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Cell Biology

Identification of a spectrin-like protein in nonerythroid cells

(nonerythroid spectrin/cell cytoskeleton/actin/myosin/peptide mapping)

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ABSTRACT **We have demonstrated the existence of a spectrin-like protein in a variety of nonerythroid cultured cells. Indirect immunofluorescence studies with monospecific antispectrin IgG indicated the presence of proteins that have common antigenic determinants to spectrin in embryonic chicken cardiac myocytes, mouse fibroblast lines (3T3, simian virus 40-transformed 3T3), and rat hepatoma lines (HTC, HMOA). Two spectrin-like peptides of 240,000 and 230,000 daltons were immunoprecipitated from octyl glucoside-solubilized embryonic chicken cardiac myocytes, along with associated cytoskeletal proteins. Immunoautoradiographic characterization of the myocyte immunoprecipitate showed that only the spectrin-like 240,000- and 230,000-dalton peptides were stained with monospecific antispectrin IgG and ^{125}I-labeled protein A. One-dimensional partial proteolytic mapping of the myocyte 240,000- and 230,000-dalton peptides showed that these peptides share substantial sequence homology with embryonic chicken erythrocyte spectrin 240,000- and 220,000-dalton peptides.**

Spectrin is the major component of a cytoskeletal protein network that is attached to the cytoplasmic surface of the erythrocyte membrane (1) through a high-affinity association with the syndeins (bands 2.1–2.6) (2–4). Spectrin, which constitutes 25–30% of the total erythrocyte membrane protein (5), appears to play a key role in the maintenance of the discoid shape of the membrane (for review, see ref. 6) and in restricting the lateral mobility of its macromolecules (for review, see ref. 7).

Spectrin has been found in all mammalian and avian red cells studied but has not been detected previously in nonerythroid cell types. Earlier attempts to demonstrate the presence of spectrin in nonerythroid cells by complement fixation (8) and radioimmunoassay (9) were unsuccessful. However, the recent demonstration of immunoreactive forms of the syndeins in diverse cells and tissues by an extremely sensitive radioimmunoassay (10) suggested to us the appropriateness of reinvestigating the question of the ubiquity of spectrin.

In the studies presented here, we demonstrate the presence of immunoreactive forms of spectrin in several cell types by indirect immunofluorescence and immunoautoradiographic characterization. Furthermore, we have immunoprecipitated two large polypeptides (240,000 and 230,000 daltons) from embryonic chicken cardiac myocytes (ECCMs) by using a monospecific antispectrin IgG. These spectrin-like peptides appear to be similar but not identical to bands 1 and 2‡ of embryonic chicken erythrocyte (ecRBC) spectrin by partial proteolytic mapping.

Preliminary reports of these observations have been presented elsewhere (11, §).

MATERIALS AND METHODS

Production and Characterization of Antibody. Human erythrocyte spectrin α–β heterodimers were purified to homogeneity by preparative rate zonal sedimentation on linear sucrose gradients as described (12). Spectrin (255 μg/ml) was emulsified in an equal volume of complete Freund's adjuvant (Difco) and injected into male New Zealand White rabbits intradermally and intramuscularly at multiple sites. The initial injection contained 80 μg of protein. Booster injections (40 μg) were made in an identical fashion. Preimmune serum was collected before the initial injection. The IgG fractions of both preimmune and immune sera were prepared as described (13).

The purified immune IgG fraction was characterized by immunoelectrophoresis (14), and immunoautoradiographic gel staining techniques as described by Granger and Lazarides (15).

Isolation of ECCMs. ECCMs were isolated from ventricles of White Leghorn chicken embryos at 8 days of incubation. Dissected hearts were minced and washed extensively with Ca^{2+}/Mg^{2+}-free Earle's balanced salt solution (GIBCO) to remove erythrocytes. The mince was digested with 0.125% trypsin prepared in the same salt solution for 20 min at 37°C and filtered through 20-μm Nitex cloth. The heart cells were collected in serum-containing medium, washed extensively with phosphate-buffered saline (P_i/NaCl), and used immediately for the immunoprecipitation experiments. For use in the indirect immunofluorescence experiments, myocytes were washed with Eagle's minimal essential medium (without L-glutamine)/10% fetal calf serum containing penicillin at 100 units/ml and streptomycin at 100 μg/ml; 5×10^5 cells were inoculated into 30-mm Falcon tissue culture plates containing 22-mm^2 glass coverslips and incubated in humid 5% CO_2/95% air.

ECCMs growing in culture on glass coverslips were processed for immunofluorescence experiments using antispectrin IgG as follows: cells were fixed either by immersion in absolute acetone at −20°C for 5 min or by treatment with 3% paraformaldehyde in P_i/NaCl for 2 min at 20°C, washed extensively with P_i/NaCl (aldehyde-fixed cells were also washed with 0.1 M glycine in P_i/NaCl to quench any unreacted aldehyde groups and permeabilized with 0.2% Triton X-100 in P_i/NaCl for 3–5 min at 20°C), incubated with 50 μl of antispectrin IgG (9 mg/ml diluted 1:100), washed extensively with P_i/NaCl, allowed to react with 50 μl of rhodamine-conjugated goat anti-rabbit IgG (19.6 mg/ml diluted 1:200; Cappel Laboratories, Cochranville, PA), washed with P_i/NaCl and examined with a Zeiss Universal microscope equipped with epifluorescence optics.

Immunoprecipitation of Spectrin from Detergent-Solubilized Cells. ECCMs, ecRBCs, and human erythrocytes (hRBCs) (0.1 ml of packed cells) were washed three times in 100 vol of

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Abbreviations: ECCM, embryonic chicken cardiac myocyte; P_i/NaCl, phosphate-buffered saline; hRBC, human erythrocyte; ecRBC embryonic chicken erythrocyte; iPr_2P-F, diisopropyl fluorophosphate.

‡ The major polypeptides of the erythrocyte are designated by Steck's nomenclature (5). Band 1 is the α-chain (240,000 daltons) and band 2 is the β-chain (220,000 daltons) of the spectrin heterodimer.

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cold 5 mM $NaPO_4$/155 mM NaCl/0.4 mM diisopropyl fluorophosphate (iPr_2P-F), pH 8.0. The cells were brought to a final volume of 0.7 ml by addition of 5 mM $NaPO_4$/155 mM NaCl/0.4 mM iPr_2P-F/40 mM octyl glucoside, pH 8.0, and incubated with detergent for 15 min at 4°C. Detergent-solubilized cells (0.1 ml) were incubated with $NaDodSO_4$ for gel electrophoresis. The remaining 0.6 ml of detergent-solubilized cells were incubated for 60 min at 22°C in (final volume, 0.9 ml) 5 mM $NaPO_4$/155 mM NaCl/0.4 mM iPr_2P-F/30 mM octyl glucoside, pH 8.0, containing monospecific anti-spectrin IgG, at 1 mg/ml, fraction V bovine serum albumin, at 500 μg/ml, and pancreatic trypsin inhibitor at 0.2 μg/ml. Protein A-bearing staphylococcus A (pansorbin, Calbiochem) (0.1 ml) was added, and incubation was continued for 60 min at 22°C. The spectrin–IgG–staphylococcus A immunoprecipitate was pelleted by a 5-min centrifugation at 3000 × *g*. The supernatant was removed and incubated with $NaDodSO_4$ for gel electrophoresis. The pellet was washed three times with 100 vol of 5 mM $NaPO_4$/155 mM NaCl/0.4 mM iPr_2P-F/40 mM octyl glucoside, pH 8.0. The spectrin–IgG–staphylococcus A complex (100 μl) was resuspended by addition of an equal volume of 5% $NaDodSO_4$/160 mM dithiothreitol/50 mM Tris, pH 8.0/5% sucrose/5 mM EDTA containing pyronin Y at 0.2 mg/ml. The antigen–antibody complex was dissociated from the staphylococcus A by a 45-min incubation of 60°C. The mixtures were placed into 0.4-ml polyethylene microfuge tubes and centrifuged at 20,000 × *g* for 30 min at 20°C. The immunoprecipitate-containing supernatant was removed for gel electrophoresis.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed by the discontinuous system of Laemmli (16). Gels were stained and destained as described by Fairbanks *et al.* (17).

One-Dimensional Partial Proteolytic Mapping. The RBC and ECCM immunoprecipitates were subjected to electrophoresis on $NaDodSO_4$/acrylamide gels as described above. The gel was stained for 6 hr in 0.025% Coomassie blue/25% isopropanol/10% acetic acid and destained for 3 hr in 10% isopropanol/10% acetic acid. The spectrin and spectrin-like peptides were cut from the gel with a razor blade and dried under a heat lamp for 6 hr. Drying of the gel facilitated the uptake of ^{125}I-labeled Bolton–Hunter reagent (140 μCi per gel slice; 1 Ci = 3.7 × 10^{10} becquerels), which was added in 0.5 ml of 5 mM $NaPO_4$/155 mM NaCl, pH 8.0. Incubation of the gel slices with ^{125}I-labeled Bolton–Hunter reagent was for 18 hr at 4°C. Gel slices were washed 10 times with 5 mM $NaPO_4$/155 mM NaCl, pH 8.0 (10 ml), and then given a final wash in 0.125 M Tris·HCl, pH 6.8/0.1% $NaDodSO_4$/1 mM EDTA and incubated in this buffer for 2 hr.

Peptide maps were obtained by limited proteolysis in $NaDodSO_4$/polyacrylamide gels by the technique of Cleveland *et al.* (18) with the modification that the separating gel contained 10% acrylamide/0.26% bisacrylamide. ^{125}I-Labeled peptide maps were visualized by autoradiography.

Autoradiography. Autoradiograms were exposed for 1–7 days at −70°C, using Kodak X-Omat XAR-5 film with a Dupont Cronex Lightning Plus intensifying screen.

RESULTS

Characterization of Antispectrin IgG. Rabbit antispectrin antibody was raised against hRBC spectrin heterodimers purified to homogeneity by well-established techniques (12). The immunoelectropherogram shown in Fig. 1 illustrates the lack of detectable reaction between purified spectrin and the IgG fraction of preimmune serum. There are no visible precipitin lines between antispectrin IgG and either actin or myosin. There is a single precipitin line observed in the case of spectrin.

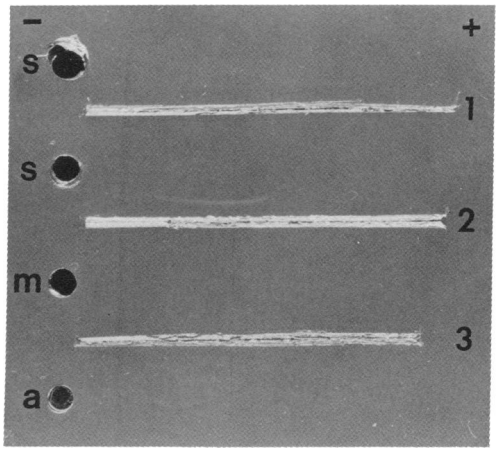

FIG. 1. Immunoelectropherogram of spectrin, myosin, and actin versus preimmune and immune IgG. Wells: s, 10 μl of purified spectrin (0.6 mg/ml); m, 10 μl of myosin (1 mg/ml); a, 10 μl of actin (1mg/ml). Troughs: 1, 50 μl of preimmune IgG (9 mg/ml); 2 and 3, 50 μl each of immune IgG (9 mg/ml). Direction of electrophoresis was from − to +. Photographed using darkfield illumination.

These results confirm those found by using Ouchterlony double-diffusion analysis (data not shown).

It was critical for these experiments that our antibody not contain any crossreacting antibodies against antigens that might by present in trace amounts in our spectrin preparation (e.g., syndeins, band 4.1, actin). This has been demonstrated by an immunoautoradiography gel staining technique (15) in which a $NaDodSO_4$/polyacrylamide gel of RBC ghost protein was fixed, equilibrated with a physiological ionic strength buffer, incubated with antispectrin IgG, and then stained with ^{125}I-labeled protein A. Comparison of the Coomassie blue-stained gel

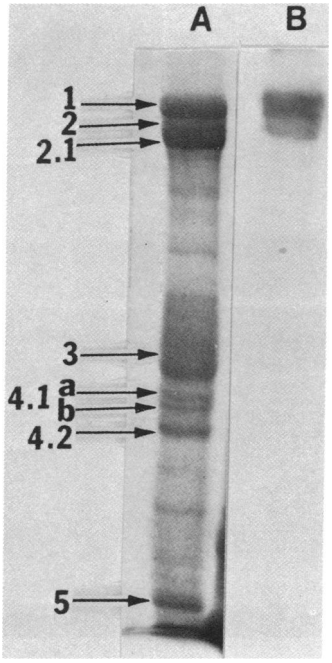

FIG. 2. Immunoautoradiographic characterization of hRBC ghost proteins using antispectrin IgG. hRBC membrane protein (64 μg) was subjected to electrophoresis on a $NaDodSO_4$/6% polyacrylamide slab gel, and the gel was autoradiographed for 5 days at −70°C with an intensifying screen. Lanes: A, Coomassie blue-stained gel; B, immunoautoradiogram.

with the corresponding immunoautoradiogram (Fig. 2) shows that antispectrin IgG is monospecific for the spectrin 240,000- and 220,000-dalton peptides. Our antibody raised against native purified hRBC spectrin reacts preferentially with the 240,000-dalton chain of spectrin.

Indirect Immunofluorescence Study of Cultured Cells Using Antispectrin IgG. Cultured ECCMs prepared by either of two procedures contain protein that reacts with antispectrin IgG (Fig. 3) as shown by indirect immunofluorescence. The staining pattern is diffuse and not localized in any particular part of the cell. The incubation of antispectrin IgG with a 10-fold excess of purified spectrin before staining reduced the fluorescence to background levels. Similar results were obtained with cultured mouse fibroblasts (3T3 cells, simian virus 40-transformed 3T3 cells) and rat hepatoma cells (HTC cells and HMOA cells) (data not shown). ecRBCs similarly stained also showed a positive reaction, which was abolished in the case of antigen-adsorbed IgG (Fig. 3).

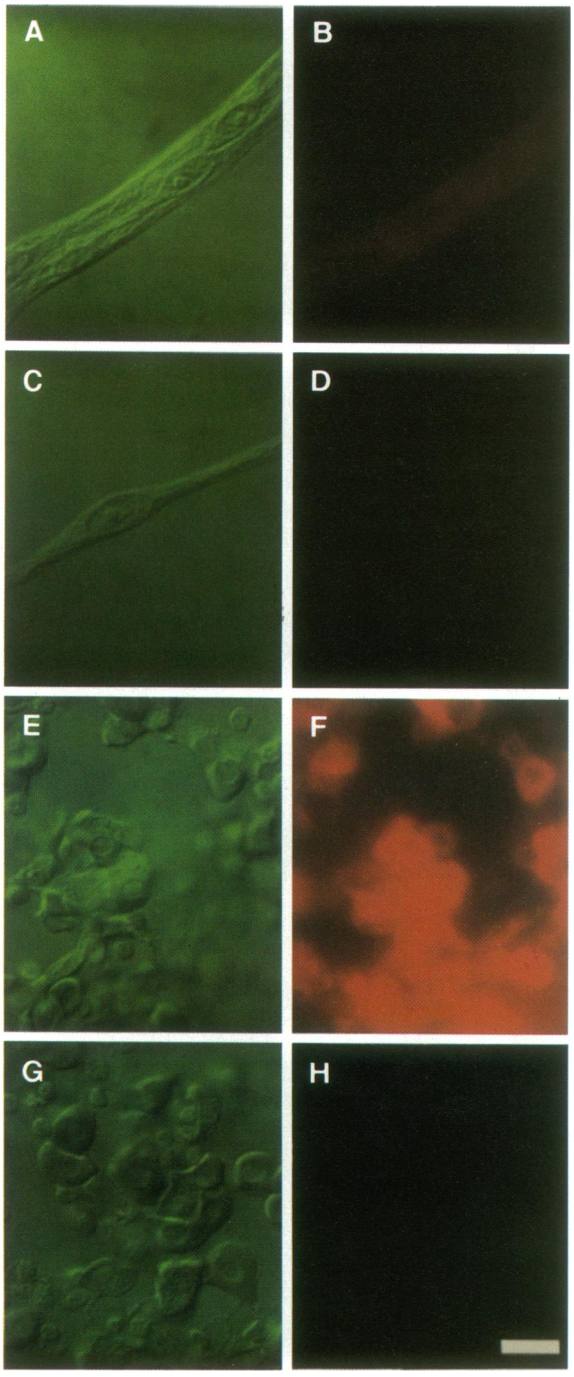

FIG. 3. Nomarski differential interference contrast (*Left*) and immunofluorescence (*Right*) micrographs of ECCMs and ecRBCs stained with antispectrin IgG or spectrin-absorbed antispectrin IgG. (*A* and *B*) ECCMs stained with antispectrin IgG. (*C* and *D*) ECCMs stained with spectrin-adsorbed antispectrin IgG. (*E* and *F*) ecRBCs stained with antispectrin IgG. (*G* and *H*) ecRBCs stained with spectrin-adsorbed immune IgG. Bar = 10 μm.

Immunoprecipitation of Spectrin and a Spectrin-Like Protein from hRBCs, ecRBCs, and ECCMs. The membrane skeleton of erythrocytes and the cell cytoskeleton of nonerythroid cells has been operationally defined as the proteinaceous residue remaining after neutral detergent extraction of intact cells (for review, see ref. 19). We therefore reasoned that our monospecific antispectrin IgG should extract intact spectrin along with associated skeletal proteins from cells solubilized with the neutral detergent octyl glucoside. In the Coomassie blue-stained $NaDodSO_4$/polyacrylamide gel (Fig. 4), we demonstrate that, although spectrin constitutes a minor component of octyl glucoside-solubilized hRBCs (lane B), it is the major component of the hRBC immunoprecipitate (lane D) and is absent in the supernatant left after removal of the immunoprecipitate with staphylococcus A (lane C). In addition to spectrin, the hRBC immunoprecipitate contains the syndeins, bands 3 and 4.1, and actin, all of which are components of the hRBC membrane skeleton (lane D). The 240,000- and 220,000-dalton spectrin polypeptides can also be immunoprecipitated, along with associated proteins, from ecRBCs with monospecific antispectrin IgG (lane G). Antispectrin IgG raised against human spectrin reacts less efficiently with ecRBC spectrin. The antispectrin immunoprecipitate from octyl glucoside-solubilized ECCMs contains spectrin-like polypeptides of 240,000 and 230,000 daltons (lane J). By gel scanning (not shown) and peak integration, the 240,000- and 230,000-dalton spectrin-like polypeptides are present in a 1.1:1.0 ratio, similar to the 1:1 ratio observed for

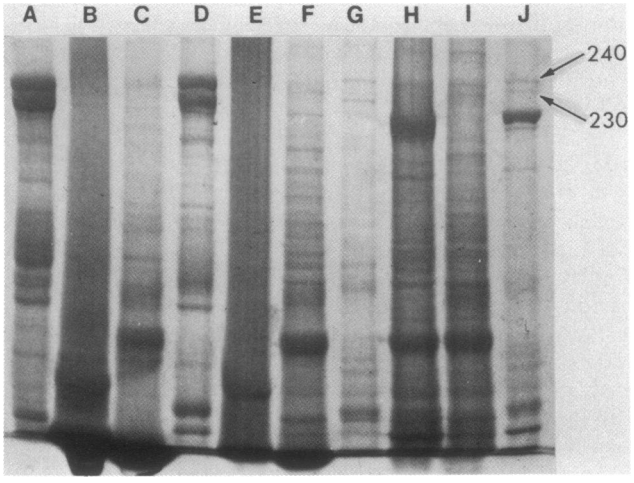

FIG. 4. Electrophoretic analysis of the immunoprecipitation of a spectrin-like protein from hRBCs, ecRBCs, and ECCMs with a monospecific antispectrin IgG. Lanes were loaded with 20 μl of undiluted fraction. Lanes: A, standard hRBC ghost protein (3.2 mg/ml); B, octyl glucoside-solubilized hRBCs; C, supernatant remaining after immunoprecipitation of hRBCs with antispectrin IgG; D, immunoprecipitate from hRBCs; E, octyl glucoside-solubilized ecRBCs; F, supernatant remaining after immunoprecipitation of ecRBCs with antispectrin IgG; G, immunoprecipitate from ecRBCs; H, octyl glucoside-solubilized ECCMs; I, supernatant remaining after immunoprecipitation of ECCMs with antispectrin IgG; J, immunoprecipitate from ECCMs. 240 and 230, 240,000- and 230,000-dalton spectrin-like peptides with the ECCM immunoprecipitate.

hRBC spectrin. Along with the spectrin-like protein, the ECCM immunoprecipitate contains major components of 195,000 (myosin) and 43,000 daltons (actin) and minor components of 190,000, 160,000, 110,000, 90,000, 67,000, 64,000, 60,000, 55,000, and 50,000 daltons.

We have shown the ability of purified hRBC spectrin to block the immunoprecipitation of ^{125}I-labeled spectrin and associated proteins by antispectrin IgG. Octyl glucoside-solubilized hRBC and ECCM protein were iodinated with ^{125}I-labeled Bolton–Hunter reagent. ECCM skeletal proteins appear to be labeled more efficiently than hRBC skeletal proteins by this reagent (Fig. 5). Preadsorption of monospecific antispectrin IgG with twice the concentration of pure hRBC spectrin effectively decreases the immunoprecipitation of ^{125}I-labeled spectrin and spectrin-like protein from hRBCs and ECCMs. In addition, the immunoprecipitation of ^{125}I-labeled spectrin-associated proteins decreased in parallel with ^{125}I-labeled spectrin in hRBCs and ECCMs.

Immunoautoradiographic Characterization of hRBC, ecRBC, and ECCM Immunoprecipitates with Antispectrin IgG. Antispectrin immunoprecipitates from octyl glucoside-solubilized hRBCs, ecRBCs, and ECCMs were isolated and subjected to electrophoresis. Immunoautoradiographic characterization of $NaDodSO_4$/polyacrylamide gels containing the immunoprecipitated protein is shown in Fig. 6. Comparison of the Coomassie blue-stained gels (lanes A, C, and E) and the corresponding immunoautoradiograms (lanes B, D, and F) shows that monospecific antispectrin IgG reacts only with the 240,000- and 220,000-dalton spectrin bands in the hRBC and ecRBC immunoprecipitates (lanes A–D). Furthermore, the antispectrin IgG reacts specifically with the 240,000- and 230,000-dalton spectrin-like polypeptides in the ECCM immunoprecipitate (lanes E and F). Although both myosin (195,000 daltons) and actin (43,000 daltons) were present in large molar excess over the spectrin-like peptides in the ECCM immunoprecipitate (lane E); there was no detectable staining of either or of any other associated protein (lane F).

Partial Proteolytic Mapping of Spectrin and the Spectrin-Like Protein. Further evidence for the occurrence of a spectrin-like protein in a nonerythroid cell was obtained by comparative peptide mapping of ecRBC spectrin and the ECCM spectrin-like protein. Partial proteolytic one-dimensional maps were obtained by the technique of Cleveland *et al.* (18) (Fig. 7). In agreement with our previous two-dimensional mapping of hRBC spectrin (2), the large and small chains of ecRBC spectrin (lanes A and C) and ECCM spectrin-like protein (lanes B and D) show distinct peptide maps demonstrating lack of substantial sequence homology between band 1 and band 2 of ecRBC and ECCM spectrin-like protein. However, the chymotryptic maps of the 240,000-dalton peptides from ECCM and ecRBC spectrin show a large number of common peptides (lanes A and B), as do the 230,000-dalton (ECCM) and 220,000-dalton (ecRBC) subunits of these spectrin-like proteins (lanes C and D). This result shows that, although ecRBC spectrin and ECCM spectrin-like protein are not identical, they share substantial sequence homology.

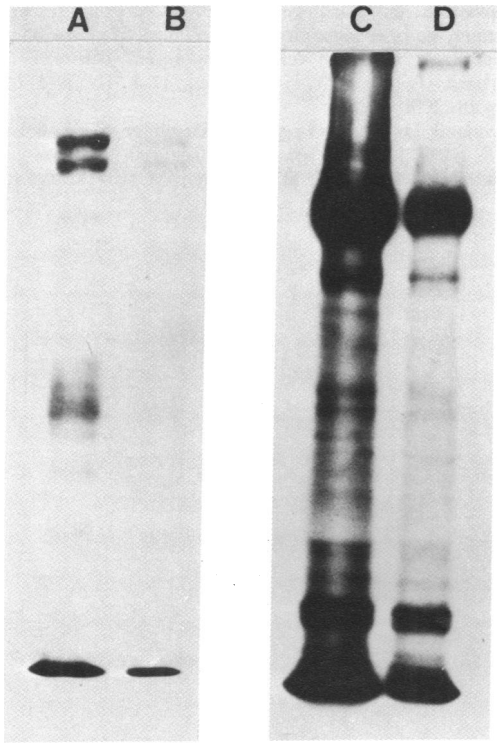

FIG. 5. Previous incubation with purified unlabeled hRBC spectrin blocks the binding of spectrin and associated cytoskeletal proteins to the IgG–staphylococcus A complex. ^{125}I-Labeled immunoprecipitate-containing supernatant was subjected to electrophoresis on a discontinuous $NaDodSO_4$/polyacrylamide slab gel, and the gel was autoradiographed for 1 day at −70°C. Lanes: A, 30 μl of antispectrin IgG immunoprecipitate of ^{125}I-labeled hRBCs; B, 30 μl of spectrin absorbed-antispectrin IgG immunoprecipitate of ^{125}I-labeled hRBCs; C, 30 μl of antispectrin IgG immunoprecipitate of ^{125}I-labeled ECCMs; D, 30 μl of spectrin absorbed-antispectrin IgG immunoprecipitate of ^{125}I-labeled ECCMs.

DISCUSSION

Five observations have led to the conclusion that a spectrin-like protein exists in nonerythroid cells. (*i*) Indirect immunofluorescence of ECCMs stained with a monospecific antispectrin IgG shows a diffuse fluorescence that is not observed with spectrin-adsorbed IgG. (*ii*) Two spectrin-like peptides can be immunoprecipitated from octyl glucoside-solubilized ECCMs

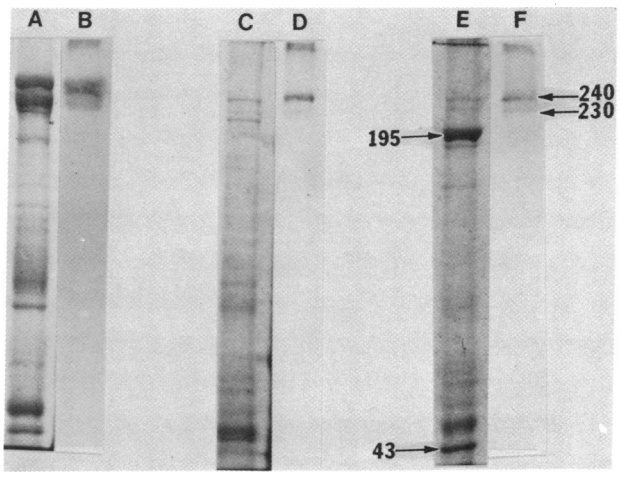

FIG. 6. Immunoautoradiographic characterization of the hRBC, ecRBC, and ECCM immunoprecipitates using antispectrin IgG. Antispectrin immunoprecipitates were subjected to electrophoresis and the gels were immunoautoradiographed as described in the legend to Fig. 2. Lanes: A, Coomassie blue-stained hRBC immunoprecipitate; B, immunoautoradiogram of the hRBC immunoprecipitate; C, Coomassie blue-stained ecRBC immunoprecipitate; D, immunoautoradiogram of the ecRBC immunoprecipitate; E, Coomassie blue-stained ECCM immunoprecipitate; F, immunoautoradiogram of the ECCM immunoprecipitate. 240 and 230, ECCM spectrin-like peptides (240,000 and 230,000 daltons, respectively); 195, myosin (195,000 daltons); 43, actin (43,000 daltons).

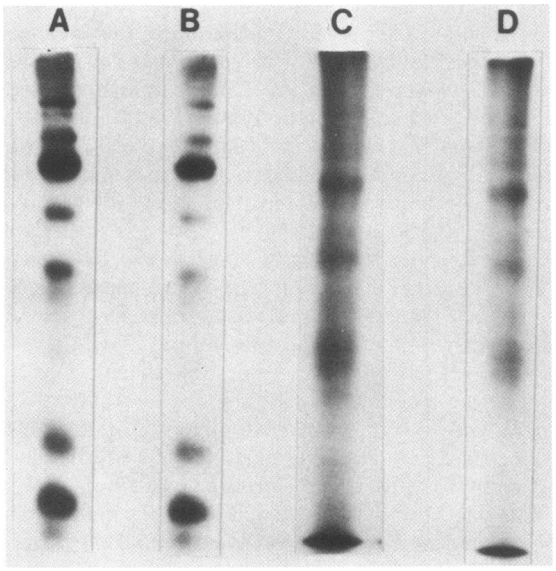

FIG. 7. Comparison of one-dimensional partial proteolytic maps of ^{125}I-labeled spectrin from ecRBCs and ^{125}I-labeled spectrin-like protein from ECCMs using chymotrypsin at 2 μg per lane. Lanes: A, ^{125}I-labeled ecRBC 240,000-dalton peptide; B, ^{125}I-labeled ECCM 240,000-dalton peptide; C, ^{125}I-labeled ecRBC 220,000-dalton peptide, D, ^{125}I-labeled ECCM 230,000-dalton peptide.

(along with associated proteins) with antispectrin IgG. The 240,000- and 230,000-dalton peptides are present in a 1:1 (mol/mol) ratio analogous to their RBC spectrin counterparts. (*iii*) Immunoprecipitation of ^{125}I-labeled spectrin-like protein and associated proteins from ECCMs is blocked by previous adsorbtion of the antispectrin IgG with purified hRBC spectrin. (*iv*) Immunoautoradiographic characterization of the ECCM immunoprecipitate with antispectrin IgG and ^{125}I-labeled protein A showed that only the 240,000- and 230,000-dalton spectrin-like peptides are stained with the antibody. (*v*) Partial proteolytic peptide mapping of the ecRBC 240,000- and 220,000-dalton spectrin chains and the ECCM 240,000- and 230,000-dalton spectrin-like peptides suggests substantial sequence homology.

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