Lymphoma Thy-1 Glycoprotein Is Linked to the Cytoskeleton via a 4.1–like Protein

Lilly Y. W. Bourguignon, Suzanne J. Suchard, and Efstathia L. Kalomiris

Department of Anatomy and Cell Biology, School of Medicine, University of Miami, Miami, Florida 33101

Abstract. In this study we have found that the phosphoprotein doublet of 68,000 and 65,000 daltons (68/65 kD) in mouse T-lymphoma cells shares several structural and functional similarities with erythrocyte band 4.1. Our evidence for identifying the 68/65-kD doublet as a lymphoma 4.1–like protein is as follows: (*a*) it displays an immunological cross-reactivity with anti–erythrocyte band 4.1 antibody; (*b*) it exhibits a Svedberg unit of sedimentation coefficient of 4 S; (*c*) it is phosphorylated in the presence of phorbol ester (phorbol-12-*O*-tetradecanoylphorbol-13-acetate) and its phosphorylation requires Ca^{2+}; (*d*) it is phosphory-lated primarily at serine residues; and (*e*) it can bind directly to fodrin (a spectrin-like actin-binding protein). In addition, this lymphoma 4.1–like protein can be both colocalized and coisolated with the major T-lymphocyte–specific glycoprotein, Thy-1 (gp 25). Therefore, all of these results strongly suggest that the lymphoma 4.1–like protein (68/65-kD doublet) may play a pivotal role in linking the Thy-1 (gp 25) glyco-protein to fodrin which, in turn, binds to the actin filaments that are responsible for recruiting Thy-1 antigens into cap structures.

THE Thy-1 antigen was first identified as a cell surface alloantigen (having two allotypic forms called Thy-1,1 and Thy-1,2) on thymus and brain cells from mice (55). The Thy-1 glycoprotein (25,000 D) is one of the most abundant membrane components in both thymocytes and neurons of rodents (7, 55). Immunoreactive forms of Thy-1 have also been detected in dog and human brain tissue (3, 27). Amino acid sequence data indicate that the Thy-1 protein shares a large amount of sequence and structural homology with a single immunoglobulin-variable domain (24).

Although the Thy-1 glycoproteins display properties of integral membrane proteins, no intramembraneous or trans-membrane hydrophobic amino acid sequence has been detected (20). Recently, it has been suggested that the Thy-1 antigens are anchored in the plasma membrane via a phospholipid, possibly one with a phosphoinositol domain (44, 65). However, this hypothesis has been challenged by cDNA sequence analysis which suggests that a segment of 31 amino acids at the carboxyl terminus of the Thy-1 molecules may be responsible for the integration of this protein into the plasma membrane (21, 57). Until these conflicting pieces of evidence can be clarified, the question of whether the Thy-1 antigens penetrate into the plasma membrane remains unresolved.

Antibodies to Thy-1 are known to induce Thy-1 antigens to form surface receptor clusters and aggregates: the so-called patching and capping phenomenon (11, 12). This antibody-induced Thy-1 antigen redistribution appears to be closely correlated with certain physiologically relevant events. For example, proliferation of mouse T-lymphocytes is known to result from the clustering of Thy-1 antigens by anti–Thy-1 antibody (35, 45, 46); and the outgrowth of neuronal processes in tissue culture is facilitated by anti–Thy-1 antibody binding to their receptors (41). In addition, anti–Thy-1 patching and capping display the following characteristics: (*a*) the patched and capped structures are closely associated with a number of cytoskeletal proteins (e.g., actin and myosin) (13, 17); (*b*) the capping process is sensitive to microfilament-disrupting agents (cytochalasins B and D), implying that the function of microfilaments is important for Thy-1 antigen movement (15); and (*c*) actin polymerization occurs concomitantly with Thy-1 cap formation (40). These results provide strong evidence for the direct involvement of the cytoskeleton in the mechanism of Thy-1 antigen movement.

Recently, immunoreactive forms of band 4.1 have been identified in brain tissue (6, 33), in fibroblasts (23), and in several blood cell types such as lymphocytes, platelets, and polymorphonuclear leukocytes (62). In these nonerythroid cells, the immunoreactive 4.1 proteins appear as either a singlet or a doublet with apparent molecular masses ranging from 68,000 to 75,000 D after SDS PAGE (8). Synapsin, one of the major components of synaptic vesicles, has been shown to display a number of biochemical properties similar to those described for both erythrocyte 4.1 and brain 4.1–like protein (6). In erythrocytes, band 4.1 has been shown to enhance the interaction between spectrin and actin (22, 52, 68). In addition, band 4.1 protein also binds to the erythrocyte transmembrane glycoproteins, glycophorin and band 3 protein (the anion transport channel protein) (1, 53). Unfortunately, the structural or functional involvement of 4.1–like

proteins in specific cellular processes of certain blood cell types such as lymphocytes, platelets, and polymorphonuclear leukocytes is unclear at the present time.

In this study we have found that a 68,000/65,000-D phosphoprotein doublet (designated 68/65-kD) in T-lymphoma cells also displays immunoreactivity with the erythrocyte membrane skeletal protein, band 4.1. The 68/65-kD doublet shares several structural and functional similarities with the erythrocyte 4.1 protein. Additionally, the lymphoma 4.1–like protein can be colocalized and co-isolated with the major T-lymphocyte–specific glycoprotein, Thy-1 by complementary techniques including double immunofluorescence staining, immunoprecipitation, and sucrose gradient centrifugation. Furthermore, our data indicates that the lymphoma 4.1–like protein (but not Thy-1) binds to fodrin which, in turn, then is able to bind to actin filaments. It is proposed that the lymphoma 4.1–like protein plays a pivotal role in linking Thy-1 to the cytoskeleton which is required for collecting Thy-1 antigens into cap structures.

Materials and Methods

Cells

The mouse T-lymphoma cell line, BW 5147, an AKR/J lymphoma line (obtained from Dr. R. Hyman, The Salk Institute, San Diego, CA) were grown in DME supplemented with 10% heat-inactivated horse serum (Gibco, Grand Island, NY) at 37°C in 5% CO_2/95% air.

Immunofluorescence Microscopy

Surface Labeling. Cells were stained for surface Thy-1 (gp 25) patching and capping by first washing with RPMI 1640 medium and then treating these cells with primary antibodies (monoclonal rat antibodies) raised against Thy-1 (gifts kindly provided by Dr. Ian Trowbridge, The Salk Institute) followed by the addition of a secondary antibody such as fluorescein-conjugated goat anti-rat IgG at room temperature or 37°C for 20 min.

Intracellular Labeling of 4.1 Protein. Fluorescein-labeled Thy-1 (gp 25) patched/capped 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 (19), and then stained with rabbit anti–erythrocyte 4.1 protein (generously supplied by Dr. Van Bennett, The Johns Hopkins University, Baltimore, MD). These samples were then incubated with rhodamine-conjugated goat antibody against rabbit IgG to visualize the distribution of protein 4.1. To detect any nonspecific antibody binding, permeabilized cells were first incubated with antisera that were preabsorbed with soluble, native erythrocyte 4.1 protein to remove anti–protein 4.1 antibodies. These samples were then incubated with rhodamine-conjugated goat anti–rabbit IgG. No label was observed in such control samples.

These fluorescein and rhodamine-labeled samples were examined under a Zeiss photomicroscope using a 63× oil-immersion lens with epi-illumination and filter system of CZ 487710 and CZ 487714, respectively. Cells were photographed on Kodak plus-X film (Eastman Kodak Co., Rochester, NY).

Phorbol Ester Treatment

Phorbol ester, such as phorbol-12-*O*-tetradecanoylphorbol-13-acetate (TPA), was purchased from Sigma Chemical Co. (St. Louis, MO). This reagent was then dissolved in DMSO (final concentration of 1%) and used at a final concentration of 10^{-7} M. In control samples, cells were incubated in either DMSO plus phosphate-free DME or in phosphate-free DME alone. After incubation, aliquots were removed and cells were processed for further analysis as described below.

Radioactive Labeling of Cellular Proteins

Labeling Proteins with ^{125}I. Surface proteins and cellular proteins were labeled with ^{125}I using the iodogen method of Fraker and Speck (30).

Labeling Proteins with [^{35}S]Methionine or [^3H]Glucosamine. Cell cultures (1 × 10^5 cells/ml) were incubated either with [^{35}S]methionine

(1,000 Ci/mmol) (for labeling total cellular proteins) or with [^3H]glucosamine (100 Ci/mmol) (for labeling glycoproteins) (ICN K & K laboratories, Inc., Plainview, NY) for 16 h at 37°C in DME supplemented with 10% heat-inactivated horse serum in 5% CO_2/95% air.

Protein Phosphorylation. (a) Labeling in vivo: To metabolically label cellular phosphoproteins, cells (1 × 10^7 cells/ml) were washed with phosphate-free DME, and $H_3^{32}PO_4$ (carrier free, ICN K & K laboratories Inc.) was then added at 0.25 mCi/ml. After 1 h of incubation with $H_3^{32}PO_4$ at 37°C (isotopic equilibrium inside the cell is reached under this condition), cells were exposed to TPA or phosphate-free DME alone for 10 min at room temperature. (b) Labeling in vitro: Isolated plasma membrane (prepared according to the procedures described below) was incubated in 20 mM KCl, 1 mM Mg acetate, 10^{-4}–10^{-6} M Ca^{2+} (pCa = $-\log_{10}10^{4-6}$ = 4.0–6.0) and 1 mM [γ-^{32}P]ATP (100–200 cpm/pmol) (ICN Chemical and Radioisotope Division, Irvine, CA) for 5 min at 30°C. As a control, 10^{-4}–10^{-6} M Ca^{2+} (pCA = 4.0–6.0, high Ca^{2+}) was replaced by 10^{-8} M Ca^{2+} (pCa^{2+} = $-\log_{10}10^8$ = 8.0, low or no Ca^{2+}) in the incubation mixture. Phosphorylation was terminated by adding an excess amount of unlabeled 0.1 M PBS (pH 7.4) at 0°C.

Plasma Membrane Isolation

Plasma membrane was isolated by the method described by Johnson and Bourne (37) with slight modifications. Radioactively labeled samples (i.e., ^{125}I-labeled, [^3H]glucosamine-labeled, [^{35}S]methionine-labeled, or $^{32}PO_4$-labeled cells) were harvested by low speed centrifugation (500 g_{av}), washed twice with Dulbecco's phosphate-buffered medium and resuspended in a solution of 20 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM 2-mercaptoethanol, pH 7.4, at 0°C. These cells were then lysed by homogenization with 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. The pellet was resuspended in 10% sucrose (wt/wt) in the same buffer, layered over a discontinuous gradient of 30 and 40% sucrose (wt/wt), and spun at 150,000 g_{av} for 90 min. The plasma membrane fraction was collected from the 30–40% interface and washed with phosphate-buffered Earle's balanced salt solution, pH 7.3. Enzyme marker assays (5' nucleotidase and/or Na^+/K^+-ATPase activities) showed that these plasma membrane preparations were at least 20-fold purified and contained negligible amounts of other membranous organelle contamination (13).

NP-40 Extraction

Isolated plasma membrane was washed and resuspended in phosphate-buffered Earle's balanced salt solution containing 1 mM PMSF and 10 mM iodoacetamide. Detergent extraction was accomplished by adding NP-40 to the phosphate-buffered Earle's balanced salt solution 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., the NP-40-soluble fraction) and the pellet (i.e., NP-40-insoluble fraction) were collected for further biochemical analysis as described previously (14, 16). Previous studies have shown that the NP-40-insoluble fraction contains the membrane-associated cytoskeleton (13, 16).

Immunoblotting and Immunoprecipitation Procedures

Isolated erythrocyte ghost membranes and mouse lymphoma membranes were electrophoresed either on an exponential polyacrylamide gel gradient (6.0–17.0) or a 12% polyacrylamide gel that contained SDS as described below. The polypeptides were transferred to nitrocellulose sheets as described previously (16). Nitrocellulose sheets were subsequently incubated with anti–erythrocyte 4.1 antibody, followed by incubation with ^{125}I-protein A, and analyzed by autoradiography as described below.

^{32}P-, ^{125}I-, or [^3H]Glucosamine-labeled detergent-extracted total mouse lymphoma plasma membranes, cytoskeletal fractions, or isolated proteins were incubated with either rabbit anti–erythrocyte 4.1 protein or anti–erythrocyte 4.1 protein-free (preabsorbed) antiserum at 4°C for 30 min. Anti-4.1 antibody was preabsorbed by an excess amount (at least 100 μg/ml) of the erythrocyte band 4.1. Subsequently, goat anti-rabbit IgG was added to the samples at 4°C for 30 min to precipitate the immune complex. The immunoprecipitates were obtained by centrifugation at 700 g_{av} for 5 min and washed three times with 0.1% NP-40 in phosphate-buffered Earle's balanced salt solution using the same centrifugation conditions. To detect Thy-1 antigens, similar immunoprecipitation procedures were carried out using NP-40-solubilized, ^{125}I-labeled plasma membranes. Either monoclonal rat

anti-Thy-1 or anti-Thy-1-free (preabsorbed) antibodies followed by rabbit anti-rat and goat anti-rabbit IgG was added to the samples at 4°C for 1 h to generate the immunocomplexes. The immunoprecipitates were subsequently solubilized by SDS, and analyzed by SDS PAGE and autoradiography as described below.

Immunobinding Procedures

Antigenically active molecules were detected by binding with rat anti-Thy-1 (gp 25)-conjugated Sepharose beads. Antibody was coupled to CNBr-activated Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) according to the method outlined by Cuatrecasas (26). ^{125}I-labeled membrane proteins were incubated with these antibody-conjugated beads for 2 h. The beads were then poured into small columns and washed extensively with 0.1% NP-40. As a control for nonspecific binding, beads were prepared with preabsorbed anti-Thy-1 antiserum (anti-Thy-1 antibodies were removed with excess Thy-1 antigens). Proteins bound to the anti-Thy-1-conjugated beads were detected by counting on a Beckman 5500 Gamma counter (Beckman Instruments Inc., Palo Alto, CA).

SDS PAGE and Autoradiographic Analysis

Electrophoresis was conducted using an exponential polyacrylamide gradient (6.0–17.0%) slab gel and the discontinuous buffer system described by Laemmli (39). All samples were dissolved in a buffer that contained 2% SDS, 0.1 M 2-mercaptoethanol, 0.003% bromophenol blue, 20 mM Tris-HCl, pH 6.8, and heated to 100°C for 2 min. Electrophoresis was conducted at a constant current of 15 mA at room temperature for 4 h. The polypeptide banding pattern was revealed by staining with Coomassie Blue. Gels containing samples labeled with ^{125}I-, ^{32}P-, [^{3}H]glucosamine, or [^{35}S]methionine were processed through fluorography (10), vacuum dried, and exposed to Kodak x-ray (X-Omat XAR-5) film at −70°C.

Phosphoamino Acid Analysis

The ^{32}P-labeled 68/65-kD protein isolated from cells that were labeled either in the presence or absence of TPA was recovered from polyacrylamide gel slices by electroelution as described by Hunkapiller et al. (36) with the exception that a Hoeffer cylindrical gel apparatus was used as the elution tank. Hydrolysis of the eluted protein was performed at 110°C for 2 h by the method of Cooper et al. (25) and the phosphoamino acids were separated by conventional two-dimensional paper chromatography on 3-mm Whatman chromatographic paper (Whatman Inc., Clifton, NJ). Separation in the first dimension was achieved at pH 3.5 for 2 h at 2,000 V, and in the second dimension at pH 1.9 for 30 min. The phosphoamino acid standards (i.e., phosphoserine, phosphothreonine, and phosphotyrosine) were mixed with ^{32}P-labeled samples, co-analyzed and identified by ninhydrin staining. The labeled phosphoamino acids were detected by exposing to Kodak x-ray (X-Omat XAR-5) film at −70°C.

Analysis of NP-40-solubilized Plasma Membrane by Sucrose Gradient Centrifugation

Isolated ^{125}I-, [^{35}S]methionine-, or [^{3}H]glucosamine-labeled plasma membrane was extracted with 1% NP-40 and then loaded onto a 36-ml linear sucrose gradient (7–48% in phosphate-buffered Earle's balanced salt solution, pH 7.2) with a 2-ml cushion of saturated sucrose. The gradient was centrifuged at 70,000 g_{av} for 12 h. Parallel gradients containing protein standards of tetrameric fodrin or spectrin (11 S) (32) and apoferritin (18 S) (61) were used to determine the S value of the gp 25-enriched peak. Thirty-six 1-ml fractions were collected from the bottom of each tube. The individual fractions from the gradient were analyzed by either liquid scintillation counting (for [^{35}S]methionine and [^{3}H]glucosamine labels) or gamma counting (for ^{125}I-surface labeled or ^{125}I-labeled total cellular proteins). Individual or peak fractions (depending upon the radioactive label) were precipitated with 10% TCA, and subsequently analyzed by SDS PAGE and autoradiography. The relative amounts of gp 25 (Thy-1) to other surface proteins in each gradient fraction were determined by scanning densitometry (Quick Scan R & D; Helena Laboratories, Beaumont, TX) of autoradiograms from ^{125}I-surface labeled or [^{3}H]glucosamine-labeled materials. To quantitate the relative molar ratio of the 68/65-kD protein and gp 25 in the 28-S complex, autoradiograms of [^{35}S]methionine-labeled or ^{125}I-labeled material were also analyzed by scanning densitometry.
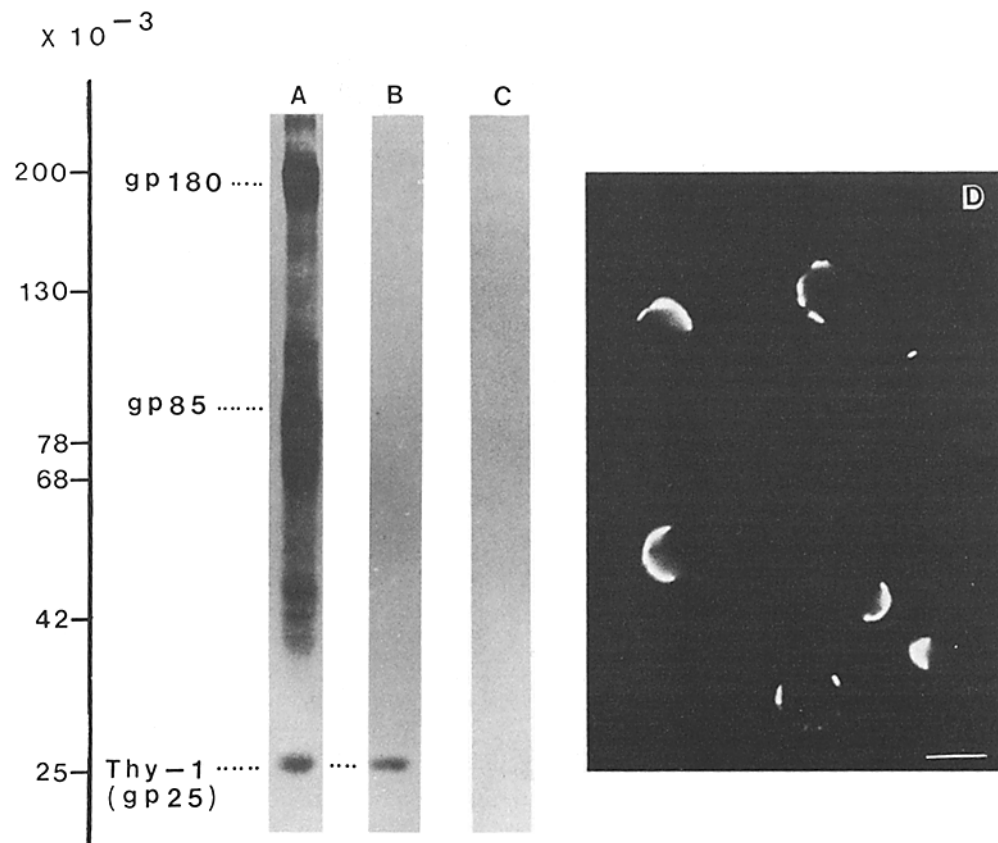

Figure 1. Identification of Thy-1 antigen in mouse T-lymphoma plasma membranes. (*A*) Autoradiogram of [^{3}H]glucosamine-labeled total plasma membranes. (*B* and *C*) Autoradiogram of [^{3}H]glucosamine-labeled plasma membranes that are immunoprecipitated by either monoclonal rat anti-Thy-1 (*B*) or preabsorbed anti-Thy-1 (anti-Thy-1-free) antisera (*C*) followed by rabbit anti-rat antibodies and goat anti-rabbit antibodies. (Molecular mass markers [×10³] are as follows: myosin, 200,000; β-galactosidase, 130,000; lactoperoxidase, 78,000; BSA, 68,000; actin, 42,000; concanavalin A, 25,000. These markers represent molecular weight values indicated in all subsequent gel figures.) (*D*) Immunofluorescence staining of Thy-1 capping induced by monoclonal rat anti-Thy-1 and fluorescein-conjugated goat anti-rat antibodies. Bar, 13 μm.
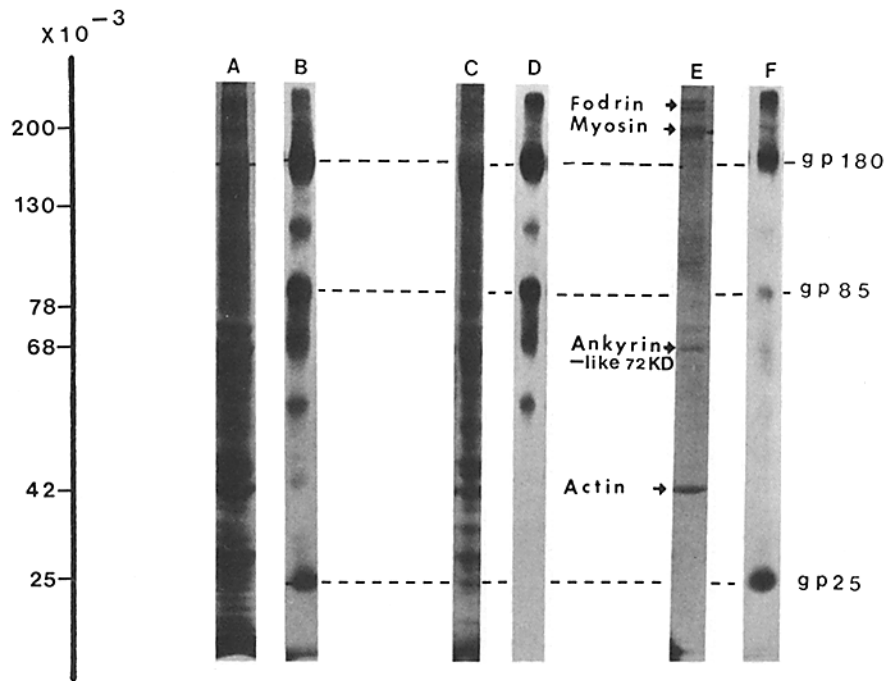
X10^{-3}

A   B   C   D   E   F

Fodrin →
Myosin →

200—

130—

78—
68—

42—

25—

Ankyrin→
—like 72 KD

Actin →

— gp 180

— gp 85

gp 25

Figure 2. NP-40–treated lymphoma plasma membranes. (*A*) Coomassie Blue–stained total plasma membrane. (*B*) Autoradiogram of ^{125}I-labeled total plasma membrane. (*C*) Coomassie Blue–stained NP-40–soluble fraction of the plasma membrane. (*D*) Autoradiogram of ^{125}I-labeled proteins associated with the NP-40–soluble fraction of the plasma membrane. (*E*) Coomassie Blue–stained NP-40–insoluble fraction (i.e., cytoskeleton material) of the plasma membrane. (*F*) Autoradiogram of ^{125}I-labeled proteins associated with the NP-40–insoluble fraction (i.e., cytoskeleton material) of the plasma membrane.

Dissociation of Lymphoma 68/65-kD Protein from 28 S (Thy-1/4.1 Complex) By Urea

Fractions containing ^{125}I-Thy-1/4.1 or total ^{125}I-labeled Thy-1/4.1 complex material (gradient fractions 16–20) were pooled, the sucrose was diluted by the addition of phosphate-buffered Earle's balanced salt solution, and centrifuged at 150,000 g_{av} for 3 h. The pelleted material was then resuspended in a small volume of phosphate-buffered Earle's balanced salt solution (100–200 µl) containing 2 M urea. The samples were incubated at 20°C for 1 h and then centrifuged in a Beckman airfuge for 40 min at 24 psi (100,000 g_{av}). The supernatants and pellets were then counted on a Beckman 5500 Gamma counter to determine the amount of radioactivity that was extracted into the supernatant during extraction. The extract was then run over a concanavalin A–Sepharose column to remove any Thy-1 molecules that were removed along with the 4.1-like proteins during extraction. The extracted material (free of contaminating Thy-1 molecules) was then dialyzed against 20 mM KCl, 10 mM Tris-HCl, pH 8.0, containing 0.5% NP-40. This material was then loaded onto a 16-ml linear sucrose gradient (7–28% in 10 mM Tris-HCl, pH 8.0, 20 mM KCl) with a 0.5-ml cushion of 65% sucrose. The gradient was centrifuged at 70,000 g_{av} for 22 h. Thirty-two 0.5-ml fractions were collected from the bottom of each tube. The fractions were then counted on a Beckman 5500 Gamma counter to determine the distribution of iodinated protein and analyzed by SDS PAGE and autoradiography. The iodinated peak material from this gradient was then immunoprecipitated with rabbit anti-erythrocyte band 4.1 (as described above) to confirm the presence of the 4.1-like 68/65-kD protein as the major protein in this peak. Protein standards of 3.7-S G-actin (58), 5-S transferrin, and 11-S tetrameric fodrin or spectrin (32) were used to determine the S value of the iodinated peak.

Reassociation of Lymphoma 4.1–like Protein with gp 25 (Thy-1) Molecules

Upon removal of 2 M urea, these unlabeled or ^{125}I-labeled lymphoma 4.1-like proteins (4-S material, 68/65-kD protein prepared according to the same procedures described above) were then incubated with ^{125}I-labeled and unlabeled Thy-1 molecules (prepared according to the procedures outlined by Williams et al. [69]), respectively for 2 h in 20 mM KCl, 10 mM Tris-HCl, pH 8.0, 0.5% NP-40 at approximately equal molar concentrations. The resulting complex was then analyzed by a sucrose gradient (7–48%) centrifugation as described above or passing over concanavalin A–Sepharose columns (6.0 × 0.5 cm) equilibrated with 20 mM KCl, 10 mM Tris-HCl, pH 8.0, containing 0.5% NP-40. After binding, the columns were washed with equilibration buffer until no more radioactivity was removed. After washing, columns were eluted with 1 M α-methyl-D-mannoside and

0.5-ml fractions were collected. A parallel concanavalin A–Sepharose column was run as a control for the nonspecific binding and/or elution of ^{125}I-labeled lymphoma 4.1. Fractions from all columns were then counted and plotted vs. fraction number to establish an elution profile for each column. Peak fractions from each column were then processed through anti-Thy-1-conjugated immunobead columns (as described in the immunobead-binding section).

Actin-binding Analysis

[^{35}S]Methionine-labeled 28-S complex material was either preincubated in the presence or absence of 0.1 µM of bovine brain fodrin (prepared according to Bennett et al. [9]) in phosphate-buffered Earle's balanced salt solution for 2 h and then added to 4.65 µM G-actin under polymerizing conditions for 2 h. After 2 h, the material was centrifuged at 20,000 g_{av} for 20 min and assayed for binding by determining the amount of [^{35}S]methionine label that became associated with the actin pellets (54). Controls were carried out to determine the amount of the 28-S material that would pellet in the absence of fodrin and actin.

Fodrin (or Spectrin) Binding Assays

Bovine brain fodrin (or human erythrocyte spectrin) (9, 18) and BSA were each conjugated to CNBr-activated Sepharose beads (Pharmacia Fine Chemicals). ^{125}I-labeled lymphoma proteins (either the 28-S Thy-1/4.1 complex, 68/65-kD [4-S material], or Thy-1) were then incubated with 200 µl of either fodrin or BSA-conjugated beads overnight at 0°C in phosphate-buffered Earle's balanced salt solution. After binding, the beads were washed extensively with phosphate-buffered Earle's balanced salt solution and then the amount of binding was assayed by gamma counting.

Results

Identification of Thy-1 Antigen and Its Associated Cytoskeletal Proteins

To analyze the major membrane components in the mouse T-lymphoma cell line (BW 5147), cells were either metabolically labeled with [^{3}H]glucosamine or surface-labeled with ^{125}I. SDS PAGE and autoradiographic analysis indicates that gp 180 (180 kD), gp 85 (85 kD), and gp 25 (25 kD) are three major glycoproteins in the plasma membrane fraction (Fig.

1 *A*). It is well known that the Thy-1 alloantigenic determinant is borne on a glycoprotein of ~25,000 D in the mouse T-lymphoma cell line (BW 5147) (64). This glycoprotein has been isolated from cultured mouse T-lymphoma cell lines and used to prepare either polyclonal or monoclonal antisera which show a strong cross-reactivity to gp 25 (Fig. 1 *B*). Control experiments using anti–Thy-1–free serum reveal no detectable gp 25 (Fig. 1 *C*) confirming the specificity of this immunoprecipitation data.

The availability of anti–Thy-1 antiserum has allowed us to examine Thy-1 (gp 25) patching and capping cytochemically (Fig. 1 *D*). Treatment of the lymphoma cells with only the primary antibody (monoclonal rat anti–Thy-1 antibody) induces only 10–15% capped cells in the total lymphoma cells. The proportion of Thy-1 patched and capped cells is enhanced to 50–60% of the total population if the cells are treated with both primary and secondary antibodies (i.e., rat anti–Thy-1 antibody followed by goat anti–rat antibody) (Fig. 1 *D*) (12). The Thy-1 patch and cap structures are always accompanied by a localized concentration of intracellular actin and myosin directly under the surface patches and caps (13, 17). Therefore, it has been suggested that the cytoskeleton is directly involved in Thy-1 patching and capping (13, 17).

Association of Cell Surface Glycoproteins with the Detergent-insoluble Cytoskeleton

One way to gain a better understanding of the interactions occurring between Thy-1 antigens and the cytoskeleton is to isolate and analyze the membrane–cytoskeleton complexes from lymphoma cells. Treatment of isolated lymphoma plasma membrane (^{125}I-labeled) (Fig. 2, *A* and *B*) with the non-ionic detergent, NP-40, solubilizes ~90% of total membrane proteins (Fig. 2, *C* and *D*) and leaves an insoluble residue that can be pelleted by centrifugation at 100,000 g_{av} (Fig. 2, *E* and *F*). Our data show that three major surface membrane proteins, gp 180, gp 85, and gp 25 (Fig. 2 *F*), are preferentially associated with the insoluble fraction which is operationally defined as the cytoskeleton fraction, since it contains actin, myosin, fodrin, and ankyrin-like/72-kD molecules (Fig. 2 *E*). Recently, we have reported that both gp 180 and gp 85 (via an ankyrin-like molecule) can be linked to the cytoskeletal protein, fodrin (16, 18). The nature of the linkage between gp 25 (Thy-1) and the cytoskeleton, however, has not been defined clearly.

Identification of the 68/65-kD Doublet as a 4.1–like Protein

Nonerythroid 4.1–like proteins (ranging in molecular mass from 68,000 to 80,000 D) have been identified in several different cell types (5, 23, 33, 34, 62). It has been demonstrated that at least one of these non-erythroid 4.1–like molecules can be phosphorylated by a cAMP-dependent kinase (6, 38, 50, 60). Fig. 3 shows the results of our SDS PAGE and autoradiographic analysis of the phosphoprotein components found in both the total plasma membrane and cytoskeletal fractions (i.e., NP-40–insoluble material) isolated from lymphoma cells that were briefly labeled with H₃³²PO₄. The 68/65-kD polypeptide doublet is detected in the total plasma membrane (Fig. 3 *A*) and the cytoskeletal fractions (Fig. 3 *B*). The membrane–cytoskeleton phosphoprotein of 130 kD has been previously identified as myo-
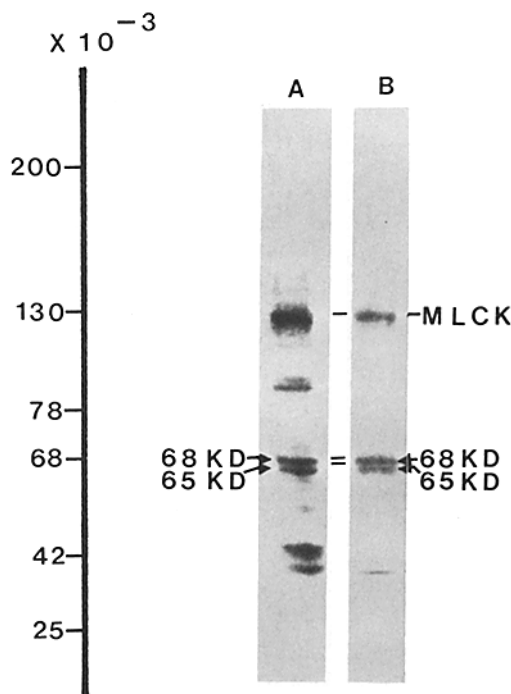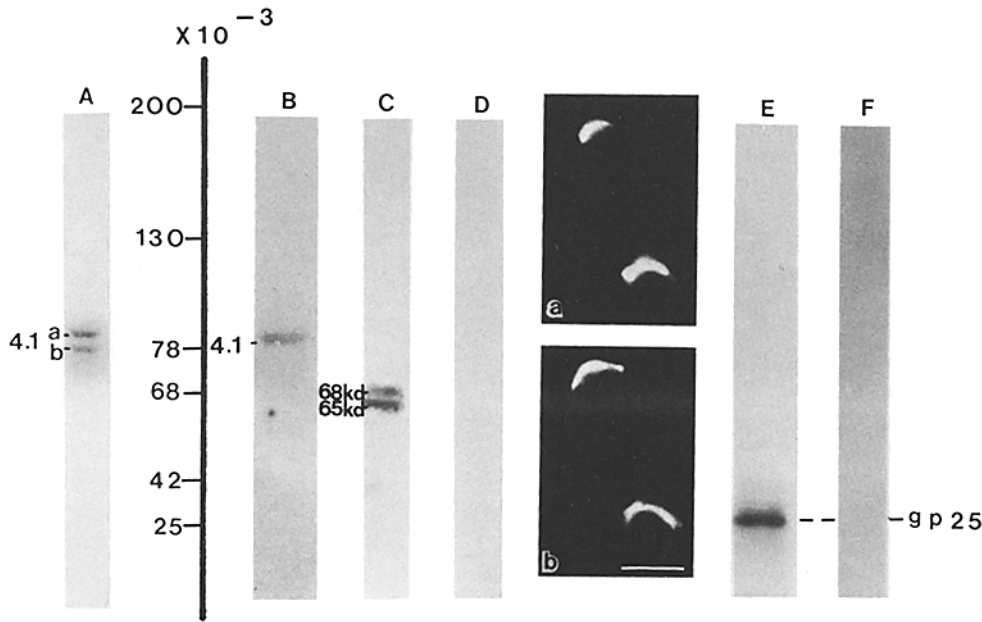

Figure 3. Identification of lymphoma phosphoproteins. (*A*) Autoradiogram of H₃³²PO₄-labeled proteins in the total plasma membrane. (*B*) Autoradiogram of H₃³²PO₄-labeled proteins associated with the NP-40–insoluble fraction (i.e., cytoskeleton material) of the plasma membrane. *MLCK*, myosin light chain kinase (14).

sin light chain kinase (Fig. 3, *A* and *B*) (14). We have now identified the 68/65-kD polypeptide doublet in the membrane–cytoskeletal fraction as a phosphorylated lymphoma 4.1–like protein by a standard immunoprecipitation technique (Fig. 4 *C*) using an antibody shown to be specific for erythrocyte band 4.1 by immunoblotting (Fig. 4 *B*). The specificity of the immunoreagent was demonstrated by the fact that (*a*) anti–erythrocyte band 4.1 recognizes only band 4.1 (82,000/78,000 D) in human erythrocyte ghosts (Fig. 4 *B*); and (*b*) an anti–4.1–free antiserum (i.e., preabsorbed with purified erythrocyte 4.1 [Fig. 4 *A*]) precipitated neither the 68/65-kD polypeptide nor any of the other phosphorylated proteins (Fig. 4 *D*). Therefore, we propose that the 68/65-kD protein doublet is an immunoreactive form of the erythrocyte band 4.1 found in mouse T-lymphoma cells.

In addition, we have found that the level of phosphorylation of the 4.1–like protein doublet (along with the 130,000-D and 85,000-D proteins but not the 45,000-D and 36,000-D proteins) is significantly enhanced during phorbol ester (TPA) treatment (Fig. 5, *A* and *B*). Furthermore, the results of our phosphoamino acid analysis indicate that phosphorylation of the lymphoma 4.1–like protein occurs primarily at serine residues (with very little in threonine and none in tyrosine residues) in both TPA-treated (Fig. 6, *a* and *b*) and untreated control samples (Fig. 6, *c* and *d*). In vitro protein phosphorylation experiments using [γ-³²P]ATP reveal that the phosphorylation of the 4.1–like protein occurs in the presence of Ca²⁺ (10⁻⁴–10⁻⁶ M [pCa=4.0–6.0]), but is greatly diminished in the absence of Ca²⁺ (10⁻⁸ M [pCa=8.0]) (Fig. 5, *C* and *D*). Therefore, we believe that the phorbol ester–activated protein kinase C may be responsible for the phosphory-

Figure 4. Immunological characterization of lymphoma 4.1-like protein. Cells were either metabolically labeled with carrier-free $H_3{}^{32}PO_4$ (*C* and *D*) or surface labeled with ^{125}I (*E* and *F*) as described in Materials and Methods. (*A*) Purified erythrocyte band 4.1 resolved into 4.1a and 4.1b subunits on a 12% SDS polyacrylamide gel. (*B*) Immunoblot of erythrocyte ghost membranes using erythrocyte anti-4.1 antibody. (In order to resolve a wide range of polypeptides, the ghost membranes were analyzed by a 6.0–17.0% SDS polyacrylamide gradient gel. Under these conditions the 4.1 protein resolves as a single band.) (*C* and *E*) Autoradiogram of either ^{32}P-labeled proteins (*C*) or surface ^{125}I-labeled protein (*E*) that can be specifically immunoprecipitated by anti–erythrocyte band 4.1 antibody from NP-40–extracted plasma membrane. (*D* and *F*) Autoradiogram of either ^{32}P-labeled proteins (*D*) or surface ^{125}I-labeled proteins (*F*) that can be precipitated by anti–erythrocyte band 4.1-free (preabsorbed with purified band 4.1 in *A*) antisera from NP-40–extracted plasma membrane. (Note that neither 68/65-kD nor gp 25 are detected in *D* and *F*.) (*a* and *b*) Double immunofluorescence staining of lymphoma cells for localization of cell surface Thy-1 cap structures (*a*) and intracellular lymphoma 4.1-like proteins (*b*). Bar, 13 μm.

lation of the lymphoma 4.1–like protein (primarily at serine residues) (Fig. 6) in a manner similar to that described in erythrocytes (29).

Structural Linkage between the Thy-1 Antigen and the 4.1-like Protein

Double-label immunofluorescence staining shows that during Thy-1 capping (Fig. 4 *a*), a 4.1–like protein concentrates directly beneath the Thy-1 cap structures (Fig. 4 *b*). This provides qualitative evidence for the co-redistribution of these two molecules during capping. To demonstrate the possible linkages between these two molecules, we analyzed plasma membrane extracts by immunoprecipitation. The immunoprecipitation of ^{125}I-labeled plasma membrane with anti–4.1 antibody reveals the co-precipitation of gp 25 (Thy-1 antigen), but not gp 180 or gp 85 (Fig. 4 *E*). In the presence of anti–4.1–free antiserum, no surface molecules are detected (Fig. 4 *F*). These data strongly suggest that there is a close association between 4.1–like molecules and the Thy-1 capped structures.

We have used sucrose gradient centrifugation in order to further analyze the nature of the linkage between gp 25 (Thy-1) and the 4.1–like molecule. This approach has recently been found to be very effective in the isolation of membrane-cytoskeleton complexes from lymphoma cells (16, 18). Centrifugation of the non-ionic detergent (NP-40)–solubilized lymphoma plasma membrane fraction (material containing either [^{35}S]methionine-labeled [Fig. 7, curve *1*], [^{3}H]glucosamine-labeled [Fig. 7, curve *2*], or ^{125}I-labeled gp 25 [determined by calculating the relative proportion of gp 25 to other surface ^{125}I-labeled proteins across the gradient; Fig. 7, curve *3*]) indicates that the majority of the membrane-associated components remain near the top of the gradient

(Fig. 7). In contrast, a very large proportion of ^{125}I-labeled gp 25 is detected in the middle of the gradient with an estimated S value of 28 (Fig. 7, curve *3*). Svedberg units of sedimentation coefficients were determined relative to 11-S tetrameric spectrin or fodrin (32) and 18-S apoferritin (61) that were used as sedimentation markers. SDS PAGE and au-
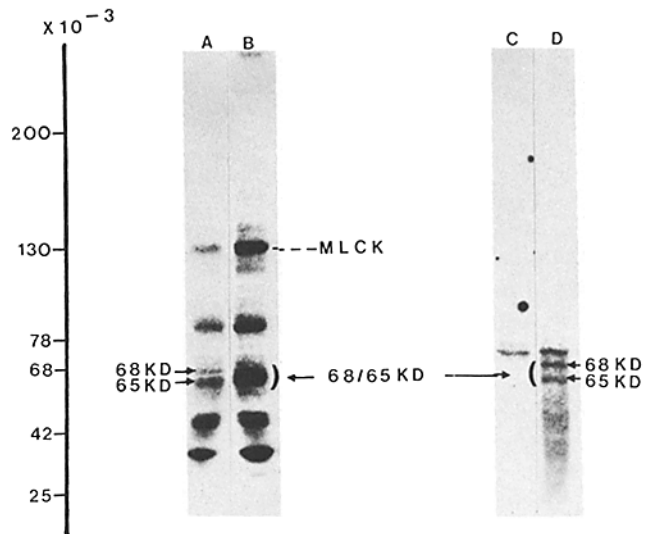

Figure 5. Phosphorylation of lymphoma proteins in the presence of TPA or Ca^{2+}. (*A* and *B*) Autoradiogram of ^{32}P-labeled plasma membrane isolated from cells that were metabolically labeled with carrier-free H_3PO_4 in the presence (*B*) or absence (*A*) of phorbol ester, TPA. (*C* and *D*) Autoradiogram of plasma membrane that was incubated with [γ-^{32}P]ATP in vitro in the presence of Ca^{2+} (10^{-4}–10^{-6} M) (*D*) or absence of Ca^{2+} (10^{-8} M) (*C*).
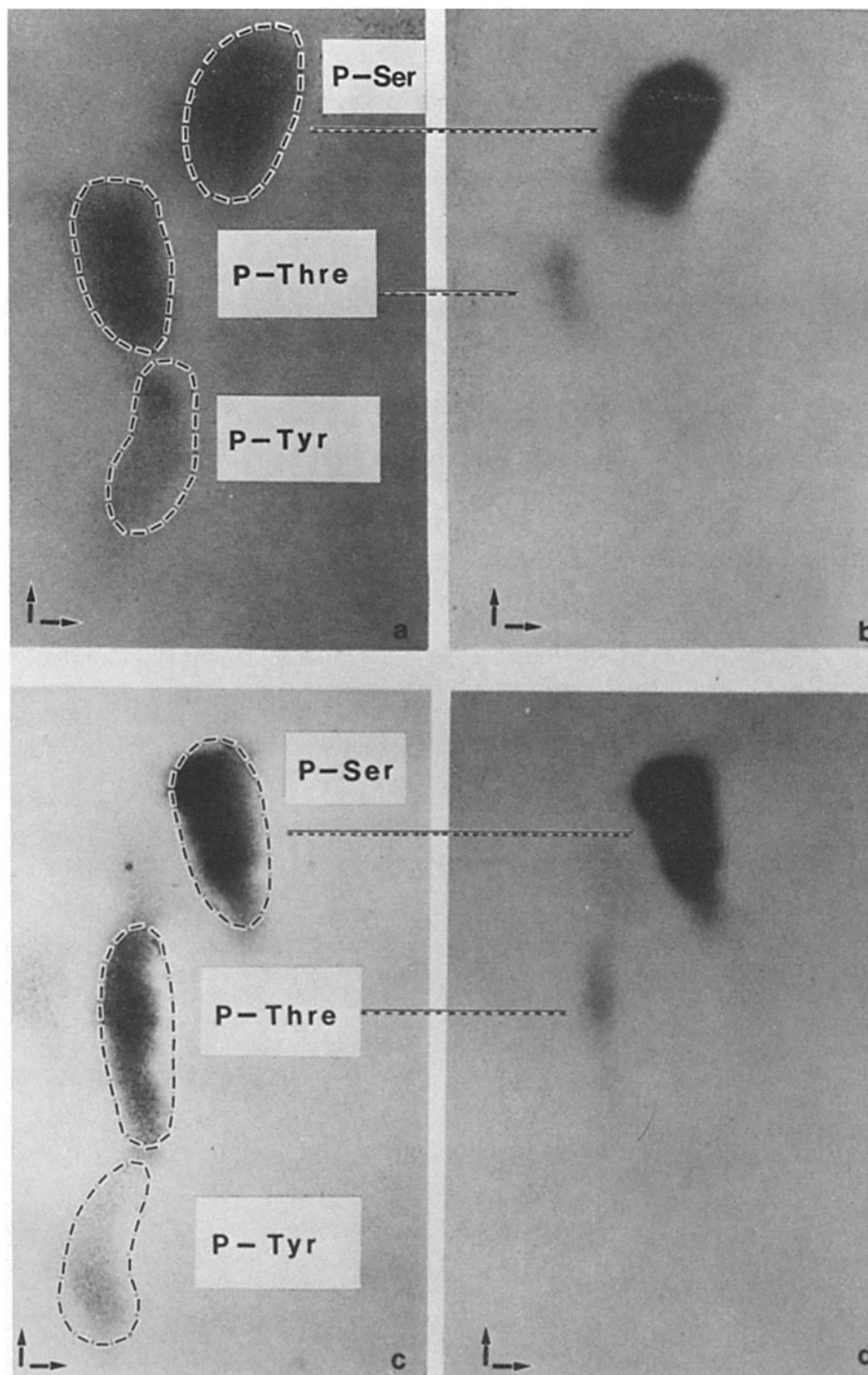
2534

Figure 6. Analysis of phosphoamino acids of the 68/65-kD protein from $H_3{}^{32}PO_4$-labeled cells treated with or without TPA. The acid hydrolysate of ^{32}P-labeled 68/65-kD protein isolated from cells that were labeled either in the presence (*a* and *b*) or absence of TPA (*c* and *d*) was resolved in two dimensions by electrophoresis as described in Materials and Methods. The P-ser, P-thre, P-tyr standards (*a* and *c*; identified by ninhydrin staining) were mixed with ^{32}P-labeled samples prepared from TPA-treated cells (*b*; autoradiogram) and untreated cells (*d*; autoradiogram), respectively. (*a* and *b* are the same sample; *c* and *d* are the same sample. The origin is designated by an arrow. *P-ser*, phosphoserine; *P-thre*, phosphothreonine; and *P-tyr*, phosphotyrosine.)

toradiographic analysis of the pooled fractions from the [^{35}S]methionine-labeled 28-S region (fraction numbers 16–20) reveals the presence of only a 68/65-kD doublet and the gp 25 (Thy-1) antigen (Fig. 7, lane *1*). Similar results were obtained after identical analysis with ^{125}I-labeled total cellular extracts (data not shown). Autoradiograms of either ^{125}I-surface labeled or [^3H]glucosamine-labeled material demonstrate that gp 25 is the only surface-labeled glycoprotein present in the 28-S peak (Fig. 7, lanes *2* and *3*).

The molar ratio of the 68/65-kD (4.1-like) protein and gp 25 (Thy-1) in the 28-S complex has been examined using autoradiograms of either ^{125}I-labeled or [^{35}S]methionine-la-

beled 28-S material (i.e., Fig. 7, lane *1*). The ratio of 68/65-kD protein to gp 25 was determined by integration of the area under the respective peaks generated by scanning densitometry of the autoradiograms (Fig. 8). For our calculations, we assumed a monomeric form of each subunit of the 68/65-kD protein (68,000–65,000 D per subunit) and a monomeric form of gp 25 (25,000 D). We also assumed equivalent labeling with either ^{125}I or [^{35}S]methionine for both polypeptides, since identical ratios of 68/65-kD to gp 25 were obtained with both isotopes. These preliminary calculations show that the 68/65-kD protein and gp 25 (Thy-1) occur in a stoichiometric ratio of ~1:4.
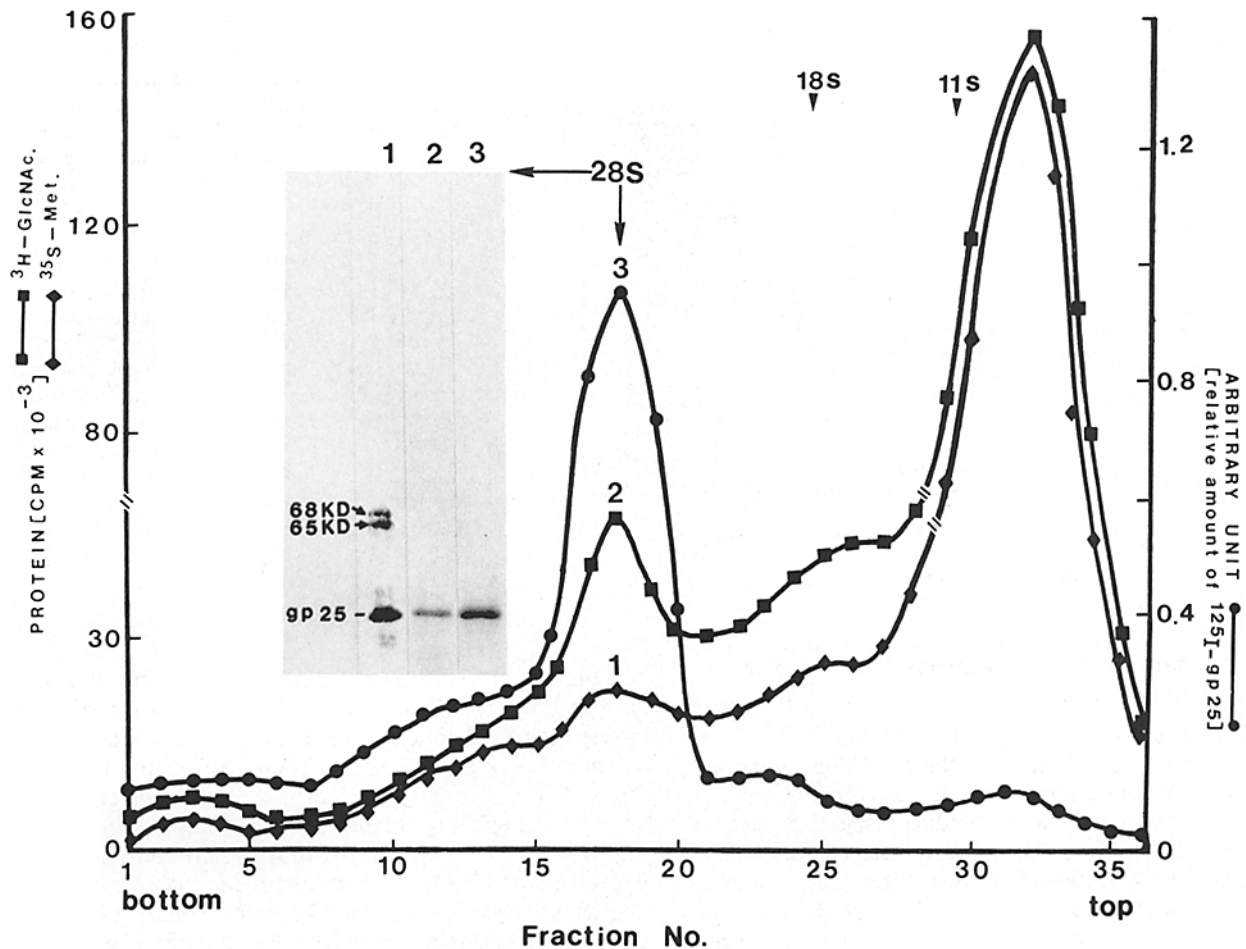
Figure 7. Sucrose gradient centrifugation of NP-40–solubilized plasma membranes. Cells were metabolically labeled with either [35S]methionine or [3H]glucosamine, or surface labeled with 125I. Radioactively labeled plasma membranes were isolated, extracted by NP-40 followed by centrifugation on a 7–48% sucrose gradient as described in Materials and Methods. (Curve *1*) [35S]methionine-labeled plasma membrane protein profile. (Curve *2*) [3H]glucosamine-labeled glycoprotein profile. (Curve *3*) Relative amount of 125I-surface labeled gp 25 (Thy-1) across the gradient (determined by calculating the relative proportion of gp 25 to other surface 125I-labeled proteins). Protein standards used are tetrameric spectrin or fodrin, 11 S; and apoferritin, 18 S. (Lane *1*) Autoradiogram of [35S]methionine-labeled proteins detected in 28-S peak. (A similar pattern was obtained using 125I-labeled total cellular proteins.) (Lane 2) Autoradiogram of [3H]glucosamine-labeled proteins detected in 28-S peak. (Lane 3) Autoradiogram of 125I-labeled surface proteins detected in the 28-S peak.

Treatment of the 28-S complex material with 2 M urea results in the disruption of the 28-S complex and the release of the 68/65-kD doublet with a Svedberg unit of sedimentation coefficient of 4 S (Fig. 9, *A* and *B*) (G-actin [3.7 S], transferrin [5.0 S], and tetrameric fodrin [11 S] were used as standard sedimentation markers [32, 58]). A large majority of the 4-S material can be immunoprecipitated with anti-erythrocyte band 4.1 antibody (Fig. 9, *C* and *D*). Upon removal of 2 M urea, the lymphoma 4.1-like protein (4 S; 68/65-kD doublet) is capable of reassociating with Thy-1 molecules as a 28-S complex (data not shown). The resulting complex is capable of binding to a concanavalin A–sepharose column and both Thy-1 and 4.1-like material coelute in the presence of α-methyl-D-mannoside (Fig. 10 *A*). In addition, the Thy-1/4.1 reassociated material can be quantitatively precipitated by anti–Thy-1 antibody (Fig. 10 *B*), confirming that both the Thy-1 and 4.1-like polypeptides form a relatively stable complex. Therefore, we believe that the lymphoma 4.1-like protein (4S−68/65-kD molecule) and Thy-1 strongly interact with each other under both in vivo and in vitro conditions.
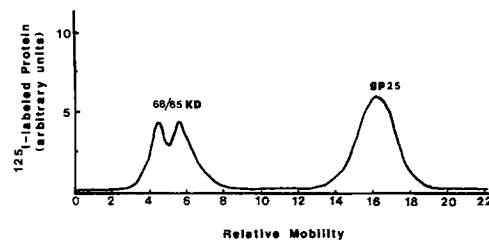

Figure 8. Analysis of molar ratio between the 68/65-kD protein and gp 25 in the 28-S complex. The 28-S complex labeled with 125I or [35S]methionine was obtained according to the procedures described in Fig. 7. Autoradiograms of the radioactively labeled 28-S complex, analyzed by SDS PAGE, were scanned on a scanning densitometer. The preliminary calculations of the stoichiometry between the 68/65-kD protein and gp 25 is 1:4. This value was determined by scanning autoradiograms of either 125I- or [35S]methionine-labeled material. We assumed a monomeric form of each subunit of the 68/65-kD protein (68,000 D/per subunit), a monomeric form of gp 25 (Thy-1) (25,000 D), and equivalent labeling of these proteins with 125I or [35S]methionine.
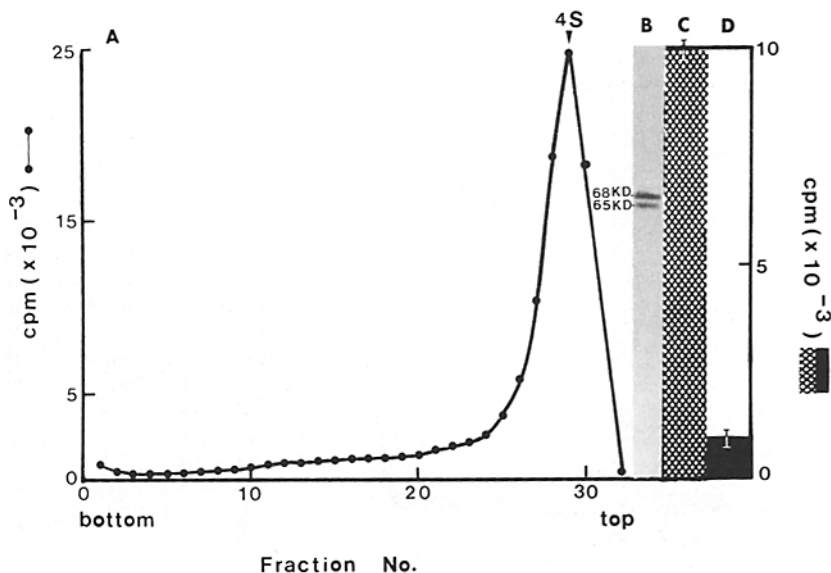
Figure 9. Isolation of the lymphoma 4.1-like protein. (*A*) First, ^{125}I-labeled 28-S complex was isolated according to the procedures described in Materials and Methods and then treated with 2 M urea. The urea-soluble material was loaded on a linear sucrose gradient (7–28%) and centrifuged at 70,000 g_{av} for 22 h. Protein standards of G-actin (3.7 S), transferrin (5.0 S), and tetrameric spectrin or fodrin (11 S) were used to estimate the S value of the ^{125}I-labeled peak as 4 S. (*B*) Autoradiogram of the ^{125}I-labeled 4-S material (primarily 68/65-kD protein). (*C* and *D*) Histogram of immunoprecipitation data of [125]-labeled 4-S material (primarily 68/65-kD protein) that specifically reacted with rabbit anti–erythrocyte band 4.1 (*C*) or rabbit anti-erythrocyte band 4.1-free (preabsorbed) antiserum (*D*). (Note that a large amount of the 4-S material can be immunoprecipitated by anti–4.1 antibody [*C*] with very little nonspecific precipitation [*D*]).

Interaction between the Lymphoma 4.1-like Protein and Fodrin (or Spectrin)

Since previous studies have suggested that the Thy-1 antigen (gp 25) becomes associated with the NP-40–insoluble cytoskeleton in mouse T-lymphoma during receptor capping (16), we have conducted F-actin–binding assays to determine if the isolated 28-S (Thy-1/4.1 complex) material has the ability to bind F-actin filaments in vitro. When 28-S complex material was incubated with actin in the absence of fodrin (or spectrin), no binding with F-actin was observed (data not shown). However, when the 28-S complex was preincubated with fodrin and then assayed for actin binding, it displayed a significant binding capability for F-actin filaments (data not shown). This set of data suggests that the Thy-1/4.1 complex

can bind to actin through its interaction with the actin-binding protein, fodrin. To test this hypothesis directly, we isolated the 28-S complex, urea-extracted to dissociate the 4.1-like (4 S—68/65-kD protein) doublet from the Thy-1 (gp 25) and then assayed for binding to fodrin (or spectrin)-conjugated Sepharose beads (Fig. 11, *A* and *B*). BSA-conjugated beads were used as a control for the nonspecific adherence of the isolated proteins to protein-conjugated beads. Our data demonstrate that the 28-S complex material has the ability to bind directly to fodrin- (or spectrin-) conjugated beads (Fig. 11 *C*). The fact that fodrin binding is mediated through the lymphoma 4.1-like protein is demonstrated by the high level of binding between fodrin (or spectrin) and the isolated 68/65-kD doublet (4 S-4.1-like material) compared
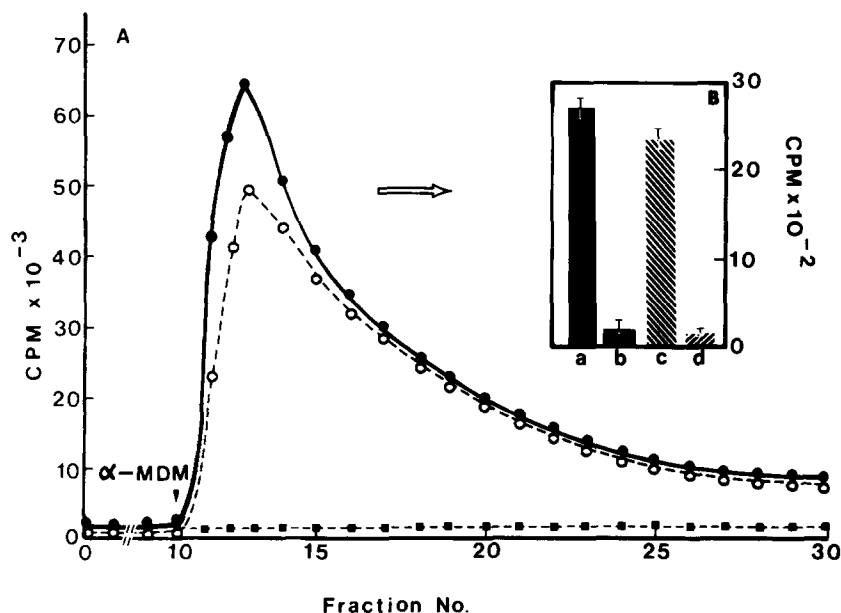

Figure 10. Reassociation of Thy-1/4.1 complex. (*A*) After the removal of 2 M urea, ^{125}I-labeled (*open circles*) or unlabeled lymphoma 4.1-like protein (4-S material, 68/65-kD protein) (as shown in Fig. 9) was incubated with an unlabeled or ^{125}I-labeled (*solid circles*) Thy-1, respectively, according to the procedures described in Materials and Methods. The reassociated Thy-1/4.1 complex can be co-bound to a concanavalin A–Sepharose column followed by co-elution with α-methyl-D-mannoside (α-*MDM*) (*solid and open circles*). ^{125}I-labeled 4.1-like protein alone (without incubation with Thy-1) fails to bind and/or specifically be eluted from a concanavalin A–Sepharose column (*solid squares*). (*B*) The α-methyl-D-mannoside-eluted material was subsequently processed through immunoaffinity columns conjugated with either monoclonal rat anti–Thy-1 antibody or anti-Thy-1-free (preabsorbed) serum. (*a*) ^{125}I-Thy-1/4.1 complex bound to an anti-Thy-1 column. (*b*) ^{125}I-Thy-1/4.1 complex bound to an anti-Thy-1-free (control) column. (*c*) Thy-1/[125]I-4.1 complex bound to an anti-Thy-1 column. (*d*) Thy-1/[125]I-4.1 complex bound to an anti-Thy-1-free (control) column.
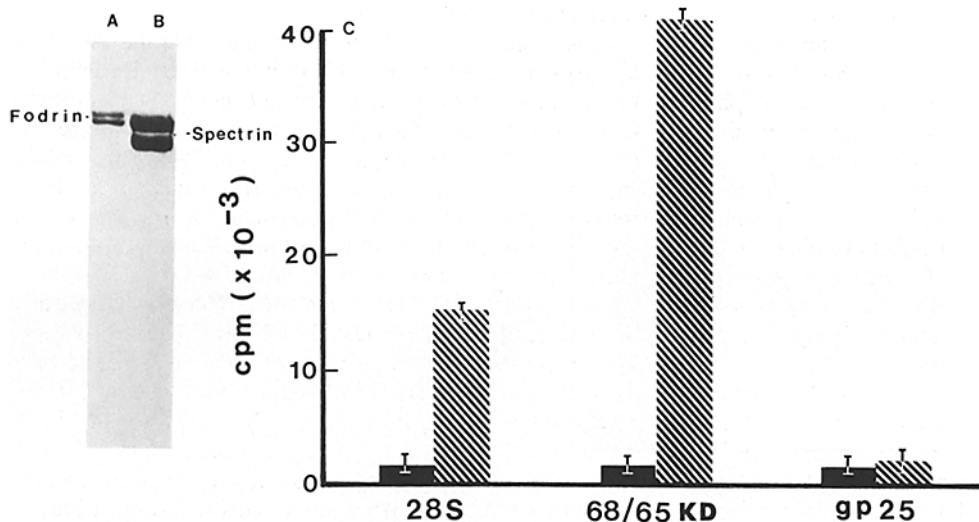
to the insignificant amount of binding between fodrin (or spectrin) and isolated Thy-1 (Fig. 11). We believe that this fodrin- (or spectrin-) binding assay is specific, since there is only a minimal amount of nonspecific binding of isolated lymphoma proteins (i.e. 68/65 kD, 28 S, or Thy-1) to BSA-conjugated Sepharose beads.

Discussion

It has been well established that actomyosin-containing components are closely associated with lymphocyte receptor patch and cap structures (11, 12). Recently, analogs of the erythrocyte membrane–cytoskeleton proteins, such as spectrin, ankyrin, and protein 4.1, have also been detected in lymphocytes (12, 16, 18, 42, 48). Most important, lymphocyte surface membranes appear to be selectively associated with the immunoreactive forms of spectrin (so-called fodrin), ankyrin, and protein 4.1, all of which co-cap with surface receptor cap structures (11, 12, 16, 18, 42, 48).

Recently, we have found that two lymphocyte transmembrane glycoproteins, gp 180 and gp 85, are closely associated with fodrin and ankyrin-like proteins, respectively (16, 18). Specifically, we have demonstrated that gp 180 (a transmembrane glycoprotein) is preferentially associated with fodrin, in a 1:1 molar ratio (16). Another lymphoma glycoprotein, gp 85, is tightly bound to an ankyrin-like protein in a stable complex that resembles the erythrocyte band 3–ankyrin complex (18). Furthermore, this gp 85–ankyrin complex is capable of binding the actin-binding protein, fodrin (18). Therefore, the functional involvement of fodrin in linking the lymphoma glycoprotein (gp 180 and gp 85/ankyrin-like complex) to actin filaments is strongly implicated.

As part of our continued interest in exploring the transmembrane interactions which occur between surface receptor molecules and the cytoskeleton, we have investigated whether there is a 4.1-like protein in lymphoma membrane–cytoskeleton preparations. Previously, immunoreactive forms of protein 4.1 have been detected in several different cell types including lymphocytes (62). Spiegel et al. (62) reported that in lymphocytes the immunoreactive form of protein 4.1 appears as a 68,000-D protein by SDS PAGE, im-

munoblotting, and autoradiographic techniques. Our immunoprecipitation experiments using a specific erythrocyte 4.1 antibody followed by SDS PAGE and autoradiographic analysis reveals that the lymphoma 4.1-like protein consists of a doublet of 68,000 and 65,000 D. It is not surprising to find a 4.1-like protein in lymphocytes that differs in molecular mass from the erythrocyte band 4.1. Molecular mass variants have been observed in a number of different cell types of both erythroid and non-erythroid origin (5, 23, 33, 34, 62). These variations clearly do not necessarily reflect differences in 4.1 function since all of the different molecular mass forms still retain their ability to bind spectrin or fodrin (6).

The lymphoma 4.1-like protein represents a component of the insoluble cytoskeletal fraction of lymphoma membranes prepared by non-ionic detergent (NP-40) extraction (Figs. 3 and 4). This 4.1-like protein can be phosphorylated either by metabolically labeling cells with $H_3{}^{32}PO_4$ (Figs. 3, *A* and *B*; 4 *A*; and 5, *A* and *B*) or by labeling isolated plasma membrane with [γ-^{32}P]ATP in vitro in the presence of Ca^{2+} (Fig. 5 *D*) (but not in the absence of Ca^{2+} [Fig. 5 *C*]). The requirement of Ca^{2+} for phosphorylation suggests the possibility that the enzyme, protein kinase C, might be involved in this phosphorylation reaction.

Recently, Eder et al. (29) have reported that erythrocyte membrane kinases and casein kinase A phosphorylate erythrocyte band 4.1 on both serine and threonine residues. Our phosphoamino acid analysis indicates that lymphoma 4.1-like protein is phosphorylated at serine residues (very little phosphorylation can be detected in threonine and none in tyrosine residues) (Fig. 6). This observation suggests that the lymphoma 4.1-like protein might share homology with erythrocyte band 4.1 serving as a substrate for protein kinase(s) (6, 38, 50, 60). At this time, the regulatory mechanism involved in the phosphorylation of this 4.1-like protein is unknown.

Phorbol esters such as TPA are known to stimulate a number of biological activities at nanomolar concentrations (28, 47). There is good evidence that phorbol esters can directly activate protein kinase C, an enzyme that normally requires both phospholipid and calcium for its activity (4, 49, 50, 51, 63). It is known that protein kinase C is activated specifically by

diacylglycerol which is generated during hormonal stimulation of phosphatidylinositol turnover (63). In vitro, TPA can substitute for diacylglycerol and directly activate protein kinase C in the presence of both Ca^{2+} and phospholipid (4, 49, 50, 63). Recently, it has been reported that the phosphorylation of band 4.1 in intact erythrocytes is stimulated by phorbol ester, suggesting the possible involvement of protein kinase C in the phosphorylation of erythrocyte band 4.1 (43). The observations that (a) phorbol ester (TPA) enhances the phosphorylation of lymphoma 4.1-like protein (Fig. 5, A and B) on serine residues (Fig. 6, a–d), and (b) phosphorylation of the lymphoma 4.1-like protein requires the presence of Ca^{2+}, in vitro, strongly suggest that the lymphoma 4.1-like protein is a substrate for protein kinase C.

Recent studies in erythrocytes indicate that the band 4.1, in addition to being a spectrin-binding protein, may also interact directly with the erythrocyte membrane after the removal of peripheral membrane proteins (1, 53, 59). At least two membrane-binding sites for 4.1 have been identified: a high affinity site on glycophorin A (1), and a low affinity site associated with band 3 (53). Interestingly, the association between glycophorin and protein 4.1 appears to be regulated by polyphosphoinositides (2). Studies done with phosphatidyl-serine-containing liposomes have also led to the speculation that phospholipid domains alone could serve as an alternative anchoring site for 4.1, as well as other cytoskeletal proteins (56).

The Thy-1 alloantigenic determinant is a glycoprotein of 25,000 D which, in the mouse, has been termed T-25 (designated as gp 25 in this paper). Both thymocyte and brain Thy-1 glycoproteins display hydrophobic properties which may be responsible for their binding to the plasma membrane (69). However, the initial determination of the sequence of rat brain Thy-1 glycoprotein failed to detect the presence of a hydrophobic segment (20). This has prompted speculation that Thy-1 insertion into the lipid bilayer may be through some hydrophobic components such as a phosphatidylinositol-like molecule that would link the Thy-1 glycoprotein to the membrane (44). Recently, DNA sequence analysis has revealed a 31-amino acid hydrophobic segment that may be important for the integration of the Thy-1 molecule into the plasma membrane (21, 57). Like purified glycophorin (31), purified Thy-1 antigen exists as an aggregate of 10–14 Thy-1 monomers (69). This aggregated form of Thy-1 (gp 25) may represent pre-existing natural clusters of receptors in the plane of the membrane.

In this study, using complementary techniques such as double-label immunofluorescence staining (Fig. 4, a and b), immunoprecipitation (Fig. 4, A–D), and sucrose gradient centrifugation (Fig. 7), we have established that a 4.1-like protein is closely associated with gp 25 (Thy-1 antigen) in the form of a 28-S complex with a stoichiometric ratio of 1:4 (Fig. 8). The fact that we are able to dissociate and then reassociate the 4.1-like protein and Thy-1 molecule, by adding and then removing 2 M urea (Figs. 9 and 10), suggests that these two proteins specifically interact with each other. Since the binding domain of Thy-1 to the 4.1-like protein has not been determined, we do not preclude the possibility that there may be other minor components (either lipid or protein molecules) responsible for linking the Thy-1 antigen to the 4.1-like protein in lymphoma membranes. Further analysis of the interactions between the lymphoma 4.1-like protein

and other transmembrane glycoproteins is currently under investigation in our laboratory.

Most importantly, we have determined that the Thy-1/4.1-like protein complex is capable of interacting with actin filaments via fodrin or spectrin (data not shown). This property is one of the best-known characteristics of erythrocyte protein 4.1 that is known to be a spectrin-binding protein (66–68). Further analysis using a fodrin- (or spectrin-) binding assay confirms that the lymphoma 4.1-like protein (not the Thy-1 antigen) is responsible for the binding of the complex to fodrin or spectrin (Fig. 11).

Our evidence for identifying the 68/65-kD doublet as a 4.1-like protein is summarized as follows: (a) it displays an immunological cross-reactivity with anti-erythrocyte band 4.1 antibody; (b) it exhibits a Svedberg unit of sedimentation coefficient of 4 S; (c) it can be readily phosphorylated in the presence of TPA and Ca^{2+} (but not in the absence of Ca^{2+}); (d) its major sites for phosphorylation are on serine residues; and (e) it can interact directly with fodrin. In conclusion, we believe our data clearly support the notion that certain linker molecules (e.g., 4.1-like and ankyrin-like proteins) as well as fodrin play a pivotal role in linking surface membrane receptors to the intracellular actin-containing microfilament network whose contraction is required for receptor capping.

References

1. Anderson, R. A., and R. E. Lovrien. 1984. Glycophorin is linked by band 4.1 protein to the human erythrocyte membrane skeleton. *Nature (Lond.).* 307:655–658.

2. Anderson, R. A., and V. T. Marchesi. 1985. Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide. *Nature (Lond.).* 318:295–298.

3. Arndt, R., R. Stark, and H. G. Thiele. 1977. Detection and molecular characterization of the thymus-brain antigen in human brain. *Immunology.* 33:101–107.

4. Ashendel, C. L., J. M. Staller, and R. K. Boutwell. 1983. Identification of a calcium and phospholipid-dependent phorbol ester-binding activity in the soluble fraction of mouse tissue. *Biochem. Biophys. Res. Commun.* 111:340–345.

5. Aster, J. C., G. J. Brewer, S. M. Hanash, and H. Maisel. 1984. Band 4.1-like proteins of the bovine lens. *Biochem. J.* 224:609–616.

6. Baines, A. J., and V. Bennett. 1985. Synapsin I is a spectrin-binding protein immunologically related to erythrocyte protein 4.1. *Nature (Lond.).* 315:410–413.

7. Beech, J. N., R. J. Morris, and G. Raisman. 1983. Density of Thy-1 on axonal membrane of different rat nerves *J. Neurochem.* 41:411–417.

8. Bennett, V. 1985. The membrane skeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* 54:273–304.

9. Bennett, V., J. Davis, and W. E. Fowler. 1982. Brain spectrin, a membrane-associated protein related in structure and function to erythrocyte spectrin. *Nature (Lond.).* 299:126–131.

10. Bonner, W. M., and R. A. Laskey. 1974. Film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.

11. Bourguignon, L. Y. W. 1986. Ligand-induced surface receptor movement and transmembrane interactions. *In* Energetics of Secretory Responses. J. W. N. Akkerman, editor. CRC Press, Inc. Boca Raton, FL. In press.

12. Bourguignon, L. Y. W., and G. J. Bourguignon. 1984. Capping and the cytoskeleton. *Int. Rev. Cytol.* 87:195–224.

13. Bourguignon, L. Y. W., and S. J. Singer. 1977. Transmembrane interac-

tions and the mechanism of capping of surface receptors by their specific ligands. *Proc. Natl. Acad. Sci. USA.* 74:5031–5035.

14. Bourguignon, L. Y. W., M. L. Nagpal, K. Balazovich, V. Guerriero, and A. R. Means. 1982. Association of myosin light chain kinase with lymphocyte membrane–cytoskeleton complex. *J. Cell Biol.* 95:793–797.

15. Bourguignon, L. Y. W., M. Nagpal, and Y. C. Hsing. 1981. Phosphorylation of myosin light chain during capping of T-lymphoma cells. *J. Cell Biol.* 91:889–894.

16. Bourguignon, L. Y. W., S. J. Suchard, M. L. Nagpal, and J. R. Glenney, Jr. 1985. A lymphoma transmembrane glycoprotein (gp 180) is linked to the cytoskeletal protein, fodrin. *J. Cell Biol.* 101:477–487.

17. Bourguignon, L. Y. W., T. Tokuyasu, and S. J. Singer. 1978. The capping of lymphocytes and other cells, studied by an improved method for immunofluorescence staining of frozen sections. *J. Cell. Physiol.* 95:239–258.

18. Bourguignon, L. Y. W., G. Walker, S. Suchard, and K. Balazovich. 1986. A lymphoma plasma membrane-associated protein with an ankyrin-like properties. *J. Cell Biol.* 102:2115–2124.

19. Butman, B. T., G. J. Bourguignon, and L. Y. W. Bourguignon. 1980. Lymphocyte capping induced by polycationized ferritin. *J. Cell. Physiol.* 105:7–15.

20. Campbell, D. G., J. Gagnon, K. B. M. Reid, and A. F. Williams. Rat brain Thy-1 glycoprotein. 1981. *Biochem. J.* 195:15–30.

21. Chang, H-C., T. Moriuchi, R. Denome, H. Ploegh, and J. Silver. 1985. A hydrophobic transmembrane segment at the carboxyl terminus of Thy-1. *Science (Wash. DC).* 227:649–651.

22. Cohen, C. M., and R. C. Langley. 1984. Functional characterization of human erythrocyte spectrin alpha and beta chains: association with actin and erythrocyte protein 4.1. *Biochemistry.* 23:4488–4495.

23. Cohen, C. M., S. F. Foley, and C. Korsgreu. 1982. A protein immunologically related to erythrocyte 4.1 is found on stress fibers on non-erythroid cells. *Nature (Lond.).* 299:648–650.

24. Cohen, F. E., J. Novotny, M. J. E. Sternberg, D. G. Campbell, and A. F. Williams. 1981. Analysis of structural similarities between brain Thy-1 antigen and immunoglobulin domains. *Biochem. J.* 195:31–40.

25. Cooper, J. A., B. M. Sefton, and T. Hunter. 1983. Detection and quantitation of phosphotyrosine in proteins. *Methods Enzymol.* 99:387–402.

26. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* 245:3059–3065.

27. Dalchau, R., and J. W. Fabre. 1979. Identification and unusual tissue distribution of the canine and human homologies of Thy-1. *J. Exp. Med.* 149:576–591.

28. Dicker, P., and E. Rozengurt. 1980. Phorbol esters and vasopressin stimulate DNA synthesis by a common mechanism. *Nature (Lond.).* 287:607–612.

29. Eder, P. S., C. J. Soong, and M. Tao. 1986. Phosphorylation reduces the affinity of protein 4.1 for spectrin. *Biochemistry.* 25:1770–1776.

30. Fraker, P. J., and J. C. Speck. 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849–857.

31. Furthmayer, H., and V. T. Marchesi. 1983. Glycoproteins: isolation, orientation, and localization of specific domains. *Methods Enzymol.* 96:268–280.

32. Glenney, J. R., P. Glenney, and K. Weber. 1982. F-actin binding and cross-linking properties of porcine brain fodrin, a spectrin-related molecule. *J. Biol. Chem.* 257:9781–9787.

33. Goodman, S. R., L. Casoria, D. Coleman, and I. S. Zagon. 1984. Identification and location of brain protein 4.1. *Science (Wash. DC).* 224:1433–1435.

34. Granger, B. L., and E. Lazarides. 1984. Membrane skeletal protein 4.1 of avian erythrocytes is composed of multiple variants that exhibit tissue-specific expression. *Cell.* 37:595–607.

35. Gunter, K. C., T. R. Malek, and E. M. Shevach. 1984. T-cell activating properties of an anti-Thy-1 monoclonal antibody possible analogy to OKT3/Leu-4. *J. Exp. Med.* 159:716–730.

36. Hunkapiller, M. W., E. Lujan, F. Ostrander, and L. E. Hood. 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. *Methods Enzymol.* 91:227–236.

37. Johnson, G. L., and H. R. Bourne. 1977. Influence of cholera toxin on the regulation of adenylate cyclase by GTP. *Biochem. Biophys. Res. Commun.* 78:792–798.

38. Johnson, E. M., T. Ueda, H. Maeno, and P. Greengard. 1972. Site-specific fluorescein-labeled cobra α-toxin. *J. Biol. Chem.* 257:5632–5636.

39. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature (Lond.).* 227:680–685.

40. Laub, F., M. Kaplan, and C. Gitler. 1981. Actin polymerization accompanies Thy-1 capping on mouse thymocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 124:35–38.

41. Leifer, D., S. A. Lipton, C. J. Barnstable, and R. H. Masland. 1984. Monoclonal antibody to Thy-1 enhances regeneration of processes by rat retinal ganglion cells in culture. *Science (Wash. DC).* 224:303–306.

42. Levine, J., and M. Willard. 1983. Redistribution of fodrin (a component of the cortical cytoplasm) accompanying capping of cell surface molecules. *Proc. Natl. Acad. Sci. USA.* 80:191–195.

43. Ling, E., and V. Sapirstein. 1984. Phorbol ester stimulates the phosphorylation of rabbit erythrocyte band 4.1. *Biochem. Biophys. Res. Commun.* 120:291–298.

44. Low, M. G., and P. W. Kincade. 1985. Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein. *Nature (Lond.).* 318:62–64.

45. MacDonald, H. R., C. Bron, M. Rousseaux, C. Horvath, and J. C. Cerottini. 1985. Production and characterization of monoclonal anti-Thy-1 antibodies that stimulate lymphokine production by cytolytic T-cell clones. *Eur. J. Immunol.* 15:495–501.

46. Maino, V. C., M. A. Norcross, M. S. Perkins, and R. T. Smith. 1981. Mechanism of Thy-1 mediated T-cell activation: roles of Fc receptors, T-200, Ia and H-2 glycoproteins in accessory cell function. *J. Immunol.* 126:1829–1836.

47. Mastro, A. M. 1983. Phorbol ester tumor promoters and lymphocyte proliferation. *Cell Biol. Int. Rep.* 7:881–893.

48. Nelson, W. J., C. A. L. S. Colaco, and E. Lazarides. 1983. Involvement of spectrin in cell-surface receptor capping in lymphocytes. *Proc. Natl. Acad. Sci. USA.* 80:1626–1630.

49. Niedel, J. E., L. J. Kuhn, and G. R. Vandenbark. 1983. Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA.* 80:36–40.

50. Nishizuka, Y. 1983. Phospholipid degradation and signal translation for protein phosphorylation. *Trends Biochem. Sci.* 8:13–16.

51. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.).* 308:693–698.

52. Ohanian, V., L. C. Wolfe, K. W. John, V. C. Pinder, S. E. Lux, and W. B. Gratzer. 1984. Analysis of ternary interaction of the red cell membrane skeletal proteins, spectrin, actin and 4.1. *Biochemistry.* 23:4416–4420.

53. Pasternak, G., R. A. Anderson, T. L. Leto, and V. T. Marchesi. 1985. Interactions between protein 4.1 and band 3: an alternative binding site for an element of the membrane skeleton. *J. Biol. Chem.* 260:3676–3683.

54. Pearl, M., D. Fishkind, M. Mooseker, D. Keene, and T. Keller III. 1984. Studies on the spectrin-like protein from the intestinal brush border, TW 260/240 and characterization of its interaction with the cytoskeleton and actin. *J. Cell Biol.* 98:66–78.

55. Reif, A. E., and J. M. V. Allen. 1964. The AKR thymic antigen and its distribution in leukaemias and nervous tissues. *J. Exp. Med.* 120:413–433.

56. Sato, S. B., and S. Ohnishi. 1983. Interaction of a peripheral protein of the erythrocyte membrane, band 4.1, with phosphatidylserine-containing liposomes and erythrocyte inside-out vesicles. *Eur. J. Biochem.* 130:19–25.

57. Seki, T., T. Moriuchi, H.-C. Chang, R. Denome, and J. Silver. 1985. Structural organization of the rat Thy-1 gene. *Nature (Lond.).* 313:485–487.

58. Sheeler, P. 1981. *In* Centrifugation in Biology and Medical Science. John Wiley & Sons, Inc., New York. p. 26.

59. Shiffer, K. A., and S. R. Goodman. 1984. Protein 4.1: its association with the human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA.* 81:4404–4408.

60. Sieghart, W., J. Forn, and P. Greengard. Ca^{2+} and cyclic AMP regulate phosphorylation of same two membrane-associated proteins specific to nerve tissue. *Proc. Natl. Acad. Sci. USA.* 76:2475–2479.

61. Smith, M. H. 1968. Molecular weights of proteins and some other materials including sedimentation, diffusion, and frictional coefficients and partial specific volumes. *In* CRC Handbook of Biochemistry. 3–30.

62. Spiegel, J. E., D. S. Beardsley, F. S. Southwick, and S. E. Lux. 1984. An analogue of the erythroid membrane skeleton protein 4.1 in nonerythroid cells. *J. Cell Biol.* 99:886–893.

63. Takai, Y., A. Kishimoto, and Y. Nishizuka. 1982. *In* Calcium and Cell Function. W. Y. Cheung, editor. Academic Press, Inc., New York. 2:386–396.

64. Trowbridge, I., and C. Mazauskas. 1976. Immunological properties of murine thymus-dependent lymphocyte surface glycoproteins. *Eur. J. Immunol.* 6:557–562.

65. Tse, A. G. D., N. Barclay, A. Watts, and A. F. Williams. 1985. A glycophospholipid tail at the carboxyl terminus of the Thy-1 glycoprotein of neurons and thymocytes. *Science (Wash. DC).* 230:1003–1008.

66. Tyler, J. M., W. R. Hargraves, and D. Branton. 1979. Purification of two spectrin-binding proteins: biochemical and electron microscopic evidence for site specific reassociation between spectrin and band 2.1 and 4.1. *Proc. Natl. Acad. Sci. USA.* 76:5192–5196.

67. Tyler, J. M., B. N. Reinhardt, and D. Branton. 1980. Associations of erythrocyte membrane proteins. Binding of purified bands 2.1 and 4.1 to spectrin. *J. Biol. Chem.* 255:7034–7039.

68. Ungewickell, E., P. M. Bennett, R. Calvert, V. Ohanian, and N. B. Gratzer. 1979. *In vitro* formation of a complex between cytoskeletal proteins of the human erythrocyte. *Nature (Lond.).* 280:811–814.

69. Williams, A. F., A. N. Barclay, M. Letarte-Mauirhead, and R. J. Morris. 1976. Rat Thy-1 antigens from thymus and brain: their tissue distribution, purification, and chemical composition. *Cold Spring Harbor Symp. Quant. Biol.* 41:51–61.