_r 235,000 (fodrin) in brain tissue, and TW-260 in intestinal brush borders—additional subunits are not detected, suggesting mutually exclusive roles. Thus, it will be important to determine (*i*) the tissue distribution of the variant polypeptides, (*ii*) whether additional variant peptides exist in other tissues, and (*iii*) the functions of these molecules in different tissues that may require a tissue-specific variant polypeptide. It has been shown, for instance, that it is the β chain of spectrin (M_r 220,000) that contains the ankyrin-binding site linking it to the inner aspect of the erythrocyte membrane (13–15). Thus, it will be interesting to determine whether the variant subunits of fodrin or TW-260/240 retain this site.

Why was spectrin not detected in other cells previously? Earlier immunological studies from this laboratory (16) as well as others (17) did not find spectrin in nonerythroid cells by antibodies against human spectrin. It could be that these antibodies had a preference for the erythrocyte-specific M_r 220,000 subunit. Alternatively, spectrin-like molecules of nonerythroid cells may be bound differently to the membrane than spectrin and only poorly extracted under previous conditions. In addition, we have found that certain antibodies against TW-260/240 and fodrin showed strong species specificity. We do, however, note that Goodman *et al.* (6) have just reported that an antibody against human spectrin precipitated related antigens from several cell lines solubilized with octylglucoside. The molecular weights reported by them indicate fodrin-like polypeptides.

Spectrin is one of the best-studied cytoskeletal proteins of the erythrocyte (for review, see ref. 5). It is bound to the inner side of the plasma membrane by a specific anchorage protein (ankyrin), which in turn is bound to the transmembrane protein, band 3. Much less is known about nonerythroid spectrin-like molecules. Immunofluorescence microscopical studies using antibodies to fodrin and TW-260/240 indicate a submembranous localization distinctly different from the stress fiber display (1, 2). Fodrin is known from axonal transport studies to migrate with several components, one of which also accounts for actin (18). In addition, immunofluorescence microscopy demonstrated the submembranous collection of fodrin antigens during lymphocyte capping (19). Of further interest is the recent report that antibodies against ankyrin, the spectrin anchorage protein in erythrocytes, detect a high molecular weight protein in various nonerythroid cells (20). Although these antigens are reported to interact with microtubules, a more definite immunoelectron microscopical study on both spectrin-like and ankyrin-like molecules of nonerythroid cells may shed light on their possible interaction.

Many questions remain concerning nonerythroid spectrin-like molecules. However, since various approaches have now established their existence, it is hoped that some aspects of their function may be developed by drawing from knowledge of the erythrocyte cytoskeleton. Of particular interest is the spectrin–actin interaction (5, 21). The calmodulin binding of the various spectrin-like molecules suggests a possible calcium sensitivity of submembranous actin organizations.

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