

**Drosophila** 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.

**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 ($\sim 30 g$ 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 dithiothreitol, 8 mM NaN₃, with 5 mM phenylmethylsulfonyl fluoride, 22 $\mu$M 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 ($g_{\text{max}} = 250,000$) 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 Sephadex (Sigma Chemical Co., St. Louis, MO) equilibrated in 500 ml of 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 NaN₃). 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 pools to 50% saturation and, after 1-2 h at $-80^\circ$C, precipitated proteins were collected by centrifugation at 8,000 $g_{\text{max}}$ for 15 min in a rotor (Sorvall GSA; DuPont Instruments, Sorvall Operations, Newport, CT). The precipitate was dissolved in 50 ml of a buffer (1 M KCl, 10% glycerol, 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 0.5 mM dithiothreitol, 8 mM NaN₃) and applied to a column (2.6 × 94 cm) of Sepharose CL-4B (Sigma Chemical Co.) equilibrated in the same buffer. Spectrin 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 (50 mM K-phosphate, pH 7.0; 0.5 mM dithiothreitol, 0.1 mM EGTA, 1 mM NaN₃). 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 Bennett (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, 1970) 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 NH₄(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. Aibhar 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 and DEAE chromatography and affinity purified by adsorption to alpha-actin-affinity/gel filtration procedure described elsewhere (Kiehart and Samloff, 1983). Purified calmodulin was stored as a standard polyethylene glycol-mediated fusion techniques (Cuello et al., 1987). Immune spleen cells were mixed with the FoxNY myosin (205 kD), beta-Drosophila spectrin fraction that was prepared from $3 cells by an actin-affinity/gel filtration procedure described elsewhere (Kiehart and Samloff, 1983). Antigen-specific hybrids were identified by ELISA assay (Engvall, 1980) on Immulon I assay plates (Dynatech Laboratories, Inc., San Diego, CA) in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM CaCl₂, 0.1% Tween 20 (Sigma Chemical Co., St. Louis, MO) or 1 mM EGTA (included during all subsequent steps). Biotinylated calmodulin was stored at 0°C in 25 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM CaCl₂, 8 mM NaN₃. Biotinylated calmodulin was detected with alkaline phosphatase conjugated streptavidin (Bethesda Research Laboratories, Gaithersburg, MD), diluted 1:100 in the same buffer) for 2-3 h at room temperature. Strips were washed in 6 changes of Tween buffer. Separate reactions were carried out in the presence of 1 mM CaCl₂ 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.

**Production of Antibodies**

Rabbit antibody BR-I, directed against chicken erythrocyd alphaspactrin (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 $3 cells by an actin-affinity/gel filtration procedure described elsewhere (Kiehart and Samloff, 1983). The rabbit was immunized by plantine injection (Sigel et al., 1983) of this spectrin fraction in Freund's complete adjuvant followed by 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 adsorption to alphaspactrin and beta-Drosophila spectrin that had been transferred to nitrocellulose from a SDS gel (Olms, 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 E. coli (Messina and Dione, 1984, Byers et al., 1987). Immune spleen cells were mixed with the FoxNY myosin (205 kD), beta-Drosophila spectrin fraction that was prepared from $3 cells by an actin-affinity/gel filtration procedure described elsewhere (Kiehart 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 subtypes 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., St. Louis, MO) 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.

**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 (Tuzynskil 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 dithio-bispropionimidate (Pierce Chemical Co., Rockford, IL) in 0.1 M triethanolamine, pH 7.5 for 30 min at room temperature. The propionimi-date 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 releasing 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 excised bands were incubated 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 homog enate by sequential chromatog raphy on phenyl-Sepharose (Sigma Chemical Co.) and DEAE Sephacel (Dedman and Kaetzel, 1983). Purified calmod ulin (25.5 mg/ml) was reacted with a 10-fold M excess of biotinyl-epsilon-aminocapric acid-N-hydroxysuccinimide ester (Calbiochem Biochemicals, San Diego, CA) in 20 mM HEPes, pH 7.4, 10 mM CaCl₂ 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 CaCl₂, 8 mM NaN₃. Biotinylated calmodulin was stored at 0°C in 25 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1 mM CaCl₂, 8 mM NaN₃. 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 CaCl₂ 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 MgCl₂ 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 (Mᵣ 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., 1982a), 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

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**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. 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 antispectrin 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 KC1. Spectrin eluted at 0.27 M KC1, 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., 1982a; 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 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-I (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.

A D. Spectrin     B Spectrin     S3 Cells     Fly Heads

D. 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-I, 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 | Antibody1 | BR-1 | 675 | Pre675 | M10-2Δ | M10-1 | 9051 | Pre905 |
|--------------------|-----------------|-----------|------|-----|--------|--------|-------|------|--------|
| Purified Drosophila spectrin | Alpha-spectrin | + +* | + + | + | + | + | + | + | - |
| Purified excess alpha-spectrin | Alpha-spectrin | + + | + + | + | + | + | + | + | - |
| Bovine brain membranes | Alpha-spectrin | + + | + + | + | + | + | + | + | - |
| S3 cell homogenate | Alpha-spectrin | + + | + + | + | + | + | + | + | - |
| Fly head homogenate | Alpha-spectrin | + + | + + | + | + | + | + | + | - |

* + and -., a reaction or no reaction of spectrin with antibody; + +, a preferential reaction with one subunit.  
Δ BR-1, anti-avian erythrocyte alpha spectrin; other antibodies were produced against Drosophila spectrin.  
9 Identical reactions were detected with M10-3, M10-4, M10-5, and M10-6.  
9 See Byers et al. (1987) for details of antibody production and characterization.
polypeptides that migrated between $M_t = 220$ 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 dithiobispropionimide (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., 1982b), 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 $^{35}$S-labeled calmodulin, which bound to a 234-kD polypeptide on nitrocellulose blots of the 3 cell lysate as obtained with $^{35}$S-labeled calmodulin, which bound to a 234-kD brain spectrin-bound calmodulin in the presence of 1 mM CaCl$_2$ (lanes 1), Tween buffer + 1 mM EGTA (lanes 2), or Tween buffer + 1 mM CaCl$_2$ + 125 nM 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.

**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 proportion 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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