Drosophilia Spectrin. I. Characterization of the Purified Protein

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Abstract. We purified a protein from *Drosophila* S3 tissue culture cells that has many of the diagnostic features of spectrin from vertebrate organisms: (*a*) The protein consists of two equimolar subunits (M_r = 234 and 226 kD) that can be reversibly cross-linked into a complex composed of equal amounts of the two subunits. (*b*) Electron microscopy of the native molecule reveals two intertwined, elongated strands with a contour length of 180 nm. (*c*) Antibodies directed against vertebrate spectrin react with the *Drosophila* protein and, similarly, antibodies to the *Drosophila* protein react with vertebrate spectrins. One monoclonal antibody has been found to react with both of the *Drosophila* subunits and with both subunits of vertebrate brain spectrin. (*d*) The *Drosophila* protein exhibits both actin-binding and calcium-dependent calmodulin-binding activities. Based on the above criteria, this protein appears to be a bona fide member of the spectrin family of proteins.

SPECTRIN plays a critical role in the structure and function of the mammalian erythrocyte (Bennett, 1985; Marchesi, 1985; Elgsaeter et al., 1986) but there is little information about its function in other cell types. By analogy to the red blood cell, it has been suggested that nonerythroid spectrin modulates membrane domain composition, regulates the distribution of cell surface components, or links other cytoskeletal elements to the plasma membrane (Baines, 1984; Bennett, 1985; Nelson and Veshnock, 1986). Recent work suggests that in chromaffin cells spectrin is involved in secretion (Perrin et al., 1987), but another set of experiments directed at assessing nonerythroid spectrin's function in several fibroblastic and epithelial cell types did not demonstrate an obvious role for this widely distributed molecule (Mangeat and Burridge, 1984).

To elucidate the role of nonerythroid spectrins, we have initiated investigations of *Drosophila* spectrin. Studies of human and mouse mutants that express altered spectrin phenotypes provide some of the most persuasive evidence regarding the role of erythroid spectrin (Shohet, 1979; Palek and Lux, 1983; Bodine et al., 1984). Similarly, the wide array of molecular and genetic tools that can be applied to *Drosophila* should facilitate investigations of nonerythroid spectrin. Here we report the identification of spectrin in *Drosophila* and describe several properties that *Drosophila* spectrin shares with vertebrate spectrins. In the accompanying paper we describe the isolation and properties of *Drosophila* spectrin cDNA clones (Byers et al., 1987).

A preliminary report of some of these findings was presented at the 1986 meeting of the American Society for Cell Biology (1986. *J. Cell Biol.* 103[5, Pt. 2]:540*a*.[Abstr.]).

Materials and Methods

Preparation and Homogenization of Cells

Drosophila cells (line S3; Schneider and Blumenthal, 1978) were cultured, harvested, and homogenized as described (Kiehart and Feghali, 1986), except that the cells were lysed in a buffer (~30 g wet wt of cells in a total volume of 100 ml) that consisted of 0.34 M sucrose, 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 0.5 mM ATP, 0.5 mM dithiothreitol, 8 mM NaN_3, with 5 mM phenylmethylsulfonyl fluoride, 22 μM pepstatin A, and 0.25 mg/ml soybean trypsin inhibitor as protease inhibitors. The cell homogenate was separated into a supernatant and pellet fraction by centrifugation at 50,000 rpm (g_{max} = 250,000) for 90 min in a 60 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA).

Spectrin Purification

The *Drosophila* cell supernatant fraction was applied to a 200-ml column of DEAE Sephacel (Sigma Chemical Co., St. Louis, MO) equilibrated in lysis buffer without protease inhibitors. The column was washed with 2 vol of lysis buffer followed by 200 ml of 0.1 M KCl in elution buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.5 mM dithiothreitol, and 8 mM NaN_3). Spectrin was eluted with an 800-ml linear gradient of 0.1–0.4 M KCl in elution buffer. Spectrin-containing fractions were identified by SDS gel electrophoresis (see below). Solid ammonium sulfate was added to these pooled fractions to 50% saturation and, after 1–2 h at −10°C, precipitated proteins were collected by centrifugation at 8,000 g_{max} for 15 min in a rotor (Sorvall GSA; DuPont Instruments, Sorvall Operations, Newtown, CT). The precipitate was solubilized in no more than 10 ml of Sepharose column buffer (1 M KCl, 10% glycerol, 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 0.5 mM dithiothreitol, 8 mM NaN_3) and applied to a column (2.6 × 94 cm) of Sepharose CL-4B (Sigma Chemical Co.) equilibrated in the same buffer. Fractions (8 ml) were collected at 30 ml/h and the fractions containing spectrin were identified by SDS gel electrophoresis.

The spectrin-containing fractions (typically a total of 60–80 ml) were pooled and dialyzed into two changes of 1 liter each of phosphocellulose column buffer (10 mM K phosphate, pH 7.0, 0.5 mM dithiothreitol, 0.1 mM EGTA, 1 mM NaN_3). The dialyzed material was applied directly to a 20–30-ml column (2.5 cm diam) of P-11 phosphocellulose (Whatman Ltd., Clifton, NJ) equilibrated in phosphocellulose column buffer. Bound proteins were eluted at 20 ml/h with 160 ml of the same buffer containing a

linear gradient of 0.01–0.4 M K phosphate. Fractions containing purified spectrin were identified by SDS gel electrophoresis and the potassium phosphate concentration in these fractions was estimated from conductivity measurements.

A membrane fraction enriched in vertebrate brain spectrin was prepared from a calf brain homogenate as described by Davis and Bennet (1983).

SDS-PAGE

Except where otherwise noted, spectrin was resolved on SDS polyacrylamide gels containing 7% acrylamide monomer and 0.08% bisacrylamide in the Laemmli buffer system (Laemmli, 1971) with a 3 or 4% acrylamide/0.1% bis-acrylamide stacking gel. We refer to these gels as our standard system: they produced a separation of *Drosophila* spectrin subunits that was more distinct and consistent than several other gel combinations tested.

Protein fractions for electrophoresis were routinely concentrated by precipitation with 10% TCA and 0.1% Triton X-100 as carrier. The precipitates were solubilized in Laemmli sample buffer and neutralized by vapor phase addition of $NH_4(OH)$ until the indicator dye turned blue. Gels were stained for protein with either Coomassie Blue (Fairbanks et al., 1971) or with silver nitrate (Merril et al., 1981). Protein standards (Sigma Chemical Co.) included myosin (205 kD), beta-galactosidase (116 kD), phosphorylase a (97.4 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD). Purified spectrin (240 and 220 kD) from human erythrocytes was provided by Dr. Athar Husain (from our laboratory). Protein concentrations were determined with the bicinchoninic acid reagent (Smith et al., 1985) using BSA as a standard. Densitometric scans of Coomassie Blue–stained gels were done with a GS 300 apparatus (Hoefer Scientific Instruments, San Francisco, CA).

Protein Blots

For antibody staining and calmodulin-binding assays, proteins separated on SDS polyacrylamide gels were electrophoretically transferred to nitrocellulose sheets (0.2 μm; Schleicher & Schuell, Keene, NH) by a modification of standard procedures (Burnette, 1981). Gels were preincubated for 15 min in 20% ethanol, 20 mM Tris, 150 mM glycine, 0.01% SDS and proteins were transferred in the same buffer for 3–4 h at 8 V/cm. To monitor the efficiency of protein transfer before further processing, the nitrocellulose blots were reversibly stained with 0.2% Ponceau red S. The complete pattern of transferred proteins was visualized by staining the blots with 0.1% India ink (Hancock and Tsang, 1983).

Production of Antibodies

Rabbit antibody BR-1, directed against chicken erythroid alpha-spectrin (Repasky et al., 1982), was generously provided by Dr. Elizabeth Repasky. Rabbit antibody 675 (anti-*Drosophila* spectrin) was produced against an enriched *Drosophila* spectrin fraction that was prepared from S3 cells by an actin-affinity/gel filtration procedure described elsewhere (Kiehart and Feghali, 1986). The rabbit was immunized by popliteal injection (Sigel et al., 1983) of this spectrin fraction in Freund's complete adjuvant followed 40 d later with a subcutaneous injection of antigen in incomplete adjuvant. Immune serum was collected 1 wk after the boost. The rabbit continued to produce high titer antibody for several months without further boosts. The IgG fraction of immune serum was purified by ammonium sulfate precipitation and DEAE chromatography and affinity purified by adsorbtion to alpha- and beta-*Drosophila* spectrin that had been transferred to nitrocellulose from a SDS gel (Olmsted, 1981). Production and characterization of a third anti-*Drosophila* spectrin serum (905) is described elsewhere (Byers et al., 1987).

BALB/c female mice were immunized with a purified *Drosophila* alpha-spectrin fusion protein produced in *Escherichia coli* (from cDNA clone 10, Byers et al., 1987). Immune spleen cells were mixed with the FoxNY myeloma line (Taggart and Samloff, 1983) and hybridomas were obtained by standard polyethylene glycol–mediated fusion techniques (Cuello et al., 1983) and selection with adenine-aminopterin-thymidine (Taggart and Samloff, 1983). Antigen-specific hybrids were identified by ELISA assay (Engvall, 1980) on Immulon I assay plates (Dynatech Laboratories, Inc., Alexandria, VA) coated with 25 ng/well of fusion protein. The secondary antibody is described below. Positive cultures were cloned at least three times in soft agar before use. Antibody subisotypes were determined by ELISA assay of culture supernatants from clonal lines using a screening kit from HyClone Laboratories (Logan, UT).

Antibody Reactions

Nitrocellulose sheets with electrophoretically blotted proteins were cut into individual strips for separate reactions and preincubated for 30 min at room temperature in Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20 (Sigma Chemical Co.), 8 mM NaN_3 (Tween buffer) with 5% newborn calf serum (HyClone Laboratories). Subsequent incubations with antibody were carried out in Tween buffer with 5% serum whereas all washes were done in Tween buffer alone. Primary antibodies were reacted for 2–4 h at room temperature, at the indicated dilutions, or overnight at 4°C. Strips were rinsed in four changes of buffer and subsequently reacted with a 1:1,000 dilution of affinity-purified alkaline phosphatase–conjugated secondary antibody (Zymed Laboratories, South San Francisco, CA) for 1–2 h at room temperature. Bound antibodies were visualized by incubating the nitrocellulose strips in a substrate solution prepared from a stock of 0.6 mg/ml nitro blue tetrazolium (Sigma Chemical Co.) in 1.5 M Tris-HCl, pH 8.8 and 60 mg/ml bromochloroindolyl phosphate (Sigma Chemical Co.) in DMSO. Immediately before use, the nitro blue tetrazolium stock was diluted 1:10 in distilled water and 0.01 vol of bromochloroindolyl phosphate stock was added. The stock solutions were stable for many weeks at −20°C. After reaction with substrate, the nitrocellulose strips were rinsed in several changes of distilled water and air dried. Stained strips were stored in commercially available "magnetic" photo album pages, which serve as a convenient, reversible mounting support for storage and reproduction of large numbers of stained strips.

Electron Microscopy

Purified spectrin molecules were viewed by low angle, rotary replication with platinum and carbon as described by Tyler and Branton (1980). The purified proteins were desalted by passage over a small G-25 spin column (Tuszynski et al., 1980) and prepared for shadowing in 0.25 M ammonium formate, pH 7.5, with 70% glycerol. Replicas were viewed on a Philips EM 301 microscope at 80 kV.

Cross-linking Experiments

Purified spectrin (10 μg/ml) was reacted with 50 μg/ml dimethyl dithiobispropionimidate (Pierce Chemical Co., Rockford, IL) in 0.1 M triethanolamine, pH 7.5 for 30 min at room temperature. The propionimidate reagent was quenched by reaction with glycine-NaOH, pH 7.5 (added to a final concentration of 0.1 M) for 15 min. Spectrin was precipitated in 10% TCA with 0.1% Triton X-100 as carrier. Cross-linked proteins were resolubilized in SDS sample buffer without reducing agent and subjected to electrophoresis on 3–10% polyacrylamide gradient gels. The major cross-linked protein band was located by copper staining (Lee et al., 1987) of a parallel lane of cross-linked sample. The band was excised with a razor blade, incubated for 15 min in SDS gel sample buffer containing 5% 2-mercaptoethanol, and placed in the sample well of a second gradient gel for electrophoresis of the reduced products.

Calmodulin Binding

Calmodulin was purified from the heat-stable fraction of calf brain homogenate by sequential chromatography on phenyl-Sepharose (Sigma Chemical Co.) and DEAE Sephacel (Dedman and Kaetzel, 1983). Purified calmodulin (2.5 mg/ml) was reacted with a 10-fold M excess of biotinyl-epsilon-aminocaproic acid *N*-hydroxysuccinimide ester (Calbiochem Biochemicals, San Diego, CA) in 20 mM Hepes, pH 7.4, 10 mM $CaCl_2$ for 2 h at 4°C (Billingsley et al., 1987). Unreacted succinimide ester was quenched and removed by dialysis against 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM $CaCl_2$, 8 mM NaN_3. Biotinylated calmodulin was stored at 0°C in 25 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1 mM $CaCl_2$, 8 mM NaN_3.

Calmodulin-binding proteins were detected on nitrocellulose blots (Flanagan and Yost, 1984) of purified *Drosophila* spectrin or partially purified calf brain spectrin. Strips cut from the nitrocellulose blots were blocked with Tween buffer, reacted with biotinylated calmodulin (diluted 1:100 in the same buffer) for 2–3 h at room temperature, then washed in six changes of Tween buffer. Separate reactions were carried out in the presence of 1 mM $CaCl_2$ or 1 mM EGTA (included during all subsequent steps). Biotinylated calmodulin was detected with alkaline phosphatase–conjugated streptavidin (Bethesda Research Laboratories, Gaithersburg, MD), diluted 1:1,000 in the above buffer, and reacted for 30 min at room temperature. Strips were washed and incubated with alkaline phosphatase substrate solution as described for antibody reactions.

Actin Cosedimentation

Cosedimentation studies were carried out in 10 mM imidazole, pH 7.0, 75 mM KCl, 0.2 mM dithiothreitol, 0.2 mM EGTA. Gel-filtered rabbit skeletal muscle G-actin (250 μg/ml; Spudich and Watt, 1971) was mixed with either *Drosophila* spectrin (70 μg/ml) or human erythroid spectrin (200 μg/ml) in the presence or absence of 25 μg/ml of human erythroid band 4.1 (human spectrin and band 4.1 were generously provided by Dr. Athar Husain). Before mixing, all protein solutions were cleared by centrifugation for 30 min at 28 psi in an Airfuge (Beckman Instruments, Inc.). Polymerization of actin was initiated by the addition of $MgCl_2$ to a final concentration of 2 mM. After 45 min at room temperature, actin–spectrin complexes were pelleted by centrifugation for 30 min at 28 psi. Pellets and supernatants were solubilized in equal amounts of SDS gel sample buffer and loaded in equal proportions onto acrylamide gels consisting of 8% acrylamide monomer and 0.08% bisacrylamide. The percentage of spectrin that cosedimented with F-actin was quantified from densitometric tracings of stained gels.

Results

Purification of Drosophila Spectrin

We initially identified a spectrinlike protein in homogenates of *Drosophila* tissue culture cells on the basis of molecular mass after SDS-PAGE, cosedimentation, or association with actin filaments, immunological cross-reactivity with anti-vertebrate spectrin antibodies, and electron microscopic appearance after rotary replication. Further purification (see below) yielded a highly enriched preparation of two polypeptide chains (M_r 234 and 226 kD in our standard gel system; Fig. 1) that closely resembled the two subunits of vertebrate spectrins and which we will refer to as alpha- and beta-*Drosophila* spectrin.

In contrast to both erythroid and nonerythroid vertebrate spectrins, which remain associated with the plasma membrane after cell lysis (Davis and Bennett, 1983; Glenney et al., 1982*a*), a large proportion of *Drosophila* spectrin remained in the high speed supernatant fraction of cell lysates (Fig. 1, *A* and *D*, compare lanes *2* and *3*). Similar results were obtained when cells were lysed in a buffer containing 0.15 M NaCl (not shown). Since the high speed supernatant fraction (Fig. 1, *A* and *D*, lane *2*) represented an enrichment of *Drosophila* spectrin relative to the total proteins of the cell lysate, it was chosen as the starting material for further purification.

The high speed supernatant was first fractionated over a column of DEAE Sephacel and proteins were eluted with a

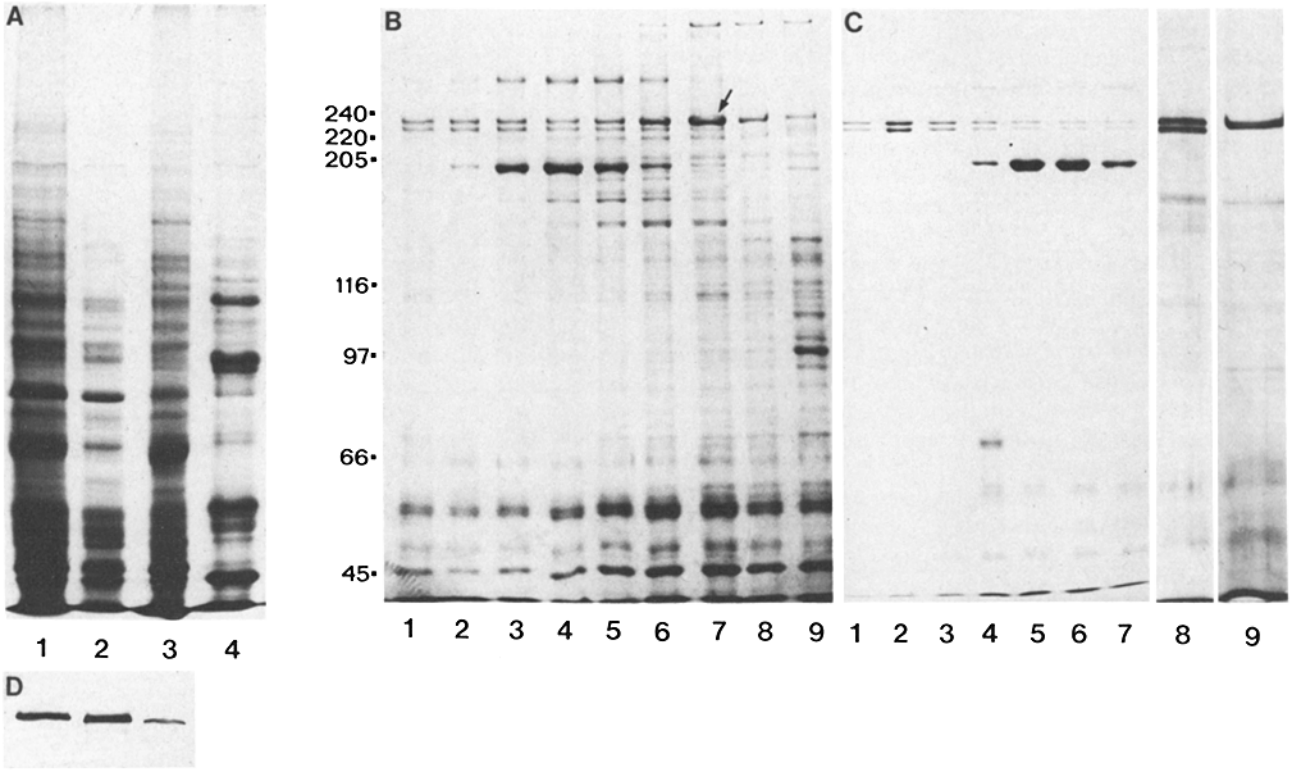

Figure 1. SDS-PAGE of various fractions obtained during purification of *Drosophila* spectrin. (See Materials and Methods for details.) (*A*) Starting materials. Lane *1*, *Drosophila* S3 cell homogenate; lanes *2* and *3*, supernatant and pellet fractions, respectively, of the homogenate; lane *4*, pooled spectrin-containing fractions from the DEAE-Sephacel column. (*B*) Fractions from the Sepharose CL-4B column. Each lane contains a sample from every second 8-ml fraction. Lanes *1–5*, early fractions containing two putative spectrin subunits; lanes *6–8*, later fractions that contained only one spectrin subunit (*arrow*), referred to in the text as excess alpha-spectrin. (*C*) Fractions from the phosphocellulose column. Lanes *1–7*, every third fraction (2.2 ml) eluted by a linear gradient of potassium phosphate. The putative *Drosophila* spectrin (lanes *1–3*) eluted with 0.11 M potassium phosphate; myosin and other low molecular mass contaminants eluted in later fractions (lanes *4–7*). Lane *8*, 1.5 μg purified *Drosophila* spectrin, pooled from lanes *2–4*, was silver-stained to demonstrate the purity of the preparation. Lane *9*, a sample of the phosphocellulose flow through fraction containing excess alpha-spectrin. The migration of protein standards is shown to the left of *B* in kilodaltons. (*D*) A nitrocellulose blot probed with anti-spectrin antibody (675, see Fig. 3). Lane *1*, S3 cell homogenate; lanes *2* and *3*, supernatant and pellet fractions, respectively, of the homogenate. Except for *D* and lanes *8* and *9* in *C*, all lanes were stained with Coomassie Brilliant Blue.

gradient of KCl. Spectrin eluted at 0.27 M KCl, slightly after the peak of cytoplasmic myosin (Kiehart and Feghali, 1986). Spectrin-containing DEAE fractions were pooled (Fig. 1 *A*, lane *4*), concentrated by ammonium sulfate precipitation, and resolubilized for gel filtration. Two forms of *Drosophila* spectrin were identified after size fractionation on Sepharose CL-4B (Fig. 1 *B*). The first form (Fig. 1 *B*, lanes *1–5*), which eluted just after the void volume, contained both the 234- and 226-kD spectrin subunits. A second form, shown in Fig. 1 *B*, lanes *6–8*, appeared to consist of only the 234-kD subunit. As described below, its structure, antigenic properties, and calmodulin-binding activity indicate that this second form represents an excess of the alpha-spectrin subunit in the supernatant fraction of the cell lysate. The Sepharose fractions containing the first form (Fig. 1 *B*, lanes *1–5*) were pooled and further purified by phosphocellulose chromatography.

A slight excess of the alpha-spectrin subunit was present in the Sepharose fractions that were pooled for further purification. The excess alpha subunit did not bind to phosphocellulose, but was enriched in the flow through fraction (Fig. 1 *C*, lane *9*). Equivalent amounts of the alpha- and beta-subunits coeluted from the column with 0.11 M potassium phosphate (Fig. 1 *C*, lanes *1–3*). Myosin and other lower molecular mass contaminants were eluted at 0.16 M potassium phosphate (Fig. 1 *C*, lanes *4–7*). Densitometric scans of the Coomassie Blue–stained SDS gels (Fig. 1 *C*, lane *2*) indicated that the 234- and 226-kD polypeptides accounted for at least 82% of the stainable material in the *Drosophila* spectrin peak. An additional 10% of the stainable material was present at the dye front and 6% was accounted for in minor bands migrating between spectrin and the dye front.

Phosphocellulose-purified *Drosophila* spectrin, similar to that shown in Fig. 1 *C*, lanes *2–4*, was used in all of the experiments that follow. Typical yields from 30 g of cells were in the range of 50–200 μg protein. Three minor contaminants found to some extent in all of our preparations were a >300-kD component, most of which elutes from phosphocellulose after spectrin (Fig. 1 *C*), a lower molecular mass component (~170 kD), and a minor band that comigrated with actin.

Electron Microscopy

Purified *Drosophila* spectrin appeared in the electron microscope as a highly asymmetric molecule composed of two closely apposed or intertwined strands (Fig. 2, *A* and *B*). The appearance was similar to that of vertebrate spectrins in which the subunits associate laterally to form heterodimers and the heterodimers in turn associate head-to-head to form the native spectrin tetramer (Shotton et al., 1979; Glenney et al., 1982*a*; Bennett et al., 1982). The average contour length of the molecule was 179.9 ± 6.1 nm (n = 78). Visible separation of the two strands (Fig. 2 *B*) was relatively infrequent, as was the occurrence of half-length molecules that may correspond to *Drosophila* spectrin dimers (not shown). An enriched fraction of the excess alpha-spectrin fraction was also examined by electron microscopy (Fig. 2 *C*). These molecules were 91.2 ± 21.8 nm long (n = 65) and did not appear to be double stranded.

Immunological Cross-Reactivity

Drosophila spectrin and vertebrate spectrins both reacted with several polyclonal and monoclonal antibodies (Fig. 3; Table I). Two sources of polyclonal antibody were used here. The first was a rabbit anti-spectrin antiserum, produced against avian alpha-spectrin and shown to react with alpha-spectrins from a variety of vertebrate sources (Repasky et al., 1982), and the second was an affinity-purified anti-*Dro-*

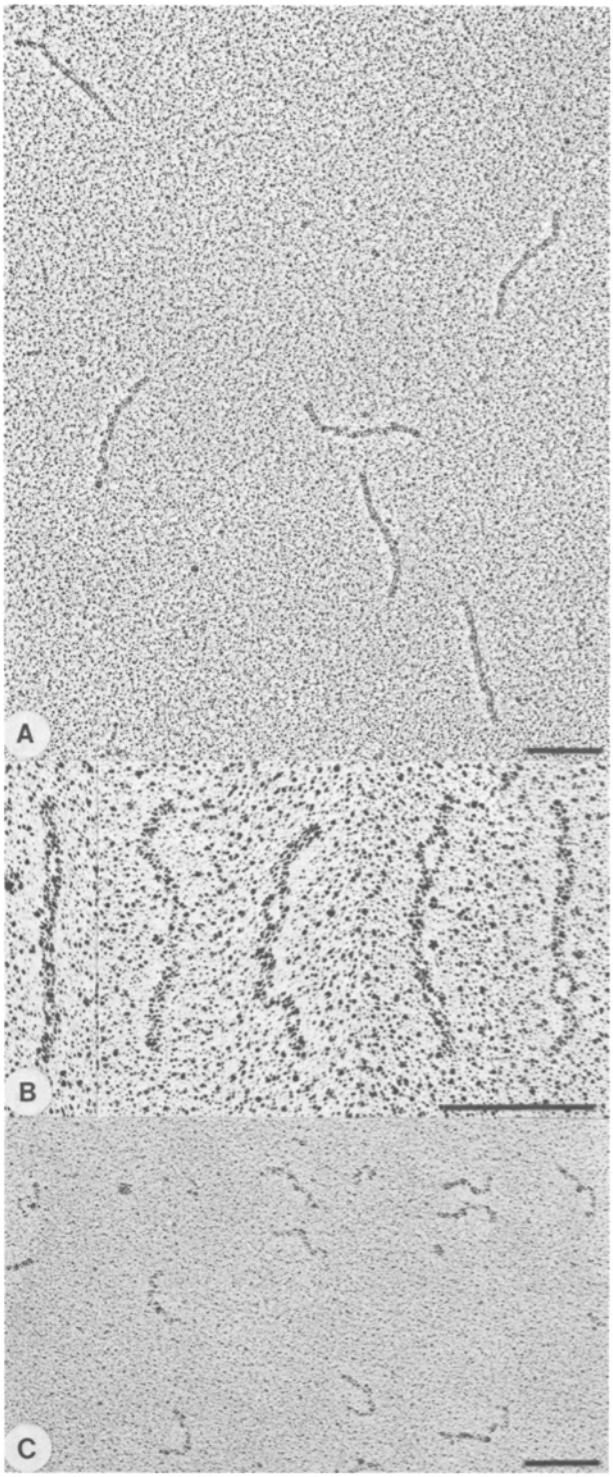

Figure 2. Electron micrographs of rotary replicated *Drosophila* spectrin. (*A* and *B*) Phosphocellulose-purified spectrin (50 μg/ml in ammonium formate/glycerol, shown in Fig. 1 *C*, lane *8*). (*C*) An enriched fraction of excess alpha spectrin (prepared from a fraction similar to that shown in Fig. 1 *B*, lane *7*). Bars, 100 nm.

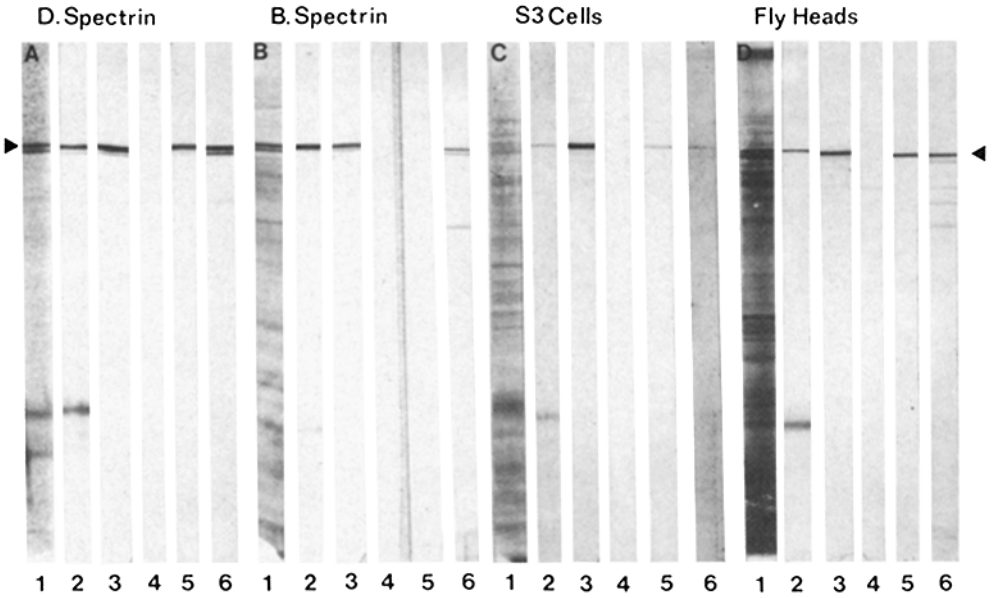

Figure 3. Immunological cross-reactivity of *Drosophila* and vertebrate spectrin. Nitrocellulose blots of (*A*) purified *Drosophila* spectrin, (*B*) a membrane fraction enriched for calf brain spectrin, (*C*) a *Drosophila* S3 cell lysate, and (*D*) *Drosophila* head proteins; lanes *1*, stained with India ink for total protein; lanes *2*, probed with anti-avian erythroid spectrin antiserum (1:1,000); lanes *3*, anti-*Drosophila* spectrin IgG (affinity-purified antibody 675); lanes *4*, preimmune 675 serum (1:2,000); lanes *5*, mAb M10-2 (undiluted culture supernatant); lanes *6*, mAb M10-1 (undiluted supernatant). Antibody reactions were visualized by reaction with an alkaline phosphatase-conjugated anti-mouse or anti-rabbit secondary antibody followed by incubation in substrate solution. Arrowheads mark the position of alpha-spectrin.

sophila spectrin antibody (675; see Materials and Methods). Both reacted extensively with the alpha-subunit of brain spectrin (240 kD) and with the 234-kD subunit of purified *Drosophila* spectrin. Anti-*Drosophila* spectrin also showed a slight reactivity toward vertebrate brain beta-spectrin as well as the 226-kD *Drosophila* subunit, but neither antibody detected the lesser amounts of *Drosophila* beta-spectrin either in blots of the S3 cell lysate or in blots of the total homogenized *Drosophila* head polypeptides. For comparison, the reactivity of a third polyclonal antiserum directed against *Drosophila* spectrin is also indicated in Table I.

Monoclonal antibodies produced against a *Drosophila* spectrin fusion protein expressed in bacteria (see Materials and Methods) were also used to compare the antigenic properties of *Drosophila* and vertebrate spectrin (Fig. 3; Table I). Of the monoclonal antibodies that reacted with the 234-kD subunit of *Drosophila* spectrin, most did not react with bovine brain spectrin (Fig. 3, lanes *5*: antibody M10-2, an IgG1) or human erythrocyte spectrin (not shown). One monoclonal antibody (M10-1, an IgM) recognized an epitope present on both subunits of *Drosophila* spectrin, brain spectrin (Fig. 3, lanes *6*), and human erythrocyte spectrin (not shown). Three

Table I. Summary of Antibody Cross-Reactions with Drosophila and Vertebrate Spectrins

Composition of blot	Reactive antigen	Antibody‡ BR-1	675	Pre675	M10-2§	M10-1	905‖	Pre905
Purified *Drosophila* spectrin	Alpha-spectrin	++*	++	−	++	++	++	−
	Beta-spectrin	+	+	−	−	+	+	−
Purified excess alpha-spectrin	Alpha-spectrin	++	++	−	++	++	++	−
Bovine brain membranes	Alpha-spectrin	++	++	−	−	++	++	−
	Beta-spectrin	−	+	−	−	+	+	−
S3 cell homogenate	Alpha-spectrin	++	++	−	++	++	++	−
	Beta-spectrin	−	−	−	−	+	−	−
Fly head homogenate	Alpha-spectrin	++	++	−	++	++	++	−
	Beta-spectrin	−	−	−	−	+	−	−

* + and −, a reaction or no reaction of spectrin with antibody; ++, a preferential reaction with one subunit.
‡ *BR-1*, anti-avian erythrocyte alpha spectrin; other antibodies were produced against *Drosophila* spectrin.
§ Identical reactions were detected with M10-3, M10-4, M10-5, and M10-6.
‖ See Byers et al. (1987) for details of antibody production and characterization.

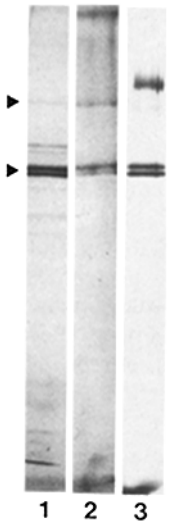

Figure 4. Chemical cross-linking of *Drosophila* spectrin subunits. Lane *1*, purified *Drosophila* spectrin (10 μg/ml in 0.1 M triethanolamine) before cross-linking; lane *2*, a parallel sample after reaction with 50 μg/ml dimethyl dithiobispropionimidate; lane *3*, the major high molecular mass cross-linked product from lane *2* (*upper arrowhead*) after reelectrophoresis under reducing conditions. Lanes *1* and *2* were stained with Coomassie Blue and lane *3* was silver stained.

Figure 5. Drosophila spectrin–calmodulin interaction. Nitrocellulose blots of purified *Drosophila* spectrin (*left*) and a membrane fraction enriched for calf brain spectrin (*right*) were reacted with 23 μg of biotin-labeled calmodulin in Tween buffer + 1 mM $CaCl_2$ (lanes *1*), Tween buffer + 1 mM EGTA (lanes *2*), or Tween buffer + 1 mM $CaCl_2$ + 125 μg unlabeled calmodulin (lanes *3*). $CaCl_2$ or EGTA was included during all subsequent steps. After reaction with calmodulin, strips were rinsed extensively in Tween buffer and biotinylated calmodulin was detected with streptavidin-alkaline phosphatase followed by incubation in substrate solution. The positions of *Drosophila* and brain alpha-spectrin are marked with arrowheads.

polypeptides that migrated between M_r = 220 and 240 kD were also detected with this antibody in fly head homogenates. This observation suggests that multiple spectrin isoforms exist in *Drosophila*.

Chemical Cross-linking

To verify the association between the 234- and 226-kD polypeptides in solution, purified *Drosophila* spectrin was reacted with the reversible cross-linking reagent dimethyl dithiobispropionimidate (Fig. 4). After cross-linking, the amount of the 234- and 226-kD subunits was diminished relative to an untreated control (*lower arrowhead*, Fig. 4, lane *1*) and the relative amount of a high molecular mass complex, barely detected in the control, was increased after cross-linking (Fig. 4, compare lanes *1* and *2*, *upper arrowhead*). Reelectrophoresis of the cross-linked complex under reducing conditions (to cleave the cross-linker) yielded equivalent amounts of the 234- and 226-kD subunits (Fig. 4, lane *3*). In parallel experiments, *Drosophila* spectrin was cross-linked into a complex that comigrated with cross-linked human erythrocyte spectrin (not shown).

Calmodulin Binding

Like the alpha subunit of most vertebrate spectrins (Palfrey et al., 1982; Glenney et al., 1982*b*), *Drosophila* spectrin bound calmodulin in a calcium-dependent manner (Fig. 5). The alpha-subunits of both *Drosophila* spectrin and bovine brain spectrin bound calmodulin in the presence of 1 mM $CaCl_2$ (Fig. 5, lanes *1*) but not in 1 mM EGTA (lanes *2*); binding was displaced by the presence of a fivefold M excess of unlabeled calmodulin (lanes *3*). Similar results were obtained with ^{125}I-labeled calmodulin, which bound to a 234-kD polypeptide on nitrocellulose blots of the S3 cell lysate as well as to the excess alpha-spectrin found in our extracts (not shown).

Interaction with Actin

Drosophila spectrin was compared with human erythroid spectrin in an actin sedimentation assay conducted in the presence or absence of purified band 4.1 from human erythrocytes (Table II). Erythroid and brain spectrins from vertebrates both are known to cosediment with F-actin and the spectrin–actin interaction is enhanced in the presence of band 4.1 (Ungewickell et al., 1979; Burns et al., 1983). We observed that *Drosophila* spectrin also cosedimented with F-actin. But, whereas the binding of human spectrin to F-actin was increased in the presence of band 4.1, the *Drosophila* spectrin–actin interaction was, if anything, slightly reduced in the presence of band 4.1. These findings are consistent with the behavior of another spectrin molecule (TW-260/240 from chicken; Coleman et al., 1987) which does not appear to interact with band 4.1 in a similar assay.

Discussion

The similarities between *Drosophila* spectrin and known vertebrate spectrins are striking. The overall structure and length of *Drosophila* spectrin (Fig. 2) is comparable to that of vertebrate spectrins. Its immunological reactivity (Fig. 3; Table I) also demonstrates homology to authentic spectrins. Its susceptibility to cross-linking agents (Fig. 4) is similar to

Table II. Cosedimentation of Drosophila Spectrin with Actin

Reactants	Percent spectrin in pellet	
	Experiment 1	Experiment 2
D. spectrin (without additions)	12	29
D. spectrin + band 4.1	8	–
D. spectrin + actin	66	72
D. spectrin + actin + band 4.1	55	44
RBC spectrin (without additions)	2	–
RBC spectrin + band 4.1	31	–
RBC spectrin + actin	46	–
RBC spectrin + actin + band 4.1	90	–

Drosophila spectrin (D. spectrin, 70 μg/ml), human erythroid spectrin (RBC spectrin, 200 μg/ml), rabbit skeletal muscle actin (250 μg/ml), and purified human erythroid band 4.1 (25 μg/ml) were combined and reacted as indicated. Actin–spectrin complexes were centrifuged and analyzed by SDS gel electrophoresis. The proportion of spectrin pelleted in each sample was determined from densitometric scans of Coomassie Blue–stained gels. See text for details.

that of erythroid spectrin and indicates that the molecule is composed of two different polypeptides, the 234- and 226-kD subunits, which are closely associated in solution. Finally, *Drosophila* spectrin is both a calmodulin-binding protein and an actin-binding protein (Fig. 5; Table II), as is the case for all vertebrate spectrins so far examined. The larger subunit (234 kD) appears to be an alpha-spectrin, based on antibody and calmodulin-binding data. The 226-kD subunit appears to be a beta-spectrin based on its copurification with alpha-spectrin, antibody reactivity (present study), and its reassociation with an alpha-spectrin fusion protein (Byers et al., 1987). The conservation of these multiple characteristics indicates that the *Drosophila* protein we have described is a member of the spectrin family of proteins. It also suggests that many of spectrin's cellular roles may have been conserved between the vertebrates and the invertebrates.

In view of these similarities between *Drosophila* and vertebrate spectrins, it is surprising that such a large porportion of the spectrin isolated from S3 cells was found in the supernatant fraction of the homogenate (Fig. 1, *A* and *D*). Generally, vertebrate spectrins have been found to be associated with a sedimentable membrane fraction, which is consistent with the observations that they are concentrated close to the cell membrane and have specific binding sites on the membrane (Bennett, 1985; Marchesi, 1985; but for exceptions see: Hirokawa et al., 1983; Mangeat and Burridge, 1984; Black et al., 1986). Indeed, when homogenized under the same salt conditions as the S3 cells, adult *Drosophila* heads released only a small percentage of their spectrin into the supernatant fraction; nearly all the spectrin could be extracted with 0.1 M NaOH (not shown). It may be that spectrin plays a different or more limited role in the economy of cultured *Drosophila* cells than it does in the intact organism where preliminary immunofluorescence studies have revealed a distinct pattern of close membrane association throughout fly development (Pesacreta, T. C., manuscript in preparation). It is also possible that the homogenization procedures we used with S3 cells destroyed the in vivo spectrin–membrane interactions, although homogenization in a variety of salt conditions appeared to have little effect on the resultant distribution of spectrin between the pellet and supernatant fractions. Distinguishing between these possibilities will require a better understanding of the localization and role of spectrin in cultured *Drosophila* cells and the intact organism.

The significance of the excess alpha-spectrin in our extracts is unclear. The presence of "excess" alpha-spectrin may be artifactual, the consequence of differential extractability or resistance to postlysis proteolysis of one subunit. Pollard (1984) suggested that differential extractability might account for his purification of only one spectrinlike polypeptide from *Acanthamoeba*, where a second immunoreactive form appears to be present in the cell. Alternatively, the amount of alpha-spectrin in our extracts may accurately reflect an excess of this subunit that is en route to degradation, as has been observed for chicken erythrocyte spectrin subunits (Moon et al., 1984; Lazarides and Moon, 1984). Finally, the excess alpha-spectrin may represent a pool of spectrin that, either as a monomer or a homodimer, functions in a manner that has not been recognized in vertebrates. We are presently developing beta-specific spectrin probes to resolve these questions by direct analysis of the quantity and distribution of each subunit.

It has been suggested that spectrin may be important in persistent, repetitive, or cyclic cell shape and motility processes (Elgsaeter et al., 1986). If so, spectrin function in nonerythroid cells may be most evident during development, as cells undergo major changes in morphology in response to environmental or hormonal stimuli. Now that spectrin has been identified in *Drosophila* and its genetic components are being investigated (Byers et al., 1987), it will be possible to explore the role or roles of this widely distributed protein using genetic and molecular approaches in a well characterized developmental system. Although such studies may reveal aspects of spectrin function that have not been detected or do not occur in vertebrates, the similarities between invertebrate and vertebrate spectrin imply that functions elucidated in *Drosophila* will be relevant to understanding function in other organisms.

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