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Authors
Bourguignon, LY
Walker, G
Suchard, SJ
et al.

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A Lymphoma Plasma Membrane–associated Protein with Ankyrin-like Properties

Lilly Y. W. Bourguignon, Gary Walker, Suzanne J. Suchard, and K. Balazovich

Department of Anatomy and Cell Biology, School of Medicine, University of Miami, Miami, Florida 33101. Address reprint requests to Dr. Bourguignon.

Abstract. In this study we have used several complementary techniques to isolate and characterize a 72-kD polypeptide that is tightly associated with a major mouse T-lymphoma membrane glycoprotein, gp 85 (a wheat germ agglutinin–binding protein), in a 16 S complex. These two proteins do not separate in the presence of high salt but can be dissociated by treatment with 2 M urea.

Further analysis indicates that the 72-kD protein has ankyrin-like properties based on the following criteria: (*a*) it cross-reacts with specific antibodies raised against erythrocyte and brain ankyrin; (*b*) it displays a peptide mapping pattern and a pI (between 6.5 and 6.8) similar to that of the 72-kD proteolytic fragment of erythrocyte ankyrin; (*c*) it competes with erythrocyte ghost membranes (spectrin-depleted preparations) for spectrin binding; and (*d*) it binds to purified spectrin and fodrin molecules. Most importantly, in intact lymphoma cells this ankyrin-like protein is localized directly underneath the plasma membrane and is found to be preferentially accumulated beneath receptor cap structures as well as associated with a membrane–cytoskeleton complex preparation.

It is proposed that the ankyrin-like 72-kD protein may play an important role in linking certain surface glycoprotein(s) to fodrin which, in turn, binds to actin filaments required for lymphocyte cap formation.

In all eukaryotic cells that have been studied, there appears to be an association between the surface membrane and the underlying cytoskeletal network (7, 21). Currently, the most well-defined membrane–cytoskeleton organization is that which exists in erythrocytes. In these cells, the linkage between spectrin (a cytoplasmic actin-binding protein) and band 3 membrane protein (an anion transport channel) is mediated by the membrane attachment protein, ankyrin (1, 40). Restricted proteolytic digestion of isolated ankyrin has been used to identify at least two functional domains of the molecule. The 72-kD chymotryptic or 65/55-kD tryptic fragments bind spectrin whereas the 82/83-kD chymotryptic and tryptic fragments bind to the cytoplasmic domain of band 3 (1, 39, 40).

Since receptor capping was first described in lymphocytes (33), a great deal of research effort has been directed toward determining the molecular mechanisms responsible for this very interesting and important phenomenon (10). It is now generally agreed that the cytoskeleton is involved, either directly or indirectly, in the lateral redistribution of surface molecules into a cap structure (10). Proteins analogous to those found in the erythrocyte membrane–cytoskeleton complex, such as spectrin (13, 26, 28), ankyrin (4, 7), and band 3 (18), have all been found in non-erythroid cells including lymphocytes. In addition, all three of the proteins have been shown to co-cap with surface receptor cap structures (7, 13, 18, 26, 28).

In this paper, we report the isolation of an ankyrin-like, 72-kD protein that is tightly associated with a wheat germ agglutinin (WGA)–binding[1] protein, gp 85, in mouse T-lymphoma cells. Biochemical analyses indicate that this protein shares several structural and functional similarities with erythrocyte ankyrin. Cytochemical studies indicate that the ankyrin-like protein is located on the cytoplasmic side of the plasma membrane and co-caps underneath receptor cap structures. It is suggested that this ankyrin-like 72-kD protein may be responsible for the linkage between certain membrane protein(s) and the cytoskeleton which is required for lymphocyte cap formation.

Materials and Methods

Cells

Mouse T-lymphoma cells (BW 5147), provided by R. Hyman (The Salk Institute, San Diego, CA) were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum (GIBCO, Grand Island, NY) and equilibrated in 5% CO_2/95% air.

Induction of Capping

Cells were harvested at a density of ~1 × 10^6 cells/ml and washed with serum-

1. *Abbreviations used in this paper:* GlcNAc, *N*-acetyl-glucosamine; PMSF, phenylmethylsulfonyl fluoride; TEAPL, 50 mM Tris-HCl (pH 7.4), 5 mM EGTA, 1 μg/ml aprotinin, 10^{-3} M PMSF, 1 μg/ml leupeptin; WGA, wheat germ agglutinin.

free RPMI 1640 medium (GIBCO). To induce cap formation, cells were incubated with either lectins (e.g., WGA and concanavalin A) or the monoclonal rat antibodies to the lymphocyte surface glycoproteins, Thy-1, T-200, and viral gp 69/71 (gifts kindly provided by Dr. Ian Trowbridge, The Salk Institute, San Diego, CA) followed by the addition of a secondary antibody (e.g., rabbit anti-rat or goat anti-rat immunoglobulin) at room temperature or 37°C for 20 min.

Radioactive Labeling of Cellular Proteins

Surface proteins and isolated cellular proteins were labeled with ^{125}I using the iodogen method of Fraker and Speck (20).

Plasma Membrane Isolation

Crude plasma membranes from capped or uncapped cells were isolated using the method described by Johnson and Bourne (23) with slight modifications. Cells labeled with ^{125}I by the iodogen method as described above were harvested by low speed centrifugation (500 g_{av}), washed with phosphate-buffered Earle's salt solution, pH 7.3, and resuspended in a solution of 20 mM Hepes (pH 7.3), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM mercaptoethanol at 0°C. These cells were lysed using a Dounce homogenizer. The lysate was centrifuged at 750 g_{av} for 5 min and the resulting supernatant centrifuged at 43,000 g_{av} for 20 min as described previously (13). The resulting membrane pellet was then used for all experiments.

Non-ionic Detergent Extraction

Crude plasma membranes from either capped or uncapped cells were washed in TEAPL buffer (50 mM Tris-HCl [pH 7.4], 5 mM EGTA, 1 μg/ml aprotinin, 10^{-3} M PMSF, 1 μg/ml leupeptin). Detergent extraction was accomplished by adding either Triton X-100 or Nonidet P-40 to the TEAPL buffer to a final concentration of 1% (vol/vol). Protein concentration in all samples was kept at ~0.5 mg/ml. The samples were incubated at 0°C for 20 min with frequent vortexing and then centrifuged at 100,000 g_{av} for 45 min. After centrifugation, the supernatant (i.e., detergent-soluble fraction) and the pellet (i.e., detergent-insoluble fraction) were collected for further biochemical analysis as described previously (12, 13). Previous studies have shown that the detergent-insoluble fraction contains the membrane-associated cytoskeleton (12, 13).

SDS PAGE and Autoradiographic Analysis

One-dimensional PAGE. Electrophoresis was conducted using an exponential polyacrylamide gradient (6.0–17.0%) slab gel and the discontinuous buffer system described by Laemmli (25).

Two-dimensional PAGE. First-dimension isoelectric focusing gels contained 2% LKB ampholytes (pH 3.5–10). Second-dimension gels were 10% acrylamide as described previously (29).

Polypeptide banding patterns were revealed by either Coomassie Blue or silver staining procedures, and quantitated by scanning densitometry. Samples labeled with ^{125}I were fluorographed (5), vacuum dried, and exposed to Kodak x-ray (X-Omat, Xar-5) film at −70°C.

WGA–Sepharose Column Chromatography

A WGA–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) column (6.0 × 0.5 cm) was equilibrated with 5 mM EGTA, 50 mM Tris-HCl (pH 7.4) containing 1% Triton X-100. Crude plasma membrane isolated from ^{125}I-labeled cells was washed in TEAPL buffer (50 mM Tris-HCl [pH 7.4], 5 mM EGTA, 1 μg/ml aprotinin, 10^{-3} M PMSF, 1 μg/ml leupeptin) and then solubilized in 1% Triton X-100 for 30 min at 4°C. The protein concentration in all samples was kept at ~2.5 mg/ml. After extraction, the samples were centrifuged at 10,000 g_{av} for 10 min, and the resulting supernatant was then passed over the WGA column at least 10 times to allow for sufficient binding. The column was then washed with same TEAPL buffer containing 1% Triton X-100 until no further radioactivity was removed. The TEAPL buffer containing 0.5 M *N*-acetyl-glucosamine (GlcNAc) was then used to elute the column. Fractions containing specifically eluted radioactive proteins were pooled and analyzed by sucrose gradient centrifugation as described below.

Isolation of 72-kD Protein and gp 85 Complex

^{125}I-labeled plasma membrane was isolated from intact cells, solubilized by 1% Triton X-100, bound to a WGA column, and eluted by 0.5 M GlcNAc as described above. The GlcNAc eluant was then loaded onto a linear sucrose gradient (7–28%) with a 0.5-ml cushion of 65% sucrose and centrifuged at 70,000 g_{av} for 22 h. Thirty-four 0.5-ml fractions were collected from the bottom of each tube. Fractions were counted on a LKB miniGamma counter to determine the distribution of surface-iodinated protein within the gradient. ^{125}I-labeled protein peaks were pooled, precipitated with 10% trichloroacetic acid, and subsequently analyzed by SDS PAGE and autoradiography. The ^{125}I-labeled peak fractions corresponded to a sedimentation value of 8 S and 16 S as calculated using protein standards of tetrameric fodrin, tetrameric spectrin (11 S), and G-actin (3.7 S) as described previously (13, 22, 30).

Effect of High Salt on the 16 S Complex

The ^{125}I-labeled 16 S material, isolated as described above, was incubated with 0.6 M NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EGTA, 1 μg/ml aprotinin, 10^{-3} M PMSF, 1 μg/ml leupeptin containing 1% Triton X-100 overnight at 4°C, rebound to a WGA column, eluted with 0.5 M GlcNAc, and then centrifuged on a linear sucrose gradient (7–28%) for 22 h at 70,000 g_{av}. The 16 S control material, incubated in the same buffer in the absence of 0.6 M NaCl, was run on a parallel 7–28% sucrose gradient. Parallel gradients containing ^{125}I-labeled G-actin and spectrin were used to determine relative sedimentation coefficients.

Isolation of the 72-kD Protein and gp 85 Protein

Both high salt–treated material and control 16 S material containing ^{125}I-labeled protein peaks were then re-bound to a WGA column and washed with 50 mM Tris-HCl (pH 8.0), 5 mM EGTA, 1 μg/ml aprotinin, 10^{-3} M PMSF, 1 μg/ml leupeptin in the presence or absence of 0.6 M NaCl, respectively. Proteins that remained tightly bound to the WGA column were then sequentially eluted with 2 M urea to obtain pure 72-kD protein followed by 0.5 M GlcNAc to obtain gp 85. Both 72-kD protein and gp 85 protein were then analyzed by either one- or two-dimensional SDS PAGE followed by autoradiography.

One-dimensional Peptide Maps

Both lymphoma 72-kD protein and the 72-kD proteolytic fragment of erythrocyte ankyrin (prepared according to a previously described method [1]) were labeled with ^{125}I using the iodogen method of Franker and Speck (20). Peptide mapping by a limited proteolytic digestion with *Staphylococcus aureus* V8 protease (0.05 μg/gel well) of these two ^{125}I-labeled proteins in a 15% SDS PAGE slab gel was performed according to the procedure described previously by Cleveland et al. (17). Gels were subsequently fluorographed, vacuum dried, and exposed to Kodak x-ray (X-Omat, Xar-5) film at −70°C.

^{125}I-WGA Western Blotting and Immunoblotting Techniques

The isolated plasma membranes, 8 S and 16 S complexes, were electrophoresed on an exponential polyacrylamide gel gradient (7.0–17.0%) containing SDS as described above. The polypeptides were transferred to nitrocellulose sheets as described by Burnette (14) for WGA binding and Bennett and Davis (4) for anti-ankyrin binding. Subsequently, these sheets were incubated with either 10 μg/ml ^{125}I-WGA or 1 μg/ml anti-ankyrin antibodies (e.g., anti-erythrocyte or anti-brain ankyrin) followed by incubation with ^{125}I–protein A. The radioactivity was analyzed by previously published autoradiographic procedures (5).

Immunoprecipitation Procedures

Isolated lymphoma plasma membranes or Nonidet P-40–insoluble cytoskeleton fractions (13) or lymphoma 72-kD protein were solubilized in 10 mM Tris-HCl (pH 8.0), 0.6 M NaCl, 0.1 mM dithiothreitol containing 0.5–1.0% Triton X-100 for immunoprecipitation. The solubilized membranes, cytoskeleton, and 72-kD protein were then iodinated (20), divided into aliquots, and incubated at 4°C for 30 min with 10 μg/ml of specific anti-ankyrin antibodies (i.e., anti-erythrocyte or anti-brain ankyrin antibodies) or preimmune serum (as a control). After the 30-min incubation, goat anti-rabbit immunoglobulin (100 μg/ml) was added to the anti-ankyrin or preimmune serum–treated samples and incubated overnight at 4°C to induce immunoprecipitation (12, 13). The resulting immunoprecipitates were pelleted by centrifugation at 700 g_{av} for 4 min and washed three times with 0.1% Triton X-100 in 10 mM Tris-HCl (pH 8.0), 0.6 M NaCl, 0.1 mM dithiothreitol using the same centrifugation conditions. The immunoprecipitates were subsequently solubilized by SDS, and analyzed by SDS PAGE and autoradiography.

Double-label Immunofluorescence Microscopy

Cells were washed with RPMI 1640 medium (GIBCO) and resuspended in the same medium. Fluorescein-labeled WGA (50 μg/ml) was added directly to cell suspensions at room temperature for 15 min to induce patch and cap formation. Fluorescein-labeled WGA–labeled cells were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 0°C for 30 min, rendered permeable by methanol treatment (15), and then stained with rabbit anti-ankyrin (e.g., anti-erythrocyte or anti-brain ankyrin antibodies). These samples were then incu-

bated with rhodamine-conjugated goat antibody against rabbit IgG to visualize ankyrin distribution. To detect any nonspecific antibody binding, cells were first incubated with antisera preabsorbed with soluble, native ankyrin to remove anti-ankyrin antibodies. These cells were then incubated with rhodamine-conjugated goat anti-rabbit IgG. No label was observed in such control samples.

The fluorescein- and rhodamine-labeled samples were examined with a Zeiss photomicroscope using a 63 × oil immersion lens and epi-illumination. Fluorescein and rhodamine fluorescence were excited with an Osram HBO 50-W bulb using the filter combination CZ 487710 and CZ 487714, respectively. Cells were photographed with Kodak plus-X film (Eastman Kodak Co., Rochester, NY).

Immunoelectron Microscopy

In this study, the technique developed by Tokuyasu (35, 36) was used with some modifications (6, 8, 9, 11). Cells (1×10^7 cells/ml) were fixed with 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 1 h. Fixed cells were rinsed with phosphate buffer and infused with 0.8 M sucrose at room temperature for at least 30 min. Small volumes (5 μl) of sucrose-treated cells were placed on top of copper stubs and immediately frozen in liquid nitrogen. Ultra-thin frozen sections (~70–100 nm in thickness) were cut with a chilled glass knife on a Sorvall MT-2B ultramicrotome with LTC-2 cryobrowl attachment (DuPont Co., Wilmington, DE) at a temperature of approximately −70°C. Frozen-thin sections were subsequently picked up by a small loop (0.5 mm in diameter) containing 2.3 M sucrose and 1% gelatin solution and brought to room temperature.

The thawed thin sections were placed on carbon-stabilized, formvar-coated copper grids and incubated with rabbit anti-ankyrin (e.g., anti-erythrocyte or anti-brain ankyrin) antibodies (10 μg/ml) or preabsorbed serum (anti-ankyrin-free serum is used as a control), followed by protein A–colloidal gold (17 nm in diameter) staining as described previously (31). Immunogold-labeled samples were further fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature, followed by staining with 2% uranyl acetate (pH 8.0) for 10 min and 0.2% uranyl acetate (pH 4.0) for 2 min (36). Finally, immunogold-labeled, uranyl acetate–stained thin sections were embedded in a thin layer (~70–100 nm thickness) of 1% methyl cellulose and air dried (36). Ultra-thin sections were examined on a Phillips 300 transmission electron microscope operating at 80 kV.

Interaction Between Lymphoma 72-kD Protein and Spectrin or Fodrin

A Spectrin-binding Assay with Spectrin-depleted Inside-out Vesicles. Erythrocyte ghost membranes from freshly drawn blood were prepared according to previously published procedures (9). Spectrin-depleted inverted (inside-out) vesicles were obtained by incubating erthrocyte ghosts in 0.3 mM sodium phosphate (pH 7.5) for 30 min at 37°C, followed by centrifugation at 42,000 g_{av} for 40 min (3). The membrane pellet was then resuspended to a protein concentration of 1 mg/ml in 20 mM KCl, 2 mM sodium phosphate (pH 7.6).

Erythrocyte spectrin was isolated from human erythrocyte ghosts as outlined by Ungewickell and Gratzer (37) with the following modifications. Ghost membranes were dialyzed against 200 vol of 0.3 mM sodium phosphate (pH 7.6) for 22 h at 4°C to remove spectrin from the membranes. After dialysis, the ghosts were pelleted by centrifugation at 80,000 g_{av} for 1 h, and the supernatant applied to a Sepharose 1000 column (90 × 2.5 cm) equilibrated with 25 mM Tris (pH 7.6), 5 mM EDTA, 0.1 M NaCl. The column was eluted with the same buffer and 8-ml fractions were collected. Protein in the fractions was monitored by absorbance at 280 nm and protein composition determined by SDS PAGE analysis on aliquots. The fractions enriched with spectrin were then labeled with ^{125}I by the iodogen method (20). Spectrin was then further purified by centrifugation at 100,000 g_{av} for 22 h on a 5–20% sucrose gradient containing the same buffer. Fractions were monitored by absorbance at 280 nm and purity of spectrin determined by SDS PAGE. The fractions containing pure spectrin were then dialyzed against 100 mM NaCl, 25 mM Tris-HCl (pH 7.6), 5 mM EDTA. The specific activity of the iodinated spectrin was 8.5×10^5 cpm/μg.

Binding studies were performed using the procedure of Bennett (1). Inverted (inside-out) vesicles (120 μg/ml) were incubated with ^{125}I-spectrin in binding buffer (20 mM KCl, 2 mM sodium phosphate [pH 7.5], 0.5 mM $MgCl_2$, 0.4 mM dithiothreitol, and 4% sucrose) for 90 min at 4°C in a final vol of 225 μl. This material was then layered over 250 μl of 20% sucrose in 0.7 mM sodium phosphate (pH 7.6), 20 mM KCl buffer, and centrifuged for 20 min at 20,000 g_{av}. The pellets were counted on a LKB miniGamma counter for radioactivity determination.

Binding studies in the presence of lymphoma 72-kD protein were performed in an identical way as outlined above except that 6 μg/ml of ^{125}I-spectrin was preincubated with varying concentrations (0.5–8.0 μg/ml) of 72-kD protein for 30 min before the addition of spectrin-depleted inside-out erythrocyte membranes. Parallel experiments using erythrocyte 72-kD proteolytic fragment of ankyrin and a nonspecific protein, bovine serum albumin (BSA), were run as internal controls.

Fodrin or Spectrin Binding to Lymphoma Proteins. Lymphoma proteins (e.g., the 72-kD protein, gp 85, and 16 S 72 kD/gp 85 complex) and BSA were each conjugated to CNBr-activated Sepharose beads (Pharmacia Fine Chemicals). During binding assays, ^{125}I-labeled fodrin or spectrin (11,000 cpm) was added to the protein-conjugated Sepharose beads in the presence of binding buffer (20 mM KCl, 2 mM sodium phosphate [pH 7.5], 0.5 mM $MgCl_2$, 0.4 mM dithiothreitol, and 4% sucrose) for 90 min at room temperature. The Sepharose beads were then centrifuged at 200 g_{av} for 2 min at room temperature and washed at least three times with the same buffer. The radioactivity associated with the 72-kD protein, gp 85, 16 S, or BSA-conjugated Sepharose beads was determined by counting the samples on an LKB miniGamma counter. Relative fodrin or spectrin binding activity was calculated by the ratio of relative amount of spectrin bound (cpm) vs. relative amount of protein (μg) conjugated to the Sepharose beads.

Results

Isolation of a Lymphoma Membrane-bound 72-kD Protein and gp 85 Complex

A great deal of effort has been made during the last several years to determine the linkages that may exist between membrane glycoproteins and the cytoskeleton in mouse T-lymphoma cells (6, 7, 10, 12, 13). In this study we have used a WGA column followed by sucrose gradient centrifugation to selectively isolate certain lymphoma glycoproteins and their associated cytoskeletal molecules. Our results indicate that a number of ^{125}I-labeled surface proteins bind to a WGA–Sepharose column and can be specifically eluted by GlcNAc (data not shown).

Sucrose gradient centrifugation analysis of this WGA-binding material reveals two distinct peaks with sedimentation coefficients of 16 S (A peak) and 8 S (B peak), respectively (Fig. 1). SDS PAGE and autoradiographic patterns indicate that the 8 S material contains a large number of surface ^{125}I-labeled polypeptides and WGA-binding proteins (Fig. 1 *B*, lanes *a–c*). An 85-kD protein, designated gp 85, appears to be one of the major surface ^{125}I-labeled and WGA-binding proteins. The 16 S peak consists of only two major polypeptides, gp 85 and 72 kD (Fig. 1 *A*, lane *a*). The gp 85, but not the 72-kD protein, is ^{125}I-labeled (Fig. 1 *A*, lanes *a–c*) and binds WGA (Fig. 1 *A*, lane *c*). Based upon the evidence that (*a*) the 72-kD protein and gp 85 both bind to a WGA–Sepharose column; (*b*) these two proteins can be co-eluted from the WGA–Sepharose column by GlcNAc; and (*c*) both 72-kD protein and gp 85 co-sediment as a single 16 S peak, we believe that the 72-kD protein is tightly associated with gp 85 in a membrane-associated complex. Since high salt treatment has been used routinely to dissociate membrane-bound proteins such as spectrin or fodrin from the erythrocyte band 3–ankyrin complex (2) or lymphoma GP 180 (32), we decided to examine the effect of high salt on the 72-kD/gp 85 complex. Our results indicate that there is no detectable change in the S value of the complex in the presence of high salt (0.6 M NaCl) as compared to the no salt condition (Fig. 2 *A*). This 16 S material (either in the presence or in the absence of high salt) was subsequently passed over a second WGA column and eluted with GlcNAc elution (Fig. 2 *B*). Our results show that both the 72-kD protein and gp 85 remain tightly associated (Fig. 2 *C*, lanes *a* and *b*). Therefore, this two-protein

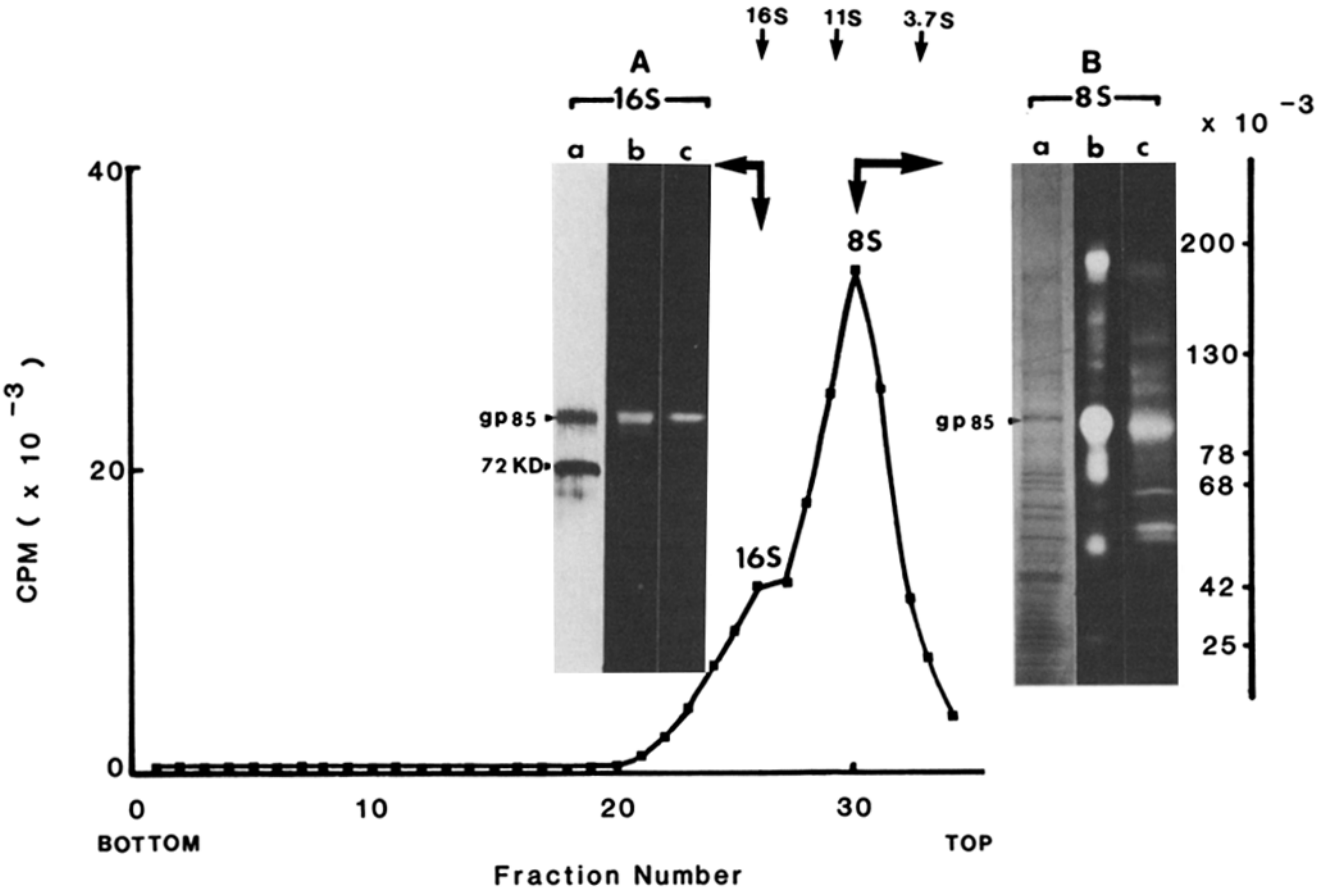

Figure 1. Analysis of ^{125}I-labeled WGA glycoproteins by sucrose gradient centrifugation. ^{125}I-labeled WGA-binding proteins were loaded onto a linear sucrose gradient (7–28%) with a 0.5-ml cushion of 65% sucrose and centrifuged at 70,000 g_{av} for 22 h. Thirty-four 0.5-ml fractions were collected from the bottom of each tube. Fractions were counted on an LKB MiniGamma counter to determine the distribution of surface ^{125}I-labeled protein within the gradient. Protein standards of G-actin (*3.7 S*) and tetrameric spectrin or fodrin (*11 S*) were used to estimate the S value of the ^{125}I-labeled peaks, *16 S* and *8 S*, respectively. SDS PAGE analysis of the pooled ^{125}I-labeled 16 S (*A*) and 8 S (*B*) peak fractions with (lanes *a*) Coomassie Blue or silver staining. Autoradiograms of (lanes *b*) ^{125}I-labeled surface iodination pattern and (lanes *c*) ^{125}I-WGA Western blot pattern. (Both gp85 and the 72-kD protein are the two major components in the 16 S peak.)

complex appears to be resistant to high salt treatment. However, the 72-kD protein can be effectively dissociated from ^{125}I-labeled gp 85 by treatment with 2 M urea (Fig. 2*D*, lanes *a* and *b*).

Identification of 72-kD Protein As an Ankyrin-like Molecule

Using a specific antibody raised against either erythrocyte or brain ankyrin and standard immunoblotting/immunoprecipitation techniques, we have found that lymphoma plasma membrane contains at least two ankyrin cross-reactive polypeptides, 215 kD and 72 kD (but not gp 85) (Fig. 3). The 215-kD protein (Fig. 3*b*) appears to have an identical molecular mass to erythrocyte ankyrin (Fig. 3*a*). The lymphoma membrane-associated 72-kD cross-reactive polypeptide (Fig. 3, *b* and *c*) may be similar to the 72-kD proteolytic fragment of erythrocyte ankyrin.

Using the same anti-ankyrin antibody and colloidal gold-conjugated protein A staining on frozen thin sections, we have determined that these ankyrin-like molecules are localized directly underneath the cytoplasmic side of the plasma membrane (Fig. 4*a*). The immunogold labeling appears to be specific since the control samples treated with preabsorbed (anti-ankyrin-free) serum followed by colloidal gold-conjugated protein A reagent does not show any significant labeling on sections (Fig. 4*b*). Because the 72-kD protein is readily co-isolated with gp 85 and the many possible interactions of the 215-kD protein with other cellular protein(s) are unknown, we decided to focus on the characterization of the 72-kD protein in this study.

Two-dimensional SDS PAGE analysis indicates that lymphoma 72-kD protein can be resolved as two spots at pI 6.5–6.8 (Fig. 5*A*). The 72-kD proteolytic fragment of ankyrin shows a major band at pI 6.5–6.8 and a minor spot at pI 7.2. Although there are some minor differences in the isoelectric focusing patterns (two spots vs. a band and a minor spot) between these two proteins, this characteristic pI 6.5–6.8 of lymphoma 72-kD protein closely resembles that previously described for the 72-kD proteolytic fragment of ankyrin (Fig. 5*B*) (38). In addition, our results from one-dimensional peptide maps show that there are some detectable similarities and differences in mapping patterns between lymphoma 72-kD protein and the 72-kD proteolytic fragment of ankyrin (Fig. 6*B*). Nevertheless, patterns of the major peptide fragments obtained from lymphoma 72-kD protein (Fig. 6*A*) are similar to those obtained from erythrocyte 72-kD ankyrin (Fig. 6*B*).

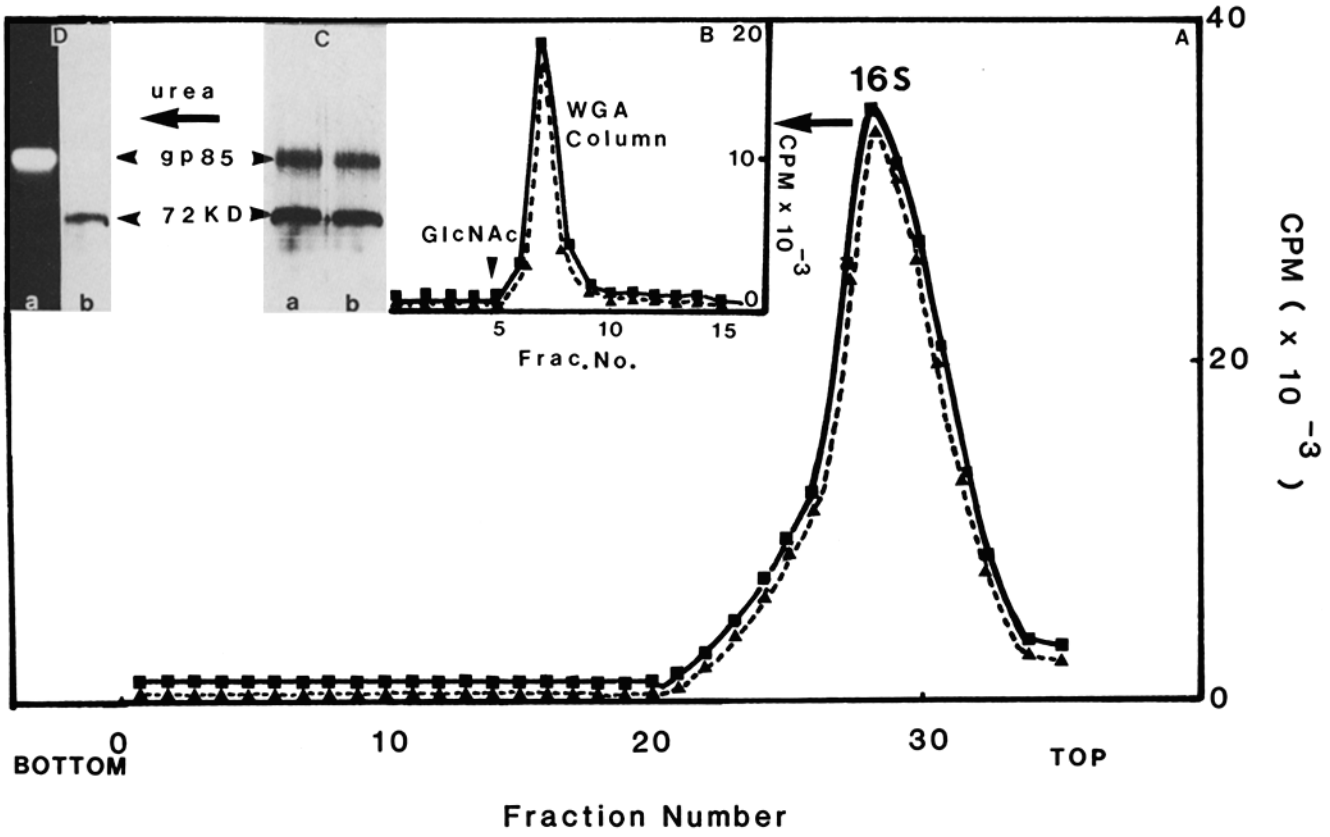

Figure 2. High salt treatment of the ^{125}I-labeled 16 S material. (*A*) Sucrose gradient centrifugation of the ^{125}I-labeled 16 S material (prepared according to the procedures described in Fig. 1) in the presence (▲) and absence (■) of 0.6 M NaCl. (*B*) WGA–Sepharose column chromatography of the ^{125}I-labeled 16 S material (with or without 0.6 M NaCl treatment) which was bound to a WGA–Sepharose column and eluted with 0.5 M GlcNAc. (*C*) SDS PAGE analysis of GlcNAc eluted WGA–Sepharose column-bound proteins (*B*) stained with Coomassie Blue or silver (lane *a*, with 0.6 NaCl treatment; lane *b*, without 0.6 NaCl treatment). (*D*) SDS PAGE and autoradiogram of WGA–Sepharose column-bound proteins eluted first by 2 M urea (lanes *b*, Coomassie Blue or silver staining) and then by GlcNAc (lane *a*, autoradiogram).

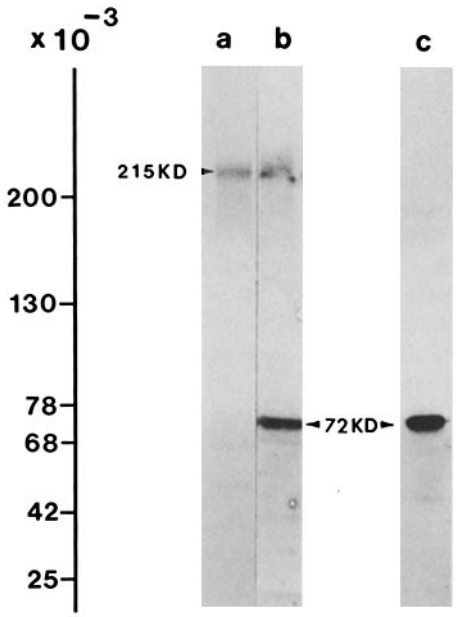

Figure 3. Immunoblotting and immunoprecipitation of lymphoma ankyrin-like molecules. Immunoblot autoradiograms of erythrocyte ghost membranes (*a*) and lymphoma plasma membranes (*b*) incubated with rabbit anti-erythrocyte ankyrin antibody followed by ^{125}I–protein A. Immunoprecipitation autoradiogram of ^{125}I-labeled lymphoma 72-kD protein with rabbit anti-erythrocyte ankyrin followed by goat anti-rabbit immunoglobulin (*c*). Similar results were obtained with anti-brain ankyrin antibody reagents (data not shown).

Since these two proteins are isolated from two different cell types, namely, mouse lymphomas and human erythrocytes, some variations in their two-dimensional gel patterns and peptide maps are expected. Given these data, we consider the 72-kD proteins to be lymphoma-specific forms of ankyrin.

The Involvement of Lymphoma 72-kD Protein Ankyrin-like Protein in Receptor Capping

A double label immunofluorescence study revealed that these ankyrin-like proteins (Fig. 7*A*) are accumulated preferentially beneath receptor cap structures (Fig. 7*B*). Biochemical analyses indicate that the amount of 72-kD protein (Fig. 7, lanes *a* and *c*) associated with a fodrin/actin-containing cytoskeleton complex (insoluble material from a mild Nonidet P-40 extraction) is significantly increased (Fig. 7, *b* and *d*) during ligand (e.g., anti-Thy-1, anti-T-200, anti-GP 69/71, concanavalin A, and polycationized ferritin)-induced capping (Table I). These data suggest that the ankyrin-like 72-kD protein may be interacting with a fodrin/actin complex on the inside surface of mouse lymphoma plasma membranes.

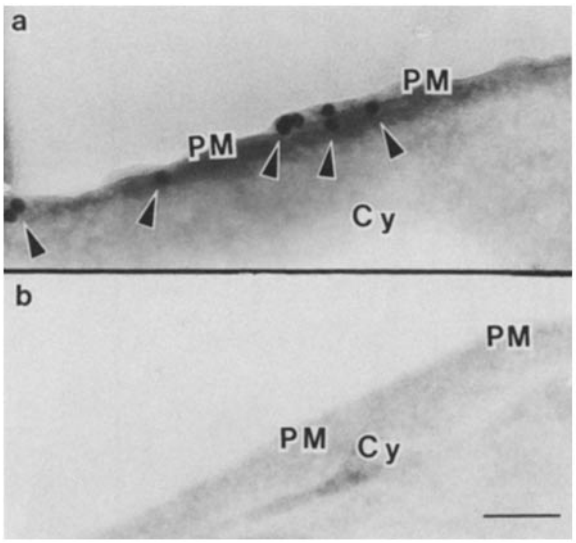

Figure 4. Immunoelectron microscopic localization of ankyrin-like molecules associated with lymphoma plasma membranes. Frozen thin sections of lymphoma cells were labeled with rabbit anti-erythrocyte or brain ankyrin followed by protein A–conjugated colloidal gold complex (*a*). As a control, frozen sections were labeled with preabsorbed serum (anti-ankyrin–free serum) followed by protein A–conjugated colloidal gold complex (*b*). Bar, 0.1 μm.

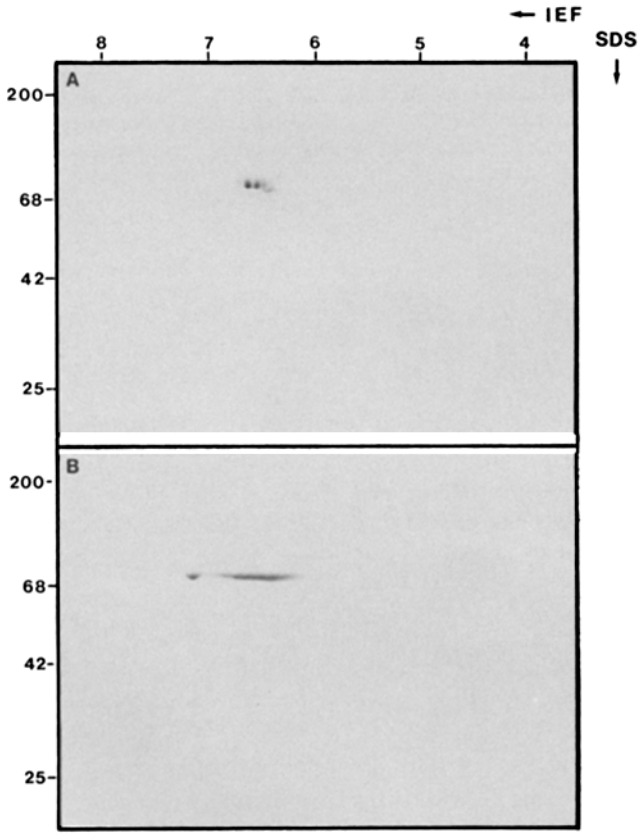

Figure 5. Two-dimensional SDS PAGE analysis of lymphoma 72-kD protein and the erythrocyte 72-kD proteolytic fragment of ankyrin. Coomassie Blue staining of isoelectric focusing (*IEF*) and SDS PAGE (*SDS*) analysis of lymphoma 72-kD protein (*A*) and erythrocyte 72-kD fragments of ankyrin (*B*). First dimension, isoelectric focusing gel with a pH gradient from pH 3.5 to pH 8.5. Second dimension, a 10% polyacrylamide gel that contains SDS.

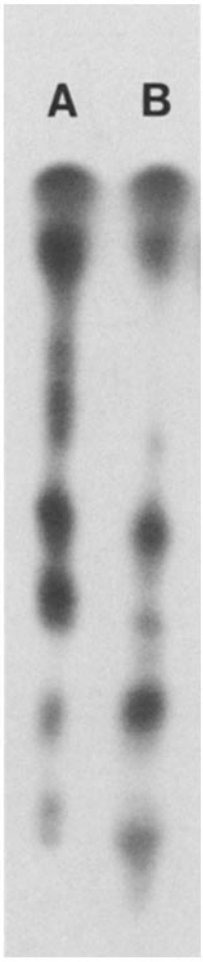

Figure 6. One-dimensional peptide maps of lymphoma 72-kD proteins and the erythrocyte 72-kD proteolytic fragment of ankyrin. Autoradiogram of peptide maps of ^{125}I-labeled lymphoma 72-kD (*A*) and erythrocyte 72-kD proteolytic fragment of ankyrin (*B*) processed through a limited proteolytic digest with *S. aureus* V8 protease (0.05 μg/gel well) in a 15% SDS polyacrylamide slab gel.

Interaction between the Lymphoma 72-kD Protein and Spectrin or Fodrin

To determine whether there is a direct interaction between the lymphoma 72-kD protein and fodrin or spectrin, we have performed the following two different binding experiments. Both the erythrocyte ankyrin and the 72-kD proteolytic fragment of ankyrin containing the spectrin-binding domain (1) have been used as an effective competitor for spectrin-binding to spectrin-depleted, inverted (inside-out) erythrocyte ghosts (3). In this study, we have investigated the ability of the lymphoma 72-kD protein to compete with erythrocyte spectrin-depleted, inside-out vesicles for spectrin binding using a spectrin/inside-out vesicle binding assay.

First, the binding pattern of ^{125}I-labeled spectrin to the spectrin-depleted inside-out vesicle preparation was found to occur in a concentration-dependent manner (Fig. 8*A*). This binding curve is similar to that obtained by Bennett (1) for ^{32}P-labeled erythrocyte spectrin binding to inverted erythrocyte vesicles, indicating that the iodination of spectrin under our experimental conditions does not interfere with its ability to bind to spectrin-depleted inside-out vesicles. The binding of ^{125}I-spectrin to spectrin-depleted inside-out vesicles is inhibited by lymphoma 72-kD protein and the 72-kD fragment of ankyrin (but not BSA) in a concentration-dependent manner (Fig. 8*B*). Under our assay conditions, erythrocyte 72,000-kD fragment of ankyrin (but not BSA) displays a 50% maximal inhibition and lymphoma 72-kD protein exhibits a con-

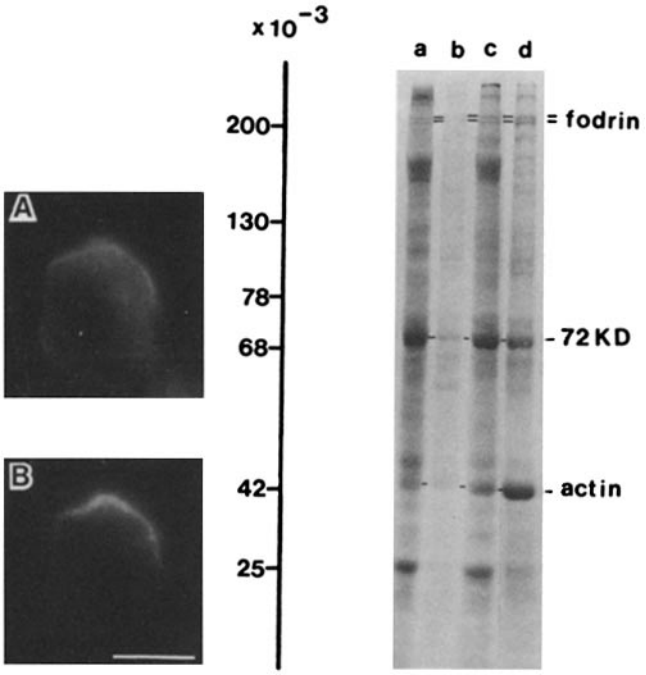

Figure 7. Association of lymphoma 72-kD protein with a membrane–cytoskeleton complex during lymphocyte capping. Double immunofluorescence staining of lymphoma cells for localization of cell surface WGA receptors (*A*) and intracellular ankyrin (*B*). (Similar accumulation patterns of ankyrin were observed underneath anti-Thy-1–, anti-T-200–, and anti-gp 69/71–induced capped structures [data not shown].) Bar, 13 μm. Coomassie Blue staining of (lanes *a* and *c*) total plasma membrane; (lanes *b* and *d*) Nonidet P-40–insoluble fraction of plasma membranes (i.e., cytoskeleton material) from uncapped (lanes *a* and *b*) and capped (lanes *c* and *d*) cells.

Table I. The Relative Accumulation of the 72-kD Protein in Capped and Uncapped Cells

Experiments	Plasma membrane (capped/uncapped)	Nonidet P-40–insoluble cytoskeleton (capped/uncapped)
Uncapped	1.0	1.0
Anti-T-200–induced cap	1.09 ± 0.11	1.60 ± 0.7
Anti-Thy-1–induced cap	0.86 ± 0.17	1.78 ± 0.21
Anti-gp 69/71–induced cap	1.13 ± 0.09	2.16 ± 0.13
WGA- or Con A–induced cap	0.95 ± 0.04	1.82 ± 0.28
Polycationized ferritin–induced cap	1.16 ± 0.12	2.56 ± 0.40

The values were obtained from scanning densitometry (arbitrary units) of Coomassie Blue–stained 72-kD protein in the plasma membrane and Nonidet P-40–insoluble (cytoskeleton) fractions similar to the gel lanes in Fig. 8.

sistent 25% maximal inhibition during the ^{125}I-spectrin binding to spectrin-depleted inside-out vesicles. Since lymphoma 72-kD protein and erythrocyte 72-kD fragment of ankyrin are derived from different cell types, the minor structural differences as indicted by two-dimensional gel and one-dimensional mapping patterns may be responsible for their differential inhibitory effects in displacing erythrocyte spectrin binding. Our second binding assay involved the addition of ^{125}I-labeled fodrin or spectrin to specific proteins (e.g., 72-kD, gp 85, 16 S complex, or BSA) that have been conjugated to Sepharose beads. Our data show that both fodrin and spectrin

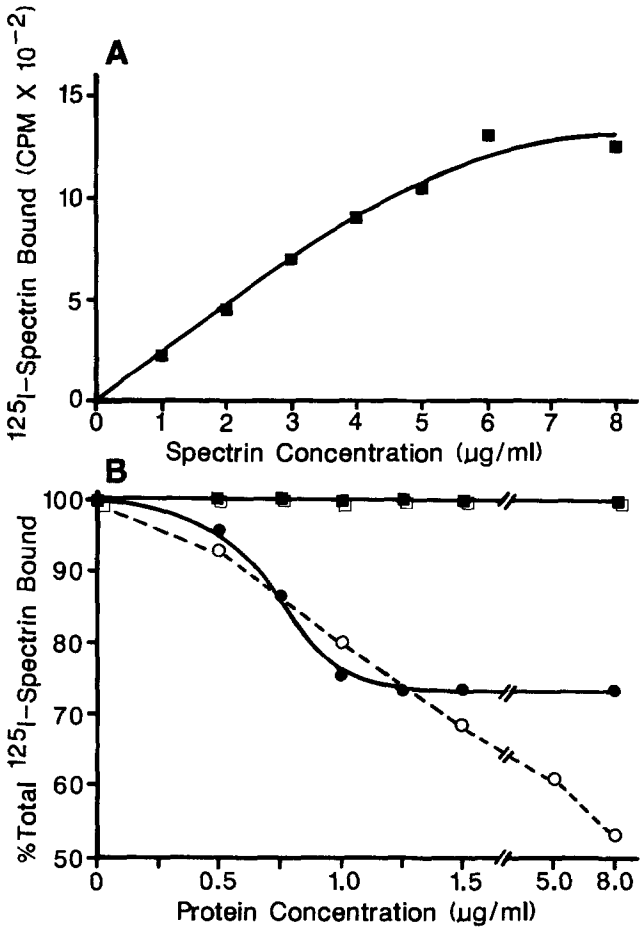

Figure 8. A spectrin-binding assay using spectrin-depleted, inside-out vesicles. (*A*) Binding curve for ^{125}I-spectrin to spectrin-depleted, inside-out erythrocyte vesicles. (*B*) Binding of 6 μg/ml ^{125}I-spectrin to spectrin-depleted, inside-out erythrocyte vesicles with (●) and without (■) preincubation with varying concentrations (0.5–8.0 μg/ml) of lymphoma 72-kD protein. The binding of ^{125}I-spectrin was also measured in the presence of various concentrations of either the 72-kD fragment of ankyrin (○) or a nonspecific protein, BSA (□).

display a preferential binding with the lymphoma 72-kD protein as compared to the 16 S complex or gp 85 alone (Fig. 9). We believe that the level of fodrin and spectrin binding to the 72-kD protein is significant, since there is only a minimal amount of nonspecific spectrin or fodrin binding to BSA-conjugated Sepharose beads (Fig. 9).

Since the lymphoma 72-kD protein shares several structural and functional similarities with the 72-kD proteolytic fragment of ankyrin, it seems reasonable to suggest that this molecule may be an ankyrin-like protein involved in the linkage between receptor molecule(s) and the cytoskeletal components in mouse T-lymphoma cells.

Discussion

Although interactions between membrane receptors and the cytoskeleton have been proposed to play an important role in lymphocyte receptor clustering and aggregation (so-called patching and capping), the molecular details concerning this transmembrane linkage are still very unclear at this time. Since analogs of proteins in the erythrocyte membrane–cy-

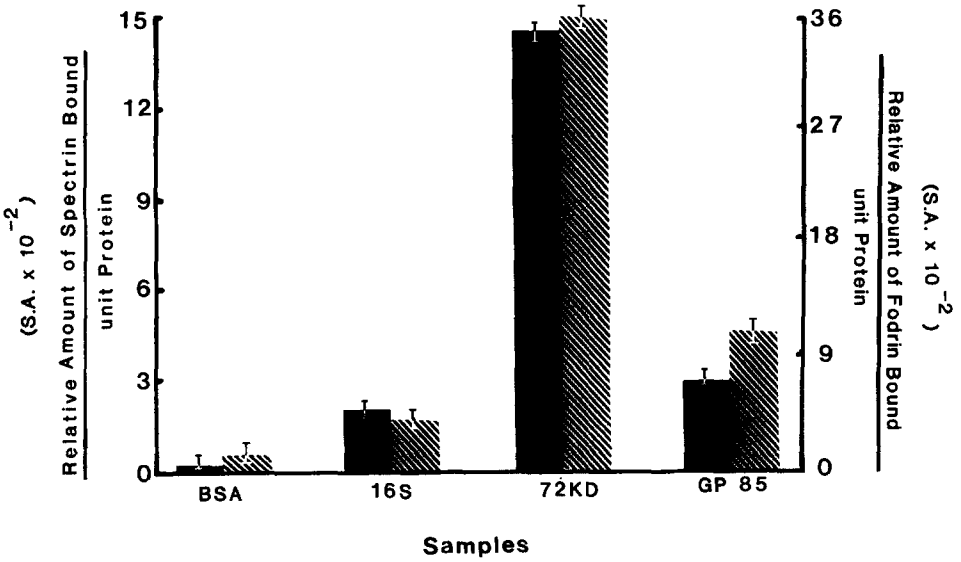

Figure 9. Direct binding of fodrin or spectrin to lymphoma proteins. ^{125}I-fodrin or spectrin was added to lymphoma protein (e.g., 72-kD protein, gp 85, or 16 S)–conjugated Sepharose beads. The Sepharose beads were centrifuged at 200 g_{av} for 2 min at room temperature. The radioactivity associated with the 72-kD protein–, gp 85–, 16 S–, and BSA– (as a control) conjugated beads was determined by counting on a LKB mini-Gamma counter. The relative fodrin (■) or spectrin (▨) binding activity was calculated by the ratio of relative amount of fodrin or spectrin bound (cpm) vs. relative amount of protein (μg) conjugated to the Sepharose beads.

toskeleton complex (i.e., spectrin, ankyrin, and band 3) have been identified in lymphocytes (7, 13, 18, 26, 28), we have sought to draw further functional analogies between the lymphocyte and erythrocyte membrane–cytoskeleton systems. The determination of common components and similar transmembrane interactions in both erythrocytes and lymphocytes should provide useful insights into how these molecules may function in the regulation of lymphocyte receptor movement.

In erythrocytes, the interaction of spectrin (a major cytoskeletal actin–binding protein) with the inner surface of the erythrocyte membrane is thought to be responsible for the maintenance of erythrocyte membrane architecture as well as cell shape (16, 19, 24, 27, 34). Spectrin-like proteins, referred to as fodrin (displaying one subunit of 240-kD and a second subunit of 235 kD) have recently been isolated and identified in a number of non-erythroid cells including lymphocytes (13, 26, 28). Both biochemical and cytochemical data indicate that when lymphocytes are induced to patch and cap, there is a preferential accumulation of fodrin molecules in the membrane-associated cytoskeleton (13) and receptor cap structures (13, 26, 28). Therefore, the participation of fodrin in linking membrane components to actin-filaments is strongly implicated (7, 13).

One of the linker molecules bridging spectrin and erythrocyte membrane components has been identified as ankyrin (4). Analyses from limited proteolytic digest experiments of the intact 215-kD ankyrin molecule indicate that (*a*) the 55–72-kD fragments of this protein contain the spectrin-binding domain (1, 39, 40), and (*b*) the 82/83-kD fragment contains the binding site for the membrane protein, band 3 (39, 40). Ankyrin-like molecules have been reported in a number of non-erythroid cell types including lymphocytes (4, 7). However, biochemical identification and characterization of this ankyrin-like protein has not been successfully performed in lymphocytes.

In this study, we have isolated a 16 S protein complex containing a membrane glycoprotein and an associated 72-kD protein. The stoichiometry for these two proteins appears to be in a 1:1 molar ratio (Figs. 1 and 2). The association between gp 85 and the 72-kD protein is rather stable based on the following evidence: (*a*) the two proteins can be co-isolated on a WGA column and co-eluted with GlcNAc (Fig. 2, *B* and *C*); (*b*) they can be co-sedimented as a single peak with a sedimentation coefficient of 16 S by sucrose gradient centrifugation (Figs. 1 and 2); and (*c*) these two molecules can not be separated in the presence of high salt but are dissociated after exposure to 2 M urea (Fig. 2). The tight association between lymphoma gp 85 and 72 kD protein (especially their co-isolation and resistance to high salt treatment) are analogous to that of the band 3–ankyrin complex in erythrocytes (2). Whether lymphoma gp 85 is an erythrocyte band 3–like protein remains to be determined.

Using anti-ankyrin antibody, we have found that there are two distinct cross-reactive forms of ankyrin in lymphoma membranes; namely, a 72-kD protein and a 215-kD protein (Fig. 4). Whether the 72-kD protein is a proteolytic fragment of a 215-kD ankyrin-like protein or a new class of non-erythrocyte ankyrin-like molecules is not clear at this time. Since our membrane extraction and 72-kD protein isolation procedures both use a variety of protease inhibitors, we feel it is unlikely that the 72-kD protein is a proteolytic fragment of the 215-kD protein. The fact that the 72-kD protein is preferentially isolated with the gp 85 membrane protein in a stable complex, and also displays a number of structural and functional similaries to erythrocyte ankyrin, implies that the 72-kD protein may be an essential part of transmembrane linkages in mouse T-lymphoma cells. Immunoelectron microscopy and immunofluorescence observations show clearly that these ankyrin-like molecules are located at the inner surface or cytoplasmic side of the plasma membrane and are co-aggregated with receptor cap structures (Figs. 4 and 7). These cytochemical results provide strong evidence for close interactions occurring between ankyrin-like molecules and the plasma membrane in intact lymphoma cells.

The two-dimensional PAGE and one-dimensional peptide mapping analyses indicate that the lymphoma 72-kD protein shares a great deal of structural homology (only minor differences) with the 72-kD proteolytic fragment of erythrocyte ankyrin. Most importantly, the results of two independent spectin-binding assays show that the lymphoma 72-kD protein has specific binding affinity for spectrin and fodrin molecules (Figs. 8 and 9). This spectrin-binding property is one

of the best known characteristics of the 72-kD proteolytic fragment of erythrocyte ankyrin (1). The fact that the amount of 72-kD protein associated with fodrin/actin–containing cytoskeletal material is proportionally increased during various ligand-induced capping (Fig. 7, and Table I) further suggests that a close interaction occurs between the 72-kD protein and cytoskeletal components in lymphoma membranes. Based on the aforementioned structural and functional similarities between the lymphoma 72-kD protein and the erythrocyte 72-kD proteolytic fragment of ankyrin, we propose that the lymphoma 72-kD protein functions analogously to erythrocyte ankyrin. Erythrocyte 72-kD ankyrin fragment has been shown to contain a spectrin-binding site but contains no membrane protein–binding region (1). In this respect, the lymphoma 72-kD protein is different from the erythrocyte 72-kD proteolytic fragment of ankyrin since the lymphoma 72-kD protein is found tightly linked to the membrane protein, gp 85. The question of whether the 72-kD protein is also capable of linking other membrane proteins awaits further investigation.

Our recent data indicates that a transmembrane glycoprotein, gp 180, is associated with fodrin (in a 1:1 molar ratio) as a complex with a sedimentation coefficient of ~20 S (13). Although an ankyrin-like protein has not been identified in the gp 180–fodrin complex, it is possible that gp 180 either contains an ankyrin-like domain or interacts with a small fraction of the 72-kD–gp 85 complex or 72-kD proteins alone which then provides for subsequent linkage between gp 180 and fodrin.

Preliminary data in our laboratory show that the membrane-associated 72-kD protein is also present in human B lymphoblasts and blood platelets. Therefore, the functional and physiological significance for this protein in transmembrane interactions is highly implicated.

In conclusion, we believe that the isolation and characterization of the lymphoma 72-kD, ankyrin-like protein offers an excellent opportunity to further our understanding of the molecular and functional organization of non-erythrocyte membranes. Furthermore, we believe that the linkage of lymphoma 72-kD protein with gp 85 and with the spectrin-like protein, fodrin (which, in turn, binds to actin filaments), may represent an important regulatory transmembrane complex required for lymphocyte receptor capping.

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