

The 4.1-like Proteins of the Bovine Lens: Spectrin-binding Proteins Closely Related in Structure to Red Blood Cell Protein 4.1

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Abstract. The superficial cortical fiber cells of the bovine lens contain membrane-associated proteins of 150,000, 80,000, and 78,000 D that cross-react with antisera prepared against red blood cell (RBC) protein 4.1 (Aster, J. C., G. J. Brewer, S. M. Hanash, and H. Maisel, 1984, *Biochem. J.*, 224:609-616). To further study their relationship to protein 4.1, these proteins were immunoprecipitated from detergent extracts of crude lens membranes with purified polyclonal and monoclonal anti-4.1 antibodies and resolved by SDS PAGE. The electrophoretic mobilities of the lens proteins of 80,000 and 78,000 D were found to be identical to bovine RBC protein 4.1a and protein 4.1b, respectively. One- and two-dimensional peptide mapping revealed that a high degree of structural homol-

ogy exists among all three of the lens 4.1-like proteins and RBC protein 4.1a and protein 4.1b. Despite the large difference in apparent molecular mass, the 150,000-D lens protein showed only minor peptide map differences. A nitrocellulose filter overlay assay showed that all three of the lens 4.1-like proteins bind to RBC and lens spectrins. We conclude that the bovine lens contains proteins of 80,000 and 78,000 D that are highly similar to protein 4.1 in structure and functional capacity. Additionally, the lens also contains a 4.1 isomorph of 150 kD. Analogous to RBC protein 4.1, these proteins may function in the lens by promoting association of spectrin with actin and by playing a role in the coupling of lens cytoskeleton to plasma membrane.

THE red blood cell (RBC)¹ membrane skeleton is a reticulum of proteins that lines the cytoplasmic face of the plasma membrane and gives this cell its characteristic shape and viscoelastic properties (for review, see references 14 and 28). The major component of this reticulum by mass is spectrin, a thread-like protein composed of subunits of 240 kD (α -spectrin) and 220 kD (β -spectrin). One end of the spectrin molecule contains a site for self-association (48), permitting the formation of tetrameric spectrin. At the end of the molecule opposite the site of self-association spectrin has an F-actin binding site (18), allowing tetrameric spectrin to function as an F-actin cross-linking protein (12). The membrane skeleton contains short actin oligomers (45) that bind multiple spectrin tetramers (13), permitting the formation of a spectrin-actin lattice. This lattice is connected to the membrane via a linker protein called ankyrin that binds simultaneously to β -spectrin (8, 9) and band 3 (10, 11), the RBC anion channel.

Protein 4.1 is another major component of the membrane skeleton, being present at a molar concentration equal to that of spectrin dimer (31). This protein binds to spectrin close to the point of spectrin-actin interaction (53, 54), thereby enhancing and stabilizing the association of spectrin with actin

(17, 26). Protein 4.1 also binds to at least one intrinsic membrane protein (1, 44), and so may serve as a second point of linkage between the membrane and the skeleton. The importance of interactions involving protein 4.1 is emphasized by the association of protein 4.1 deficiency (25, 51) and defective binding of protein 4.1 to spectrin (29, 57) with distinct types of hemolytic anemia.

It is now appreciated that the membrane skeleton contains a family of 4.1-like proteins. Using discontinuous buffer systems, protein 4.1 can be resolved by SDS PAGE into polypeptides of 80 and 78 kD that have been designated 4.1a and 4.1b, respectively (41). These proteins are closely related in sequence and function (31). It appears that 4.1a is probably produced by some sort of posttranslational modification, since reticulocytes and young RBCs contain mostly 4.1b, while 4.1a predominates in old RBCs (47). Other minor, membrane-associated 4.1-like proteins of 67 kD (56), 85 and 87 kD (30), and 150 kD (3, 32) have also been detected.

A variety of non-erythroid cell types, including fibroblasts (16), platelets (19, 49), neutrophils and monocytes (49), neurons (6, 27), and lenticular cells (5, 32) have recently been shown to express proteins serologically related to protein 4.1. The superficial cortical cells of the bovine lens contain 4.1-like proteins of 150, 80, and 78 kD (3). In this paper, we demonstrate that these proteins are closely related in structure to one another and to RBC 4.1a and 4.1b and bind spectrin.

1. Abbreviation used in this paper: RBC, red blood cell.

Materials and Methods

Materials

Bovine eyes were removed from freshly killed 1–2-yr-old animals at a local slaughterhouse and transported on ice to the laboratory for immediate use. Human blood was obtained by venipuncture from normal volunteers with informed consent. Fresh bovine blood, kindly provided by the Large Animal Clinic (Michigan State University), was processed within 24 h. Mice were obtained from Jackson Laboratory (Bar Harbor, ME). Acrylamide, bis-acrylamide, SDS, ammonium persulfate, EDTA, Bradford reagent, and Affi Gel 10 were from Bio-Rad Laboratories (Richmond, CA). Heat-killed, fixed *Staphylococcus aureus* cells bearing protein A (Pansorbin) were obtained from Behring Diagnostics (San Diego, CA). All other chemicals and reagents, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Proteins

Human RBC protein 4.1 was purified according to Tyler et al. (53) with the modifications of Cohen and Foley (15). Human RBC spectrin was prepared as described by Gratzer (34). Bovine lens spectrin was isolated by a procedure previously used for preparation of brain spectrin (20). 50 lenses were decapsulated. The superficial fiber cells were then removed and disrupted in 200 ml of ice-cold 50 mM Tris, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5, with a Dounce homogenizer. A crude membrane fraction was isolated by centrifugation at 48,000 *g* for 10 min and washed twice with homogenization buffer. The final pellet was then extracted with 100 ml of 0.2 mM Tris, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5, for 60 min at 37°C. This suspension was then centrifuged at 100,000 *g* for 30 min. Solid $(\text{NH}_4)_2\text{SO}_4$ was slowly added to the resultant supernatant to 45% saturation at 4°C, and precipitated protein was pelleted at 7,000 *g* for 10 min. The pellet was resuspended in 10 ml of 10% sucrose (wt/vol) dissolved in 10 mM NaPO_4 , pH 8.2, containing 1 M NaBr, 1 mM EGTA, 15 mM Na pyrophosphate, 0.4 mM dithiothreitol, and 1 mM NaN_3 and allowed to mix for 16 h at 4°C. The suspension was then spun at 100,000 *g* for 30 min, and the supernatant was applied to a 1.5×105 -cm Sepharose 4B column equilibrated against the buffer above. Fractions (5 ml) containing spectrin were detected using OD_{280} and SDS PAGE. Spectrin was then purified from pooled fractions by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by chromatography on a 0.5×10 -cm diethylaminoethane-cellulose column as described (20).

Purified protein 4.1 used as immunogen or in enzyme-linked immunosorbent assays (ELISA) was stored at -70°C . Proteins used in overlay experiments were labeled with ^{125}I -Bolton-Hunter reagent within 24 h of preparation and stored at 4°C until use. Protein concentration was determined with the Bio-Rad Bradford reagent assay using bovine IgG as a standard.

Figure 1. Monoclonal antibodies prepared against RBC protein 4.1 recognize the 4.1-like proteins of the bovine lens. Proteins precipitated from fiber cell extracts with polyclonal anti-4.1 antibody were electrophoresed in 7.5% polyacrylamide gels containing 0.1% SDS and then transferred electrophoretically to nitrocellulose filters. The filters were then treated with 2 μg of anti-4.1 monoclonal antibody IF3.7 (1) or 2 μg of anti-4.1 monoclonal antibody 3G11.4 (2) followed by second antibody-horseradish peroxidase conjugate at 1:1,000. Blots were stained with 4-chloro-1-naphthol. HC, heavy chain.

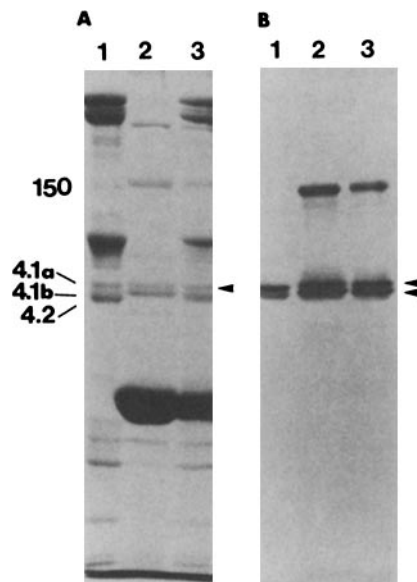
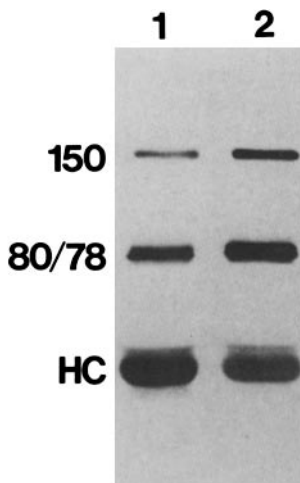


Figure 2. Bovine RBC 4.1a and 4.1b and the bovine lens 4.1-like proteins of 80 kD and 78 kD co-migrate when subjected to SDS PAGE. Solubilized bovine RBC membranes (40 μg , lane 1), precipitates prepared from lens membrane extracts with monoclonal anti-4.1 antibody 3G11.4 (50 μl of solubilized protein, lane 2), or a mixture of RBC membranes (20 μg protein) and immunoprecipitated lens proteins (25 μl , lane 3) were electrophoresed on 7.5% gels. (A) Coomassie Blue staining pattern; (B) the corresponding immunoblot after treatment with 3G11.4 (2 $\mu\text{g}/\text{lane}$) and protein A-horseradish peroxidase conjugate at 1:2,000. Staining was carried out with a horseradish peroxidase substrate, 4-chloro-1-naphthol.

Production of Polyclonal Antibodies

Antibody against native human RBC protein 4.1 was raised in New Zealand White rabbits and purified by affinity chromatography on an Affi Gel 10-protein 4.1 column according to Cohen et al. (16). This antibody has been previously described (3). Unbound, nonspecific IgG was purified from the affinity column flow-through using protein A-Sepharose CL-4B chromatography. Flow-through was applied at 20 ml/h to a 1×5 -cm protein A-Sepharose column equilibrated against 10 mM NaPO_4 , pH 7.4, containing 154 mM NaCl (PBS). The column was washed with PBS until the OD_{280} was <0.01 . IgG was then eluted with 0.2 M glycine, pH 2.7. After immediate neutralization by addition of 0.3 vol of 1 M NaPO_4 , pH 8.0, fractions containing IgG were pooled, dialyzed against PBS, and stored at -20°C .

Production of Monoclonal Antibodies

(A/J \times CB20)F₁ mice (Jackson Laboratory) were injected with purified human protein 4.1 at axillary, inguinal, and toe pad sites (8 $\mu\text{g}/\text{site}$, 50 μg total protein). The antigen was given in Freund's complete adjuvant on day 1, in Freund's incomplete adjuvant on day 5, and then in saline on days 9, 13, 17, 21, and 25. Mice were bled from the suborbital sinus on days 12 and 24. Titers were monitored by immunoblot analysis. On day 29 the mice were transferred to the University of Michigan Hybridoma Facility where cells from popliteal, inguinal, and subaxillary lymph nodes were fused with X63-Ag8,653, a nonsecreting mouse myeloma cell line (39), according to the procedure of Kearney et al. (38). Clones were expanded in culture at the Hybridoma Facility and screened for production of anti-4.1 antibody using an ELISA. 10 μg of purified protein 4.1 was adsorbed to 96-well microtiter plates (Costar, Cambridge, MA). Wells were then washed three times with PBS and quenched with PBS containing 1% bovine serum albumin (BSA) for 30 min at 23°C. After washing three times with PBS, a 100- μl aliquot of clone-conditioned culture medium diluted 1:10, 1:100, or 1:1,000 in PBS with BSA was added for 2 h at 23°C. Some wells incubated with known quantities of purified polyclonal anti-4.1 antibody served as positive controls. The wells were again washed three times with PBS, and then in-

cubated with anti-mouse IgG- or anti-mouse IgM-alkaline phosphatase conjugate at 1:2,000 in PBS with BSA for 4 h at 23°C; control wells received anti-rabbit IgG-alkaline phosphatase conjugate. After further washing, 100 μ l of 0.2 M NaCO₃, pH 9.5, containing 1 mg/ml *p*-nitrophenolphosphate was added and the plates were incubated at 37°C for 30 min. Color development was stopped by addition of 10 μ l of 2 N NaOH. Strongly positive clones were further screened using immunoblot analysis. Clones producing anti-4.1 antibody were recloned, subjected to a second round of screening, then expanded in tissue culture and propagated in Pristane-primed (BALB/c \times A/J)F₁ mice. Ascites was collected and centrifuged at 2,000 g for 10 min. Supernatants were stored at -20°C until use. IgG monoclonal antibodies were purified from pooled ascites by protein A-Sepharose chromatography according to Ey et al. (24). Protein purity was assessed by two-dimensional SDS PAGE (43).

Immunoprecipitation of 4.1-like Proteins

Immunoprecipitates were prepared by a modification of the method of Werth and Pastan (55). Crude lens membrane fractions prepared from superficial fiber cells were mixed vigorously with ice-cold 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% Na deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride pH 7.2 (buffer B), at a ratio of 1.0 ml/1 g membrane pellet. A supernatant was obtained by centrifugation at 48,000 g for 10 min and incubated with 0.02 vol of a 10% suspension of fixed heat-killed *Staphylococcus aureus* cells (Pansorbin) at 0°C for 60 min. The cells

were removed by centrifugation at 48,000 g for 5 min, and purified polyclonal or monoclonal anti-4.1 antibody that had been pre-adsorbed to Pansorbin was added to a concentration of 20 μ g/ml. After 2 h at 0°C, the cells were pelleted at 3,000 g for 7 min and washed with ice-cold buffer B, buffer B containing 2.5 M KCl, and once more with buffer B. Adsorbed proteins were then solubilized in 10 mM Tris, 3% SDS, 5% β -mercaptoethanol, 10% glycerol, pH 6.8, by boiling for 3 min or by incubation at 43°C for 60 min when used in overlay experiments.

To immunoprecipitate bovine RBC protein 4.1, white membranes were prepared as described by Tyler et al. (53). The membranes were then mixed with 1 vol of ice-cold buffer B, and 4.1a and 4.1b were immunoprecipitated from the resultant extract using the same method employed to precipitate the lens 4.1-like proteins.

Peptide Mapping

Immunoprecipitated proteins were solubilized and subjected to SDS PAGE on 7.5% (wt/vol) gels that were then stained with Coomassie Blue R. Protein bands of interest were excised and transferred to 50-ml screw-top glass tubes that were filled with 25% isopropanol and placed on a rocker. The gel slices were washed with six changes of 25% isopropanol over 24 h, and then with six changes of 10% methanol over 24 h. The gel slices were then dried with a heat lamp. The proteins within the dried gels were iodinated with chloramine T as described by Elder et al. (23) or ¹²⁵I-Bolton-Hunter reagent (New England Nuclear, Boston, MA) as follows. Dried gel slices

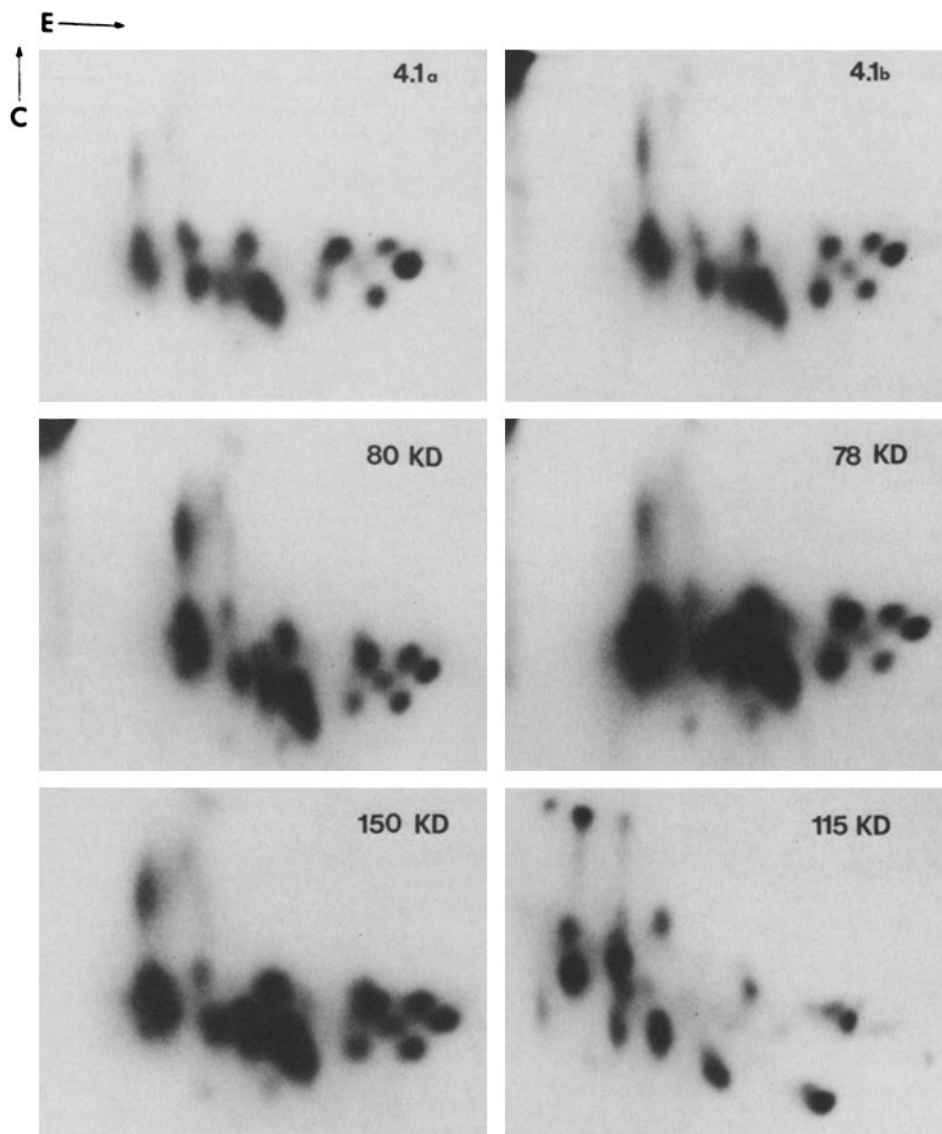


Figure 3. Comparison of ¹²⁵I-tyrosine two-dimensional peptide maps prepared from RBC 4.1a and 4.1b, the lens 4.1-like proteins of 150, 80, and 78 kD, and an unrelated lens protein of 115 kD. Proteins were labeled with ¹²⁵I using chloramine T, cleaved with trypsin, and subjected to high voltage electrophoresis (E) and rising phase chromatography (C) after spotting on thin layer chromatography plates coated with cellulose. Autoradiograms were prepared at -70°C; exposure time was 12 h.

were transferred to conical vials containing 250 μ Ci of dried Bolton-Hunter reagent (New England Nuclear). 20 μ l of ice-cold 125 mM Na borate buffer, pH 8.2 was added and the gel slices were allowed to swell for 2 h on ice. The reaction was stopped by adding 10 μ l of 1 M glycine, pH 8.2, and continuing the incubation for 30 min at 0°C. Both chloramine T and Bolton-Hunter treated gel slices were then washed against at least 10 changes of 10% methanol over 48 h as described above, redried, and hydrated with 0.5 ml of 50 mM NH_4HCO_3 , pH 8.0, containing 20 μ g of diphenylcarbamoyl chloride-trypsin (Sigma, type XI) or 20 μ g of α -chymotrypsin (Worthington Biochemical Corp., Freehold, NJ). After incubation at 37°C overnight, 20 μ g more protease was added and the digestion was allowed to continue for an additional 8 h. The supernatants were then removed and lyophilized twice. The labeled peptides were then dissolved in 15% formate-5% acetate, and $\sim 10^6$ cpm was spotted onto cellulose-coated thin layer chromatography plates (Eastman Kodak Co., Rochester, NY). High voltage electrophoresis and ascending phase chromatography were then carried out as described (23).

Electrophoresis and Western Blotting

SDS PAGE was carried out according to Laemmli (40). Gels were stained with Coomassie Blue R. Proteins were transferred electrophoretically from gels to nitrocellulose filters (Millipore Corp., Bedford, MA, 0.45- μ m pore) for 18 h at 60 V (~ 200 mA) using the buffer of Towbin et al. (52). Blots were quenched and treated with specific antibodies as described (5). The blots were then incubated with second antibody or protein A conjugated to horseradish peroxidase and stained with 4-chloro-1-naphthol as described by Hawkes (36).

Blot Overlays

The overlay procedure of Davis and Bennett (20) was used. Proteins were transferred electrophoretically to nitrocellulose from polyacrylamide gels. Blots were quenched for 15 min with 10 mM NaPO_4 , 150 mM NaCl, 1 mM EDTA, 0.2% (vol/vol) Triton X-100, 4% (wt/vol) BSA at 23°C, and then were incubated with ^{125}I -Bolton-Hunter-labeled lens spectrin, RBC spectrin, or RBC protein 4.1 in the same buffer for 18 h at 4°C with gentle shaking. The blots were then vigorously washed five times (1 min/wash) with the same buffer without BSA and dried, and an autoradiogram was prepared.

Results

Cross-reaction of Anti-4.1 Monoclonal Antibodies with the Lens 4.1-like Proteins

Immunoprecipitates prepared from detergent extracts of crude superficial fiber cell membranes using purified polyclonal anti-4.1 antibody are enriched in 4.1-like proteins of 78, 80, and 150 kD (4). After fractionation of PAGE and transfer to nitrocellulose, two out of two monoclonal antibodies produced against human RBC protein 4.1 were found to cross-react with all three of these lens proteins (Fig. 1); the lens proteins of 80 and 78 kD are not resolved well on this heavily stained blot. Both antibodies belong to the IgG class. One, 3G11.4, binds to protein A with high affinity and dissociates from 4.1-like proteins under relatively mild conditions (43°C, 3% SDS, pH 6.8). This antibody proved particularly useful when precipitating 4.1-like proteins for subsequent assessment of spectrin-binding on nitrocellulose filters, since this method requires renaturation of binding domains. Full characterization of these antibodies will be described elsewhere.

Comparison of the Molecular Mass of the Lens 4.1-like Proteins and RBC Protein 4.1

SDS PAGE was used to compare molecular mass. Coomassie Blue staining revealed that protein 4.1a and the 80-kD lens 4.1-like protein co-migrate (Fig. 2 A). It was difficult to

evaluate the relative mobility of protein 4.1b and the 78-kD lens protein by Coomassie Blue staining because these proteins are not well-resolved from bovine RBC band 4.2. Immunoblot analysis, however, showed that protein 4.1b and the 78-kD lens protein also have identical apparent molecular masses (Fig. 2 B).

Structural Comparison of Protein 4.1 and the Lens 4.1-like Proteins

Peptides containing tyrosine were compared following a method of Elder et al. (23) that uses chloramine T to promote ^{125}I -labeling of proteins in gel slices. Tryptic ^{125}I -peptide maps prepared from the bovine lens 4.1-like proteins of 150, 80, and 78 kD were identical to one another and showed strong homology to maps prepared from bovine RBC protein 4.1a and protein 4.1b (Fig. 3). A control map produced from a 115-kD protein found in lens beaded filaments (37) contained no homologous spots. Chymotryptic maps produced from the lens 4.1-like proteins and RBC 4.1a and 4.1b after chloramine T-catalyzed ^{125}I -labeling were also highly homologous (not shown).

To further evaluate structural relationships, proteins of interest were labeled at lysyl residues with ^{125}I -Bolton-Hunter reagent and cleaved with chymotrypsin. Again, peptide mapping demonstrated a high degree of homology between the lens 4.1-like proteins of 150 and 80 kD and RBC protein 4.1a (Fig. 4). In three out of three experiments, maps prepared from the 150-kD protein contained one prominent peptide

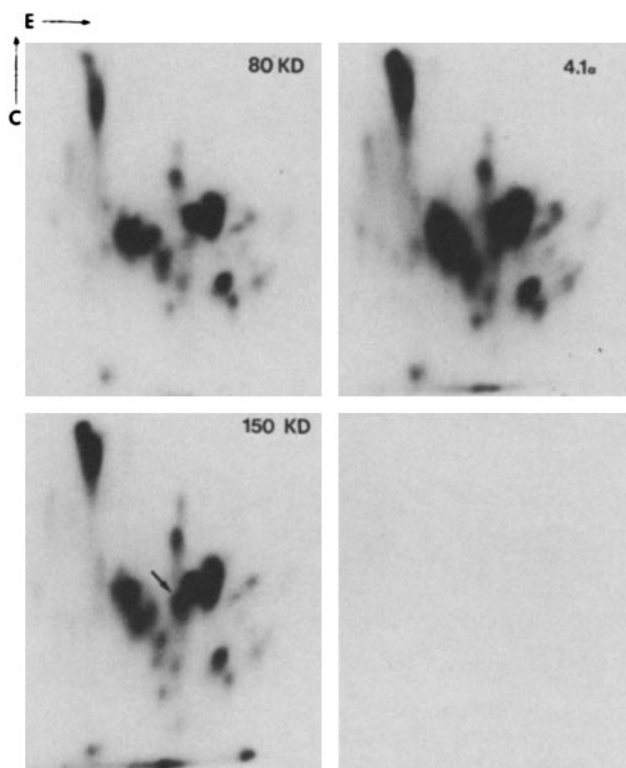


Figure 4. Comparison of ^{125}I -lysine two-dimensional peptide maps of RBC 4.1a and the lens 4.1-like proteins of 150 and 80 kD. Proteins were labeled with ^{125}I -Bolton-Hunter reagent and cleaved with chymotrypsin. Peptides were resolved on thin layer chromatography plates coated with cellulose by electrophoresis (E) in the first dimension and chromatography (C) in the second. Autoradiograms were then prepared at -70°C with an exposure time of 16 h.

¹²⁵I-RBC SPECTRIN OVERLAY

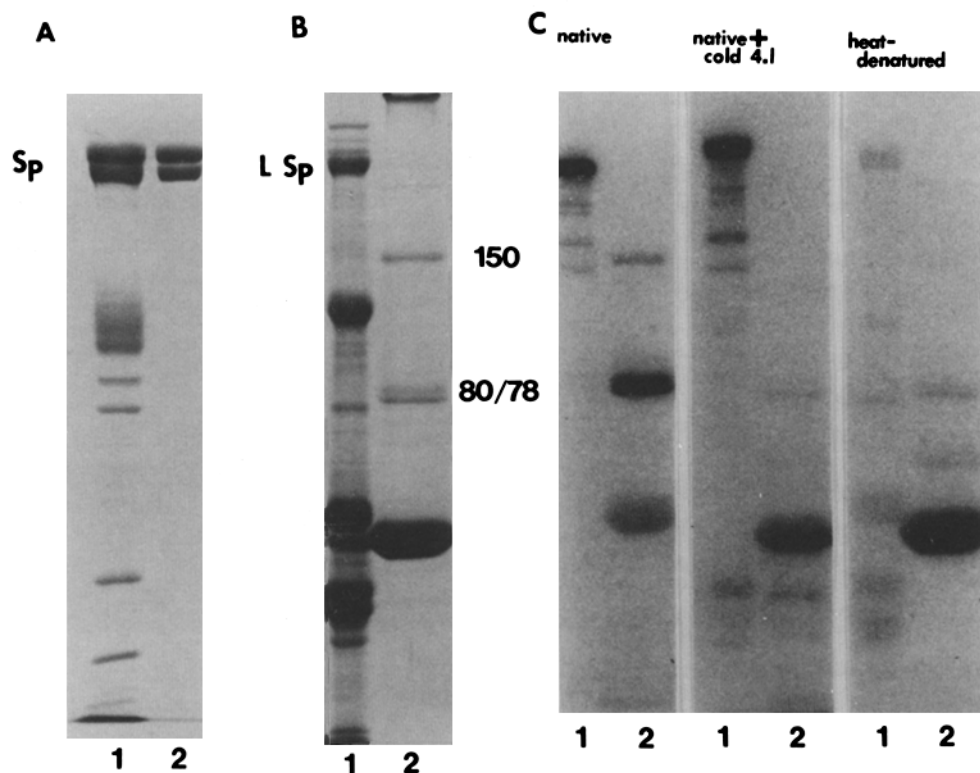


Figure 5. The 4.1-like proteins of the lens bind human RBC ¹²⁵I-spectrin. (A) Shows that the spectrin used in binding experiments was electrophoretically pure (lane 2); RBC ghosts were electrophoresed alongside as a reference (lane 1). (B) The Coomassie Blue staining pattern of bovine lens membranes (lane 1) and immunoprecipitates prepared from lens extracts with anti-4.1 monoclonal antibody 3G11.4 (lane 2). In binding experiments (C), these proteins were transferred to nitrocellulose and overlaid with a buffer containing 1.2 nM ¹²⁵I-spectrin, 1.2 nM ¹²⁵I-spectrin plus 25 nM cold RBC protein 4.1, or 1.2 nM ¹²⁵I-spectrin that had been heat-denatured. The overlays were processed and dried as described under Materials and Methods, and an autoradiogram prepared. Electrophoresis was performed in 7.5% polyacrylamide gels.

missing from maps prepared from the 80-kD lens 4.1-like protein or RBC 4.1a. Additional experiments (not shown) demonstrated that all three of the lens 4.1-like proteins also yield highly similar CNBr fragment patterns (35) on one-dimensional SDS polyacrylamide gels stained with silver (46, 50).

The Lens 4.1-like Proteins Bind Spectrin

Spectrin binding was assessed using the nitrocellulose overlay method of Davis and Bennett (20). Crude lens membrane fractions were found to contain a major ¹²⁵I-RBC spectrin-binding protein of 240 kD (Fig. 5) that probably corresponds to lens spectrin; self-association of spectrin on nitrocellulose filters has been reported previously (20). A series of minor binding proteins that may be proteolytic fragments of spectrin, and/or ankyrinlike proteins (21) were also observed. Immunoprecipitates prepared from crude lens membrane fractions contained major spectrin-binding proteins of 150, 80, and 78 kD. Co-migration of these proteins with the lens 4.1-like proteins was confirmed by subsequent staining of the same filter with anti-4.1 antibody (not shown). Binding appeared to be specific since it was inhibited by heat-denaturation of spectrin. Addition of a 20-fold molar excess of cold purified RBC protein 4.1 also produced marked inhibition of ¹²⁵I-spectrin-binding, indicating that the lens 4.1-like proteins probably bind to spectrin at the previously characterized site of RBC protein 4.1 interaction with spectrin (53, 54). Some binding of ¹²⁵I-RBC spectrin to IgG heavy chain was also observed; this was judged to be nonspecific, since it was unaffected by heat-denaturation of spectrin or addition of cold protein 4.1.

Similar experiments were also conducted with ¹²⁵I-labeled lens spectrin (Fig. 6). Binding to the lens 4.1-like proteins of 150, 80, and 78 kD was again observed. This binding appeared to be specific, since it was abolished by heat-denaturation of the ¹²⁵I-labeled spectrin.

We also screened crude lens membrane fractions for 4.1-binding proteins using the overlay procedure. The only major 4.1-binding proteins detected in crude bovine and avian lens membrane fractions resolved on 5% (Fig. 7) or 10% polyacrylamide gels (not shown) corresponded to the molecular mass of spectrin. Controls included incubation with heat denatured ¹²⁵I-protein 4.1 and addition of 20-fold molar excess of cold protein 4.1.

Discussion

Current studies suggest that proteins serologically related to mammalian RBC protein 4.1 fall into two distinct classes. The first class is exemplified by the complex family of 4.1-like proteins found in the avian RBC (32). Individual members of this family are easily resolved by discontinuous SDS PAGE, but peptide mapping reveals a high degree of structural homology between all proteins belonging to this family and mammalian RBC protein 4.1. Indirect evidence suggests that these proteins are all products of the same gene.

A second class of 4.1-like protein has been described by Baines and Bennett (6), who detected a cross-reactive protein in bovine brain with limited structural homology to protein 4.1 as judged by comparison of peptide maps. This protein appears to be identical to synapsin I, a previously

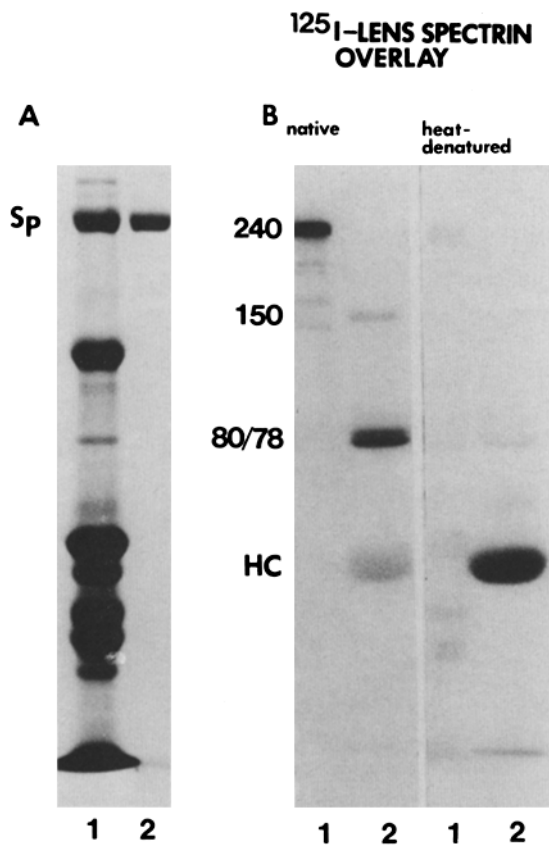


Figure 6. The 4.1-like proteins of the lens bind ^{125}I -labeled lens spectrin. (A) Lens spectrin (lane 2) was purified from crude bovine lens membranes (lane 1) as described under Materials and Methods. (B) The Coomassie Blue staining pattern of the lens membranes (lane 1) and lens proteins immunoprecipitated with anti-4.1 monoclonal antibody 3G11.4 (lane 2). Membrane proteins and proteins present in immunoprecipitates were electrophoresed in parallel lanes on the same 7.5% polyacrylamide gel, transferred to nitrocellulose, and overlaid with buffers containing native 2 nM ^{125}I -spectrin or heat-denatured 2 nM ^{125}I -spectrin. Binding was assessed by autoradiography.

characterized neuron-specific protein (22), and is clearly the product of a gene distinct from that coding for protein 4.1.

Our work shows that bovine lens contains proteins of 80 and 78 kD that are closely related or identical to RBC protein 4.1a and protein 4.1b based on (a) cross-reaction with polyclonal and monoclonal anti-4.1 antibodies, (b) identical electrophoretic mobility, (c) homologous peptide maps, (d) ability to bind to erythroid and non-erythroid spectrin, (e) ionic-strength dependency of retention on membranes (3), and (f) phosphorylation by endogenous protein kinases (4). Like RBC protein 4.1, these proteins appear to be associated with plasma membrane based on co-sedimentation with membrane fractions and immunofluorescent localization (unpublished results). Analogously, Granger and Lazarides (32) have previously localized the 4.1-like proteins of avian lens to fiber cell membranes using immunofluorescence. It thus seems reasonable to henceforth refer to these proteins as bovine lens protein 4.1a and protein 4.1b.

The bovine lens also contains a spectrin-binding 4.1-like protein with an apparent molecular mass of 150 kD (3, 5, 32).

Given the large difference in electrophoretic mobility, this protein shows a surprising degree of structural homology to protein 4.1, and thus appears to be analogous to the 4.1 isomorphs first described in avian red blood cells (32). The altered electrophoretic mobility of this protein may stem from an unusual posttranslational modification producing a large change in conformation or net charge, or from a difference in amino acid sequence, such as insertion or addition of a repeat sequence, that substantially increases molecular mass yet results in only subtle differences in peptide map patterns.

A 4.1-like protein of 150 kD has also been detected in mammalian RBCs (3, 32), but the ratio of this protein to protein 4.1 is much lower in the RBC than in the lens. This indicates that the distribution of mammalian 4.1 isomorphs varies in a tissue-specific fashion. A similar finding has also been made in avians by Granger and Lazarides (32), who showed that chicken RBCs, lenticular cells, and leukocytes contain tissue-specific sets of 4.1 isomorphs. As noted in reference 32, 4.1 isomorphs may subsume cell type-specific functions. With respect to bovine lens, this idea requires that the 150-kD isomorph differ from protein 4.1 in its molecular interactions. Previously noted differences in phosphorylation (4) and extractability (3) support this idea, but direct testing awaits purification of the 150-kD isomorph.

The close structural relationship of the lens 4.1s and RBC protein 4.1 implies similarity in functional capacity. Thus, lens 4.1s likely act *in vivo* as spectrin-binding proteins, but may additionally interact, like RBC protein 4.1 (1, 44), with specific intrinsic membrane proteins. Failure to detect such interactions in nitrocellulose filter overlay assays is not surprising, since RBC protein 4.1 also fails to associate with known 4.1-binding intrinsic membrane proteins when this assay is used (unpublished results). Interaction with proteins other than spectrin appears likely since lens 4.1a and 4.1b are retained in crude lens membrane fractions after extraction of spectrin (3). It will now be possible to identify the presence of 4.1-binding proteins in purified lens membranes using RBC protein 4.1 as a probe.

Speculation into the importance of 4.1s in lens function must take into account observations showing that these proteins initially accumulate during fiber cell differentiation (33), but then disappear, along with spectrin and many other structural proteins, during fiber cell maturation and aging (3). Since protein 4.1 appears important in maintenance of RBC membrane stability, one possible function for lens 4.1s might be to assist transiently in the stabilization of newly synthesized fiber cell membranes before the elaboration of cell-cell interdigitations (42) and synthesis of gap junctions (7). A second possibility is that 4.1s might play a more dynamic role in the process of fiber cell elongation, perhaps by linking cytoskeletal elements to specific intrinsic membrane proteins either directly or through spectrin. Phosphorylation could serve as a means of regulating these interactions, as could changes in the metabolic state of the cell through alterations in the membrane polyphosphoinositide pool (2). Further studies designed to look at specific protein-protein interactions, as well as detection and investigation of lens 4.1 mutants, will help to elucidate the true importance of these proteins.

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¹²⁵I-4.1 OVERLAY

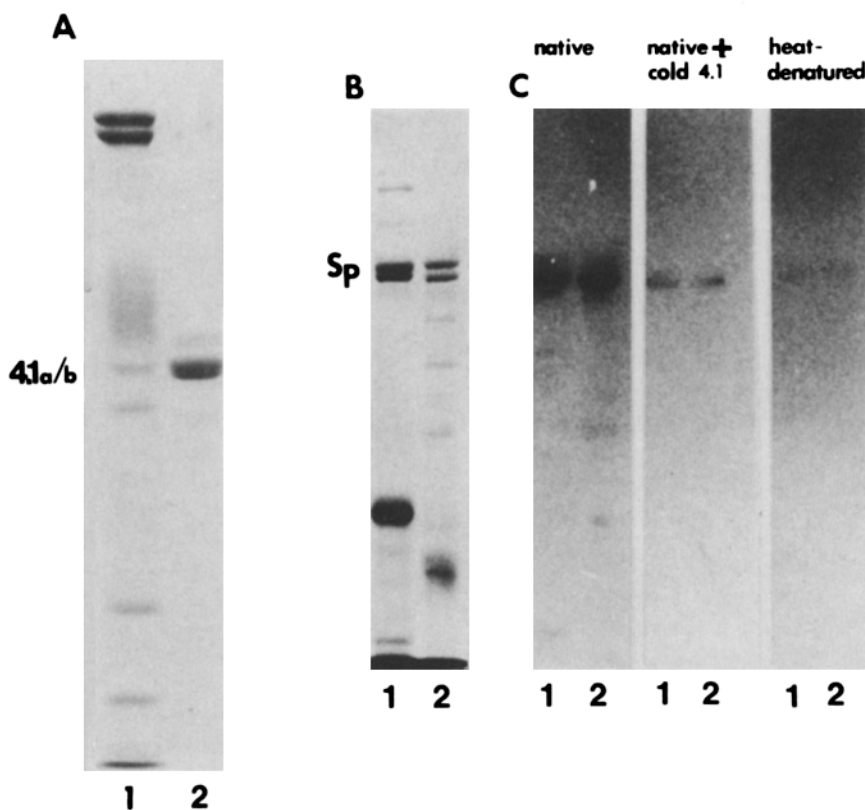


Figure 7. Detection of bovine and chicken lens 4.1-binding proteins using nitrocellulose filter overlays. (A) Human RBC protein 4.1 (lane 2) was purified from RBC membranes (lane 1) and iodinated with ¹²⁵I-Bolton-Hunter reagent. (B) The Coomassie Blue-staining pattern of bovine (lane 1) and avian lens membranes (lane 2) after electrophoresis on 5% polyacrylamide gels. To study binding to protein 4.1 (C), these proteins were transferred to nitrocellulose and incubated with buffers containing native ¹²⁵I-protein 4.1 (1.5 nM), native ¹²⁵I-protein 4.1 (1.5 nM) plus cold protein 4.1 (30 nM), or heat-denatured ¹²⁵I-protein 4.1. After washing and drying, an autoradiogram was prepared.

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