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A T-Lymphoma Transmembrane Glycoprotein (gp180) Is Linked to the Cytoskeletal Protein, Fodrin

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ABSTRACT A major mouse T-lymphoma surface glycoprotein (gp180) has been identified by labeling cells with ^{125}I and [^{3}H]glucosamine. After ligand-induced receptor patching and/or capping, the amount of gp180 in the membrane-associated cytoskeleton fraction increases in direct proportion to the percentage of patched/capped cells. There is a parallel increase in the amount of fodrin in the membrane-associated cytoskeleton fraction. Evidence is presented that gp180 is the same as or very similar to the T-lymphocyte-specific glycoprotein T-200. An immunobinding assay of Nonidet P-40–solubilized plasma membrane selectively co-isolates gp180 and fodrin. After induction of receptor rearrangement, double-label immunofluorescence reveals that fodrin accumulated directly beneath gp180 patches and caps. Membrane extraction with Triton X-114 followed by sucrose gradient centrifugation permits isolation of a gp180–fodrin complex with a 1:1 molar ratio and sedimentation coefficient(s) of approximately 20. This complex remains stable during isoelectric focusing and exhibits a pI in the range of 5.2–5.7. On the basis of our results we conclude that gp180, an intergral membrane glycoprotein, and fodrin, a component of the membrane-associated cytoskeleton, are closely associated into a complex. Furthermore, we contend that, through fodrin's association with actin, this complex is of functional significance in ligand-induced patching and capping of gp180. We also propose that, through lateral interactions in the plane of the membrane, the gp180-fodrin complex might be responsible for linking other surface receptors to the intracellular microfilament network during lymphocyte patching and capping.

The involvement of contractile microfilaments in the redistribution of cell surface receptors during patching and capping was first described by Taylor et al. in 1971 (1). Since then, numerous reports have confirmed this observation for a variety of different surface receptors of mouse and human lymphocytes (2, 3). Double-label immunofluorescence microscopy reveals that cytoplasmic actin and myosin accumulate directly beneath patches and caps; that is, beneath aggregated surface receptors (4–7). More conclusively, isolation of the plasma membrane (PM)[1] and associated cytoskeletal elements with non-ionic detergents demonstrates that, during capping, the surface receptors form a specific association with the actin-containing cytoskeleton (8, 9). However, the nature of the linkage between membrane surface receptors and the cytoskeleton is not understood.

Recently, analogs to proteins of the erythrocyte membrane–cytoskeleton complex (such as spectrin, ankyrin, and glycophorin) have been identified in nonerythroid cells (10–14). Two distinct, spectrin-like proteins have been identified: fodrin (15–18) isolated from brain; and TW 260/240 (16, 17) isolated from epithelial cells of the intestinal brush border. Like spectrin (19–21), each of these proteins has one 240,000-mol-wt subunit and a second subunit of differing molecular weight: 220,000 for spectrin (22); 235,000 for fodrin (15–18); and 260,000 for TW 260/240 (16, 17). Immunological assays and peptide mapping data indicate that the 240,000-mol-wt subunit of spectrin, fodrin, and TW 260/240 are very similar (15–17, 23, 24). In addition, all three proteins bind calmodulin and actin (15–17).

In the past several years, we have studied receptor patching and capping in the mouse T-lymphoma cell line, BW 5147

[1] *Abbreviations used in this paper:* IEF, isoelectric focusing; NP-40, Nonidet P-40; PBES, phosphate-buffered Earle's balanced salt solution; PM, plasma membrane.

(25–32). The major advantages of these cells are the ease of PM isolation (33) and the existence of detailed biochemical and serological characterization of several of the cell surface glycoproteins: T-200, a major T-lymphocyte–specific glycoprotein; Thy-1, a common T-lymphocyte surface marker; and viral glycoprotein gp 69–71, a known surface component of lymphoma cell membranes (29, 31, 34). Previous studies showed that a number of different membrane proteins are associated with the cytoskeleton in murine cells (35, 36).

In this study, we chose to investigate the relationship between surface membrane receptors and underlying cytoskeletal elements during receptor patching and capping. Biochemical and immunological analyses of isolated PM and the membrane-associated cytoskeleton indicate a close interaction between fodrin, a component of the membrane-associated cytoskeleton, and gp180, a cell surface glycoprotein. Furthermore, our double-immunofluorescence data also show that intracellular fodrin appears to be accumulated preferentially under gp180 patched/capped structures. Analysis of the unchallenged lymphoma PM, using a selective Triton X-114 extraction method followed by sucrose gradient centrifugation, reveals that gp180 and fodrin are tightly associated into a complex with a stoichiometry of 1:1 and a sedimentation coefficient of ~20. Further analysis of the complex by isoelectric focusing (IEF) indicates that gp180 and fodrin remain in a stable complex that exhibits a pI in the range of 5.2–5.7. We therefore propose that this structural interaction between gp180 and fodrin is important in the functional association of the PM and the cytoskeleton during receptor patching and capping.

MATERIALS AND METHODS

Cell Culture

All experiments were conducted on the permanent mouse T-lymphoma cell line, BW 5147, obtained from Dr. R. Hyman (Salk Institute). Cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum (Gibco Laboratories, Grand Island, NY) and equilibrated in 5% CO_2/95% air.

Induction of Patching and Capping

Cell cultures were harvested at a density of ~1×10^6 cells/ml and immediately chilled to 0°C. Cells were washed with ice-cold, serum-free RPMI 1640 medium (Gibco Laboratories) and resuspended in the same medium. Ligands were added directly to chilled cell suspensions. Ligands used include the monoclonal rat antibodies to the lymphocyte surface glycoproteins Thy-1 and gp180 (T-200) (gifts kindly provided by Dr. Ian Trowbridge [Salk Institute]). For fluorescence visualization, monoclonal antibody–labeled cells require a secondary incubation with fluorescein-labeled goat anti-rat IgG. To induce patch and cap formation, ligand-labeled cells were subsequently incubated at room temperature or at 37°C for 20 min. To demonstrate receptor distribution prior to ligand-induced patching/capping, cells were washed with RPMI 1640 medium and fixed with 2% paraformaldehyde prior to a two-stage incubation with monoclonal antibody and fluorescein-labeled goat anti-rat IgG. These cells form neither patches nor caps because their receptors are immobilized by fixation prior to ligand incubation.

Double-label Immunofluorescence Microscopy

Fluorescein-labeled gp180 (T-200) patched/capped cells were rendered permeable by methanol treatment (32) and stained with rabbit antibody against pig brain fodrin (anti-fodrin) (17). These cells were then incubated with rhodamine-conjugated goat antibody against rabbit IgG to visualize fodrin distribution. To detect any nonspecific antibody binding, cells were first incubated with antisera preabsorbed with soluble, native fodrin to remove all anti-fodrin antibodies. These cells were then incubated with rhodamine-conjugated goat anti-rabbit IgG. No labeling was observed in such control samples.

The fluorescein- and rhodamine-labeled samples were examined with a Zeiss photomicroscope using a 63x-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).

Radioactive Labeling of Cellular Proteins

Surface proteins were labeled with ^{125}I using either the lactoperoxidase and glucose oxidase methods described by Hubbard and Cohn (37) or by the iodogen method of Fraker and Speck (38). To metabolically label proteins with [^{35}S]methionine or to label glycoproteins with [^{3}H]glucosamine, cell cultures at ~1×10^5 cells/ml were incubated with either [^{35}S]methionine (1,000 Ci/mmol) or [^{3}H]glucosamine (100 Ci/mmol) (ICN K&K laboratories Inc., Plainview, NY) in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum for 16 h at 37°C in 5% CO_2/95% air.

PM isolation

PM was isolated using the method described by Johnson and Bourne (33) with slight modifications. Cells labeled with ^{125}I, [^{3}H]glucosamine, or [^{35}S]-methionine were harvested by low speed centrifugation (500 g_{av}), washed with Dulbecco's phosphate-buffered saline (PBS), and resuspended in a solution of 20 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, pH 7.4, 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. The resulting pellet was resuspended in 10% sucrose (wt/wt) in the same buffer, layered over a discontinuous gradient of 20%, 30%, and 40% sucrose (wt/wt), and spun at 100,000 g_{av} for 2 h. The PM fraction was collected from the 30–40% interface and washed with Dulbecco's PBS. Enzyme marker assays (5′ nucleotidase and/or Na^+/K^+-ATPase activities) showed that these PM preparations were at least 20-fold purified and contained negligible amounts of other membranous organelle contaminants (32).

Non-ionic Detergent Extraction

NONIDET P-40 (NP-40) EXTRACTION: Isolated PM was washed and resuspended in Earle's balanced salt solution buffered to pH 7.2 with 10 mM sodium phosphate (phosphate-buffered Earle's balanced salt solution) (PBES) containing 1 mM phenylmethylsulfonyl fluoride and 10 mM iodoacetamide. Detergent extraction was accomplished by adding NP-40 to the PBES 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 and cytochemical analysis as described previously (32). Previous studies have shown that the NP-40–insoluble fraction contains the membrane-associated cytoskeleton (9, 32, 36).

TRITON X-114 EXTRACTION: Isolated ^{125}I- or [^{35}S]methionine-labeled lymphocyte PM was washed in PBES and then resuspended in 200 μl of 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 1.0% (vol/vol) Triton X-114. The protein concentration in all samples was kept at ~0.5 mg/200 μl. The samples were incubated at 0°C for 30 min with vortexing at 5-min intervals and partitioned according to the method of Bordier (39). The resulting lower "detergent" phase and upper "aqueous" phase were then analyzed by SDS PAGE combined with autoradiography. The lower phase is not an exclusively detergent-containing phase as it contains hydrophilic cytoskeletal proteins that co-sediment with the detergent micelles.

Negative Staining Procedures for Electron Microscopy

For negative staining, three successive drops of either a solution of 0.1 M KCl, 0.01 M immidazole, pH 7.0, containing intact PMs or a 1% NP-40–extracted insoluble fraction of the PMs from cells were added to 4% Formvar-coated grids. Then three drops of 1% uranyl acetate in water (pH 4.5) were applied to the samples for 30 s and removed with filter paper. These negatively stained samples were examined in a Philips 300 electron microscope.

Sucrose Gradient Centrifugation Analysis

Isolated ^{125}I- or [^{35}S]methionine-labeled PM was extracted with 1.0% (vol/vol) Triton X-114 for 30 min and partitioned into "aqueous" and "detergent"

phases. The ^{125}I- or [^{35}S]methionine-labeled aqueous phase was then loaded onto an 11-ml linear sucrose gradient (6–26% in 150 mM NaCl, 10 mM Tris-HCl, pH 7.2) with a 0.5-ml cushion of 65% sucrose. The gradient was centrifuged at 70,000 g_{av} for 22 h. Twenty-six 0.5-ml fractions were collected from the bottom of each tube. The fractions from the gradient that contained the ^{125}I-labeled aqueous phase were then counted on an LKB MiniGamma gamma counter (LKB Instruments, Inc., Gaithersburg, MD) to determine the distribution of surface iodinated protein (i.e., gp180) within the gradient. The total protein concentration in each fraction was determined by the absorbance at 280 nm. Fractions that corresponded with either the 280-nm protein peak or ^{125}I-labeled gp180 peak were pooled and precipitated with 10% TCA, and subsequently analyzed by SDS PAGE and autoradiographic treatment as described below. The [^{35}S]methionine-labeled fractions that corresponded to the ^{125}I-labeled gp180 peak were TCA precipitated and the individual fractions analyzed by SDS PAGE, autoradiography, and scanning densitometry as described below.

IEF

Isolated ^{125}I-labeled PM was extracted with 1.0% (vol/vol) Triton X-114 for 30 min and the aqueous phase loaded onto a sucrose gradient as described above. The fractions that contained the ^{125}I-labeled gp180 were then pooled and dialyzed against 10 mM Tris-HCl, pH 7.2, 50 mM NaCl, 15% glycerol, and 0.01% Triton X-114 for 4 h. IEF was carried out in an LKB 2117 Multiphor flat-bed electrofocusing unit (LKB Instruments, Inc.) according to the procedure of Lee et al. (40) with a few minor modifications. 100 ml of a 4% Ultradex that contained 5% Biorad 3/10 ampholines in 15% glycerol with 0.01% Triton X-114 was poured into the bed and evaporated to 36% of the original wet weight. The dialyzed sample (~3 ml) was then applied as a narrow zone approximately mid-way between the anode and the cathode. The sample was focused for 9 h at 4°C, and the gel bed was divided into 30 fractions by a fractionating grid. A small portion of each gel fraction was used to determine pH, and the rest was eluted with 5 ml of 10 mM Tris-HCl, pH 7.2, 50 mM NaCl, and 0.01% Triton X-114. Five consecutive aliquots, starting from the anode, were pooled; the proteins were precipitated with 10% TCA, analyzed by SDS PAGE, and then silver stained (41).

SDS PAGE and Radioactivity Analysis

Electrophoresis was conducted using either an exponential polyacrylamide gradient (6.0–17.0%) or a 6% polyacrylamide slab gel and the discontinuous buffer system described by Laemmli (42). 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 then heated at 100°C for 2 min. For each sample, ~150–200 μg of protein was applied to the gel. Electrophoresis was conducted at a constant current of 20 mA at room temperature for 3 h. The polypeptide banding pattern was revealed by staining with Coomassie blue. Gels containing samples labeled with ^{125}I, [^{3}H]glucosamine, or [^{35}S]methionine were processed through fluorography (43), vacuum dried, and exposed to Kodak X-ray (Omat XAR-5) film. To quantitate the relative amounts of gp180 and fodrin in each gel lane, autoradiograms of [^{35}S]methionine-labeled proteins were scanned on a Quick Scan R & D (Helena Laboratories, Beaumont, TX) scanning densitometer. For quantitative measurements of [^{3}H]glucosamine-labeled proteins, the specific peptide bands were cut out of the gel and the relative amounts of radioactivity determined using Aquasol (New England Nuclear, Boston, MA) and a liquid scintillation counter. Two-dimensional gel electrophoresis, i.e., IEF, followed by SDS PAGE, was performed according to the method of O'Farrell (44). The second dimension was run on a 7% polyacrylamide slab gel. Autoradiography of two-dimensional gels was carried out as described above.

Immunoblotting Technique

Isolated total PM and the NP-40–isolated fraction (i.e., the membrane-associated cytoskeleton) were electrophoresed either on an exponential polyacrylamide gel gradient (6.0–17.0%) or a 6% polyacrylamide gel that contained SDS as described above. The polypeptides were transferred to nitrocellulose sheets as described by Burnette (45). Nitrocellulose sheets were subsequently incubated with anti-fodrin (10 μg/ml), followed by incubation with ^{125}I–protein A, and analyzed by previously published autoradiographic procedures (17).

Immunobinding Procedures

In these experiments, antigenically active molecules were detected by binding with anti-gp180 (anti-T-200)-conjugated gel beads. To prepare anti-gp180–conjugated gel beads, rabbit antibody (IgG fraction) raised against gp180 (34) was added to glutaraldehyde-activated Ultrogel beads (ACA 22; LKB Instruments, Inc.) (46) and incubated at 4°C for 6 h. A small column of these conjugated immunobeads was prepared and samples of NP-40–solubilized, ^{125}I-labeled PM were passed through. This was followed by extensive washing with 0.1% NP-40. As a control for nonspecific binding, a column was prepared with preabsorbed anti-gp180–conjugated gel beads (anti-gp180 antibodies absorbed with excess gp180, anti-gp180–free IgG-conjugated gel beads). Cellular materials bound to the anti-gp180–conjugated beads were subsequently solubilized by SDS and analyzed by SDS PAGE and autoradiography.

Immunoprecipitation Procedures

In the direct immunoprecipitation procedure, the NP-40–solubilized, [^{35}S]-methionine-labeled PM was first centrifuged at 700 g_{av} for 5 min to pellet down large filamentous material. The supernatant was then incubated at 4°C for 30 min with 10 μg/ml of either monoclonal rat anti-gp180 (anti-T-200) or monospecific rabbit anti-fodrin. After the 30-min incubation, goat anti-rabbit immunoglobulin (100 μg/ml) was added to the anti-fodrin–treated samples and incubated overnight at 4°C to induce immunoprecipitation (34). To immunoprecipitate those proteins that interact with the monoclonal anti-gp180, it was necessary to add rabbit anti-rat immunoglobulin (100 μg/ml) for 30 min at 4°C followed by the overnight incubation with goat anti-rabbit immunoglobulin. The resulting immunoprecipitates were pelleted by centrifugation at 700 g_{av} for 4 min and washed three times with 0.2% NP-40 in PBES using the same centrifugation conditions. The immunoprecipitates were subsequently solubilized by SDS, analyzed by SDS PAGE and autoradiography, and quantitated by scanning densitometry.

RESULTS

Identification of Fodrin in Mouse T-Lymphoma Cells

In this study, when cells are exposed to multivalent ligands (i.e., antibodies raised against specific cell surface antigens) and undergo receptor redistribution, they are defined as "patched/capped" since such a sample contains a mixed population of patched and capped cells. Cells that are not treated with any ligand are called "unchallenged" cells. PM isolated from unchallenged mouse T-lymphoma cells contains vesicles of various sizes but no obvious contamination by nuclei, mitochondria, or rough endoplasmic reticulum (Fig. 1 *D*). SDS PAGE analysis resolves a number of membrane polypeptides with molecular weights ranging from ~300,000 to 17,000 (Fig. 1 *C*). Although the amount of actin associated with patched/capped membranes is greater than in unchallenged cell membranes, no other obvious differences exist in the polypeptide banding patterns (28). Treatment of the isolated PM with the non-ionic detergent, NP-40, solubilizes most of the total membrane proteins (91% in unchallenged cells and 87% in patched/capped cells) and leaves an insoluble residue that contains the membrane-associated cytoskeleton (47). Electron microscopic examination of such preparations reveals that this fraction is composed primarily of 50–70 Å actin-like filament bundles (Fig. 1 *H*). Indeed, SDS PAGE analysis of this membrane-associated cytoskeleton shows that actin, myosin, and a high molecular weight doublet (>200,000-mol-wt proteins) are among the major protein components (Fig. 1 *E*). We determined the molecular weights of the lymphocyte doublet to be 240,000 for the upper band and 235,000 for the lower band (Fig. 1, *C* and *E*) by comparison with purified spectrin-like proteins such as brain fodrin (Fig. 1 *a*). Immunoblotting with anti-fodrin reveals a strong cross-reactivity with the 240,000-mol-wt subunit but not with the 235,000-mol-wt subunit (Fig. 1 *b*). Using this same antibody, we can demonstrate that these fodrin-like molecules accumulate directly underneath receptor caps (Fig. 1, *c* and *d*). Given these data we consider the high molecular weight doublet to be a lymphoma-specific form of fodrin. Further

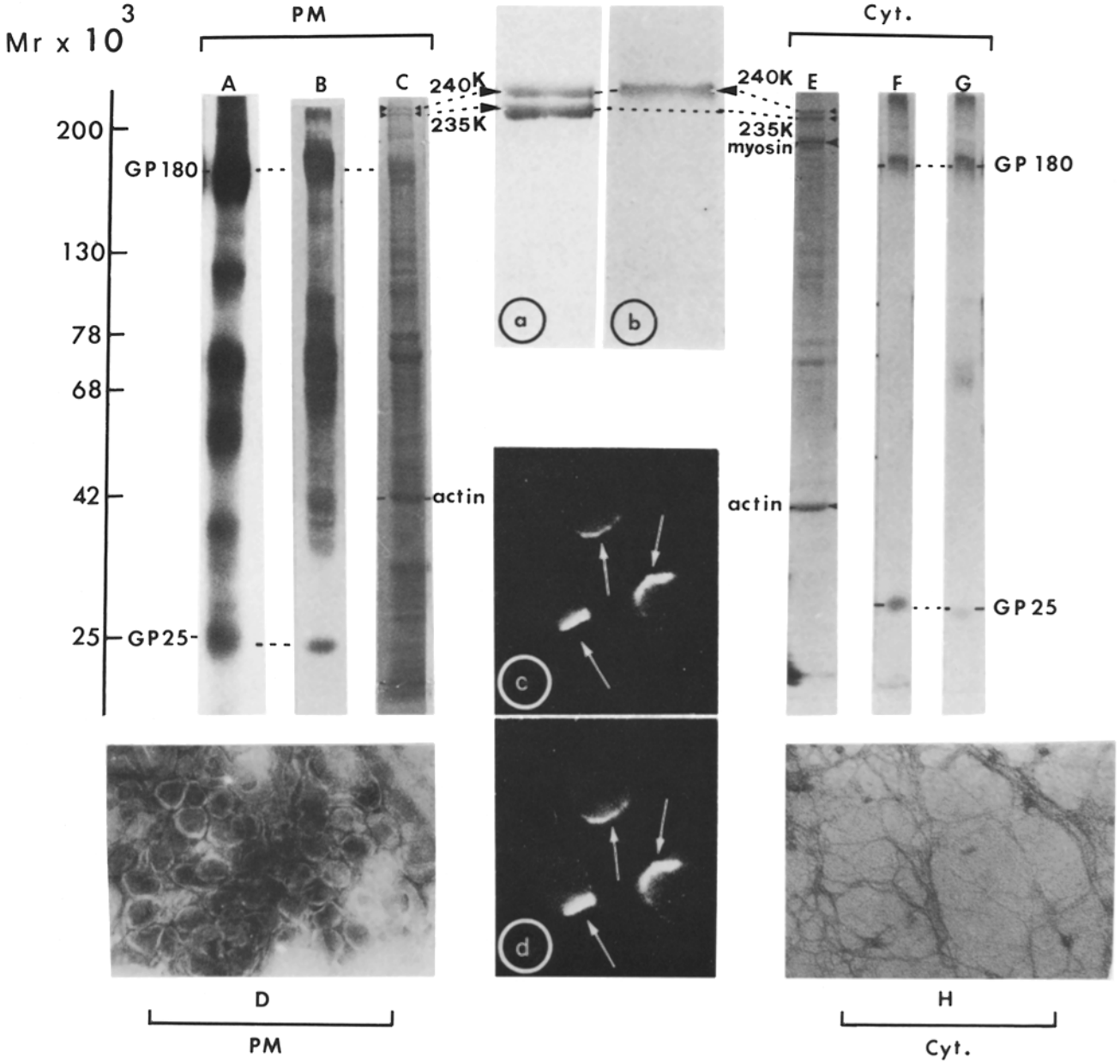

FIGURE 1 Biochemical and cytochemical analyses of isolated total PM (*A–D*) and NP-40-treated PM (primarily NP-40-insoluble fraction, referred to as cytoskeleton material [*cyt.*] hereafter) (*E–H*) from unchallenged mouse T-lymphoma cells. Arrowheads and dots indicate the position of the 240,000- and 235,000-mol-wt subunits (*240 K* and *235 K*) of lymphoma fodrin. (*A*) Autoradiogram of ^{125}I-labeled total PM proteins. (*B*) Autoradiogram of [^{3}H]glucosamine-labeled total PM proteins. (*C*) Coomassie Blue-stained total PM preparation. (*D*) Electron micrograph of isolated PM prepared by negative staining with 1% uranyl acetate. (*E*) Coomassie Blue-stained NP-40-insoluble fraction of PM (i.e., cytoskeleton material). (*F*) Autoradiogram of ^{125}I-labeled proteins which are associated with cytoskeleton material. (*G*) Autoradiogram of [^{3}H]glucosamine-labeled proteins which are associated with cytoskeleton material. (*H*) Electron micrograph of cytoskeleton material prepared by negative staining with 1% uranyl acetate. (*a*) Pig brain fodrin. (*b*) Autoradiogram of immunoblot of cytoskeleton material from unchallenged cells incubated first with anti-fodrin and then with ^{125}I-protein A. Comparable results were obtained with the cytoskeleton material from patched/capped cells and with total PM from unchallenged and patched/capped cells. Molecular weight markers (× 10^3) are as follows: myosin, 200,000; B-galactosidase, 130,000; lactoperoxidase, 78,000; bovine serum albumin, 68,000; actin, 42,000; concanavalin A, 25,000. (*c* and *d*) Double-immunofluorescence staining of mouse T-lymphoma cells for localization of cell surface receptors (*c*) and intracellular fodrin (*d*). Capped cells showing (*c*) surface distribution of fluorescein-conjugated-anti-T-200 and (*d*) intracellular rhodamine-conjugated-anti-fodrin. × 800.

analysis of the cytoskeletal material by two-dimensional gel electrophoresis (i.e., IEF) and SDS PAGE reveals that fodrin has a pI in the range of 6.1–6.4 (Fig. 2*A*) which is similar to that shown for spectrin (48). These data indicate that (*a*) the membrane-associated cytoskeleton of T-lymphoma cells contains a form of fodrin; and (*b*) there may be a specific interaction between membrane proteins (e.g., gp180) and the cytoskeletal protein (fodrin) in mouse T-lymphoma cells.

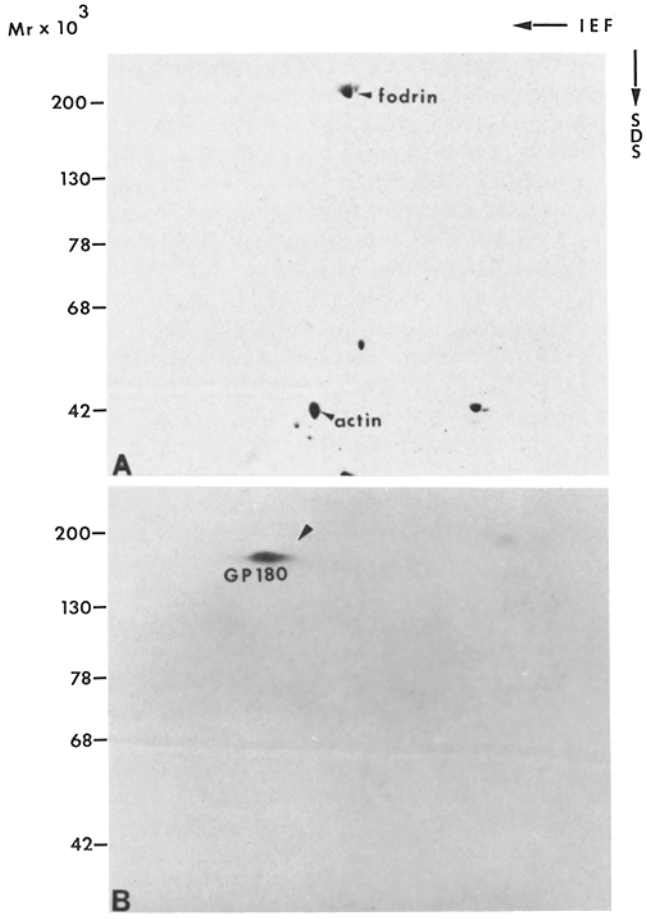

FIGURE 2 Two-dimensional gel electrophoresis: IEF and SDS PAGE analysis of unchallenged mouse T-lymphoma cytoskeleton material. Coomassie Blue staining pattern (*A*) and autoradiogram (*B*) of cytoskeleton material. First dimension, IEF gel with a pH gradient from pH 4.5 (left side) to pH 8.0 (right side). Second dimension, a 7% polyacrylamide gel that contains SDS.

Identification of PM Proteins Preferentially Associated with the Cytoskeleton

To determine whether any particular PM proteins become associated with the cytoskeleton during patching/capping, mouse T-lymphoma cells were either surface-labeled with ^{125}I or metabolically labeled with [^{3}H]glucosamine. SDS PAGE and autoradiographic analysis of the total PM from unchallenged cells reveals many ^{125}I and [^{3}H]glucosamine-labeled polypeptides with molecular weights ranging from 250,000 to 25,000 (Fig. 1, *A* and *B*). While most of these proteins are extracted into the NP-40–soluble fraction (not shown), two major cell surface glycoproteins (as determined by their ability to be labeled by ^{125}I and [^{3}H]glucosamine) with molecular weights of about 180,000 and 25,000 (designated gp180 and gp25, respectively) are resolved in the cytoskeleton fraction (Fig. 1, *F* and *G*). Autoradiography of the same two-dimensional gel used to resolve fodrin in the cytoskeleton reveals that ^{125}I-labeled gp180 has a pI in the range of 5.6–5.8 (Fig. 2*B*). After ligand-induced surface receptor patching/capping, there is a noticeable increase in the amount of gp180, gp25, and several other minor proteins present in the membrane-associated cytoskeleton fraction (see Fig. 4) (28). Antibody raised against the T-lymphocyte-specific glycoprotein T-200 (anti-T-200) shows strong cross-reactivity to gp180 (Fig. 3*C*). T-200 has been shown to be intimately associated with a number of molecularly independent cell surface components such as H-2, TL, and Thy-1 antigens in mouse T-lymphoma cells (10). Kinetic studies show that the amount of gp180 in the NP-40–insoluble fraction is directly proportional to the percentage of capped cells (Fig. 4). We therefore decided to focus our attention on gp180 and its interaction with the cytoskeleton.

Demonstration of Specific Interaction Between gp180 and Fodrin

Immunobinding analysis was used to identify those proteins with associations with gp180. NP-40–solubilized PM from unchallenged cells was incubated with beads conjugated with anti-T-200, and the bound material was analyzed by SDS PAGE. When the protein banding of this anti-T-200–bound material (Fig. 3, *C* and *D*) was compared to that of unchallenged total PM (Fig. 3, *A* and *B*), we found that both fodrin and actin are bound (Fig. 3*D*). Quantitation by scanning densitometry of the ratio of fodrin to actin in total PM (Fig. 3*B*) and immunobead-bound PM (Fig. 3*D*) detects approximately five times more fodrin than actin (Fig. 3*D*). SDS PAGE and autoradiographic analysis shows that, of all the ^{125}I-labeled membrane proteins, only gp180 is selectively bound to anti-T-200–conjugated immunobeads (Fig. 3*C*). Control results confirm the specificity of this immunobinding technique. After incubation of NP-40–solubilized PM with immunobeads prepared using preabsorbed anti-T-200 antisera (anti-T-200-free, IgG fraction), SDS PAGE analysis of bound material reveals some actin and other proteins (Fig. 3*F*), but no fodrin is detected. Similarly, analysis of the same sample by autoradiography detects neither gp180 nor any other ^{125}I-labeled protein (Fig. 3*E*).

An additional demonstration of the association between gp180 and the cytoskeleton was obtained by treating total unchallenged PM with the non-ionic detergent Triton X-114. After Triton X-114 extraction of total ^{125}I-labeled PM (Fig. 5*A*), SDS PAGE revealed that most of the integral membrane proteins become readily partitioned with the "detergent" phase (Fig. 5*B*) away from peripheral cytoskeletal proteins which remain in the "aqueous" phase (Fig. 5*D*). Most importantly, gp180, unlike the other integral membrane proteins, was selectively isolated in the aqueous phase with the cytoskeletal proteins (Fig. 5, *C* and *D*; also compare lane *B* with *C*). As demonstrated by Coomassie Blue staining, fodrin and actin are the major proteins in the aqueous phase (Fig. 5*D*).

Double-label immunofluorescence microscopy using anti-T-200 and specific antibody against brain fodrin provide additional corroboration of the interaction between gp180 and fodrin. After surface receptor aggregation of gp180 (Fig. 1*C*), fodrin accumulates directly underneath the gp180 caps (Fig. 1*D*). This data is strengthened by a kinetic analysis of capping that demonstrates the parallel recruitment of fodrin and gp180 into the NP-40–insoluble fraction (Fig. 4). The amount of fodrin in the NP-40–insoluble fraction, like gp180, is directly proportional to the percentage of capped cells. These results taken together demonstrate a specific interaction between the integral membrane glycoprotein (gp180) and the cytoskeletal protein, (fodrin) in unchallenged and patched/capped mouse T-lymphoma cells.

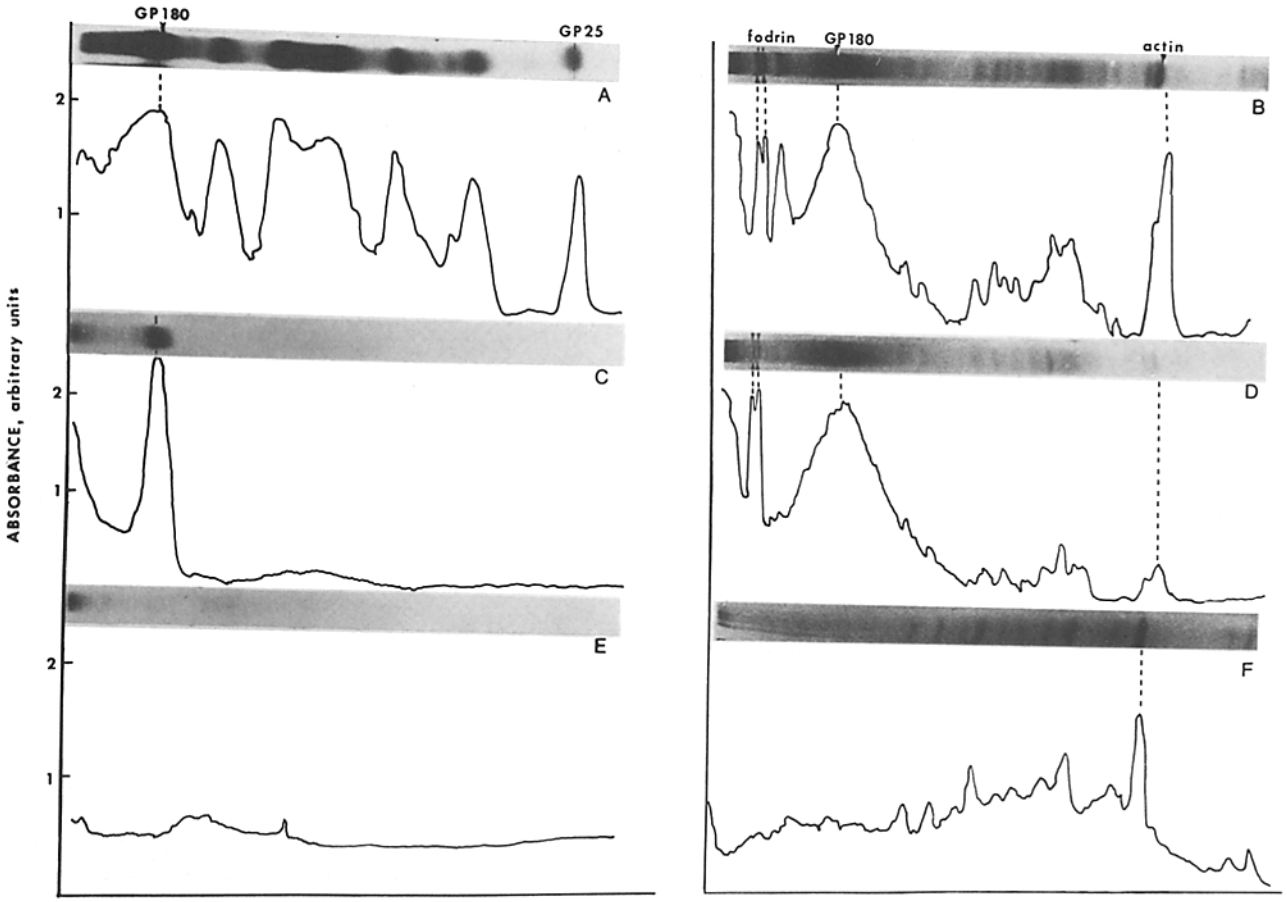

FIGURE 3 SDS PAGE analysis of total PM and cytoskeleton material from unchallenged mouse T-lymphoma cells that has been bound to anti-gp180 (anti-T-200) immunobeads. Cell surface gp's of these membranes were previously labeled with ^{125}I. Arrowheads and dots indicate the position of the two subunits of lymphoma fodrin (*240 K* and *235 K*), gp180, and actin. (The molecular weight markers used are the same as described in Fig. 1.) (*A* and *B*) Proteins from total unchallenged PM. *A* and *B* are the same lane. ([*A*] ^{125}I-labeled proteins, autoradiogram/densitometric tracing; [*B*] Coomassie Blue-stained gel.) (*C* and *D*) Proteins from cytoskeleton material that bind to anti-gp180-conjugated immunobeads. *C* and *D* are the same lane. ([*C*] ^{125}I-labeled proteins, autoradiogram/densitometric tracing; [*D*] Coomassie Blue-stained gel.) To demonstrate most dramatically the absence of gp180 and fodrin, the amount of protein loaded in the control lanes (*E–F*) greatly exceeds that which was loaded in the lanes containing proteins that were bound to anti-gp180-conjugated immunobeads. (*E* and *G*) Proteins from cytoskeleton material bound to immunobeads prepared using preabsorbed anti-gp180 antisera (anti-gp180-free). These control samples detected nonspecific binding to the immunobeads. *E* and *F* are the same lane. ([*E*] ^{125}I-labeled proteins, autoradiogram/densitometric tracing; [*F*] Coomassie Blue-stained gel.) Note that some actin is detected in *F*, but neither gp180 nor the 240,000- or 235,000-mol-wt subunits of fodrin are detected in *E* or *F*.

Demonstration That Fodrin and gp180 Exist As A Complex and Characterization of That Complex

Further analysis of the aqueous phase from Triton X-114–extracted ^{125}I-labeled PM by sucrose density centrifugation shows that the majority of the proteins in the aqueous fraction, as measured by absorbance at 280 nm, stays at the top of the gradient (Fig. 6). In contrast, the peak activity of ^{125}I–gp180 is detected a distance from the top of the gradient and is always coincident with a calculated S value of 20 (Fig. 6). The S value was calculated using 3.7S G-actin (49) and 11S tetrameric spectrin and fodrin (16) as protein standards. Examination of the pooled fractions from the 20S region by SDS PAGE reveals the presence of both fodrin (Fig. 5 *E*) and gp180 (Fig. 5 *F*) but no actin.

To demonstrate the tight association of gp180 and fodrin as a complex, we further analyzed the fractions from the gp180 peak by IEF (Fig. 7). Our preliminary data demonstrate that after IEF (under nondenaturing conditions), gp180 and fodrin elute together from fractions 11–15, as indicated from the silver stain pattern in Fig. 7, and in this complexed form have a pI in the range of 5.2–5.7. The pI of the complex coincides with the relative pI of gp180, but is much lower than the pI assigned to fodrin.

To determine the molar ratio of gp180 to fodrin under varying experimental and extraction conditions, cells were labeled metabolically with [^{35}S]methionine (Table I and Fig. 8). Metabolic labeling with [^{35}S]methionine was chosen for the quantitation of these proteins since gp180 is a glycoprotein and does not stain as well as fodrin using Coomassie blue. In all cases, the stoichiometry of gp180 and fodrin was determined by integration of the area under the respective peaks on autoradiograms of SDS PAGE separations. In our calculations, we assumed that gp180 had a monomeric form with a molecular weight of 180,000 and fodrin a tetrameric form with a combined molecular weight of 950,000. We also assumed that the specific activity of the [^{35}S]methionine-labeled polypeptides was the same. We consider the latter a valid

assumption since the same relative proportions of gp180 and fodrin are obtained by both ^{125}I-labeling and silver staining. We determined a constant stoichiometry of ~1:1 for gp180:fodrin in the isolated NP-40–insoluble cytoskeleton, immunoprecipitates for anti-gp180 and anti-fodrin, and the 20S complex from either unchallenged or patched/capped cell membranes (Table I). Additionally, the 1:1 stoichiometry of gp180 to fodrin is constant across the ^{125}I-labeled peak in Fig. 6 (data not shown). Thus, we consider that our data provide strong evidence that gp180 and fodrin are specifically associated and form a stable complex in mouse T-lymphoma cells.

DISCUSSION

As part of our continued interest in the structural and functional interactions between the cell surface and the cytoskeleton, we have investigated the proteins of mouse T-lymphoma PM and the membrane-associated cytoskeleton. By biochemical and immunofluorescent analysis of this system, we have identified a form of the cytoskeletal protein, fodrin. We have also identified a membrane glycoprotein, which we have designated gp180, which is affiliated with the membrane-associated cytoskeleton. Our results suggest that fodrin and gp180 are associated as a complex and that this complex may be functionally involved in receptor patching and capping.

In this paper, we present evidence that gp180 is very similar if not equivalent to T-200 (a major T-lymphocyte–specific glycoprotein). Omary and Trowbridge (50) have shown that T-200 is a transmembrane protein. Our results show that gp180 is iodinated by ^{125}I and glycosylated by [^{3}H]glucosamine which indicates its exposure on the outer cell surface. The presence of gp180 in the NP-40–insoluble fraction suggests its attachment to the cytoskeleton on the cytoplasmic side of the membrane. Furthermore, gp180 is phosphorylated by metabolic labeling with inorganic $^{32}PO_4$, which presumably takes place on the cytoplasmic side of the membrane (data not shown; 50, 51). Most conclusively, our results show that gp180 has strong cross-reactivity with antibody raised against T-200. The discrepancy between the molecular weights determined for these two proteins (200,000 for T-200 and 180,000 for gp180) could be a result of the different PAGE systems used by Trowbridge's (34) and our laboratories. Therefore, based on similarity of cellular localization, molecular weight, and especially on immunological cross-reactivity, we assume that gp180 is the same as or very similar to T-200.

During the analysis of the membrane cytoskeleton complex we have observed that only actin (but not fodrin) is recruited to the PM from the cytoplasm during capping (28). Fodrin appears to be soluble in NP-40 in unchallenged cells, but becomes associated with the NP-40-insoluble fraction during capping (Fig. 4) (28). Most importantly, we have noted that gp180 is also accumulated in the NP-40-insoluble fraction along with fodrin during capping (Fig. 4). These data suggest that there is a close relationship between fodrin, gp180, and the cytoskeleton. The selective enrichment of fodrin relative to actin in the anti-180–mediated immunobinding assay implies that the co-detection of gp180 and fodrin is specific. The control for this assay (where anti-T-200 is absent) provides additional support for the specificity of gp180–fodrin interaction. Even though some trapped actin is present, neither gp180 nor fodrin is detected.

An additional indication that gp180 and fodrin exist as a complex is provided by a stoichiometric analysis using cells

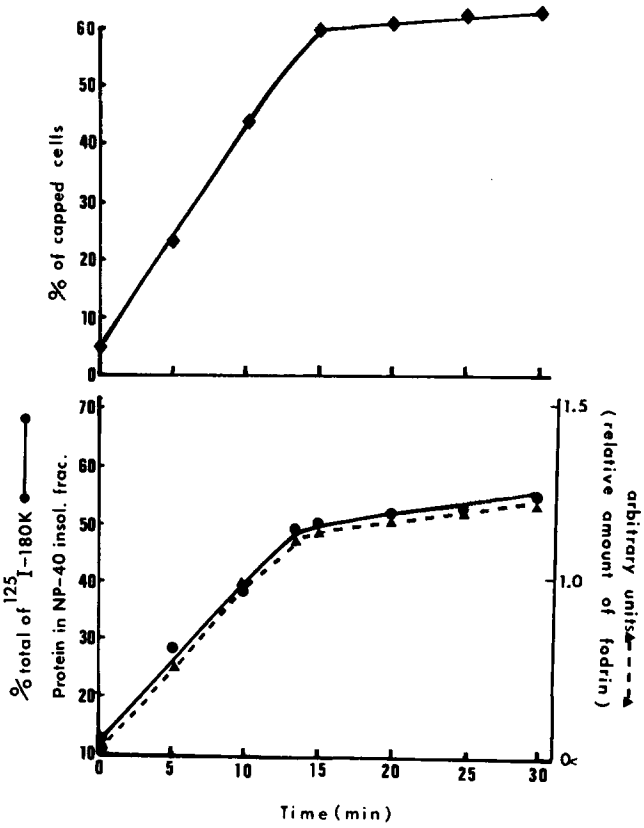

FIGURE 4 Kinetics of the accumulation of cells in the capped state (*A*) and the concomitant accumulation of the 180,000-mol-wt protein (gp180) and fodrin (*B*) in the membrane-associated cytoskeleton fraction of anti-Thy-1 patched/capped cells. Mouse T-lymphoma cells were preincubated with rat monoclonal antibody against Thy-1 followed by incubation in secondary antibody (goat anti-rat IgG) which would act as a cross-linker. Finally, the unbound ligand was washed out. At time zero, the temperature was raised and capping allowed to proceed. At various time intervals, the percentage of capped cells was determined and the cytoskeleton material (NP-40-insoluble fraction of ^{125}I-labeled PM) isolated. When monoclonal antibodies to gp180 (T-200) or gp69/71 were substituted for anti-Thy-1, the capping kinetics were comparable. (*A*) ♦, with ligand treatment. (*B*) Data plotted from SDS PAGE analysis of the Coomassie Blue–stained cytoskeleton material (^{125}I-labeled PM). The amount of protein loaded per gel lane per time point remained constant. The relative amount of fodrin was determined by scanning densitometry of the Coomassie Blue staining pattern and is expressed as arbitrary units (▲). Bands representing gp180 were cut out and the amount of radioactivity in each determined by a gamma counter counting. These values are expressed as a percent of the total gp180 contained in the PM (●).

metabolically labeled with [^{35}S]methionine (Table I). Our data show a consistent stoichiometry of ~1:1 for gp180:fodrin in the NP-40-insoluble fraction, in immunoprecipitates for anti-fodrin or anti-gp180, and in the 20S complex from both unchallenged and patched/capped cell membranes (Table I). We realize that the validity of the aforementioned ratio is highly dependent on the purity of both gp180 and fodrin in the preparation. The purified fractions (i.e., the NP-40-insoluble fractions, the immunoprecipitates for anti-gp180 or anti-fodrin, and the 20S complex) contain only fodrin and gp180 in the high molecular weight range, therefore making an accurate determination of stoichiometry possible. Although slightly different ratios were obtained for either anti-gp180–

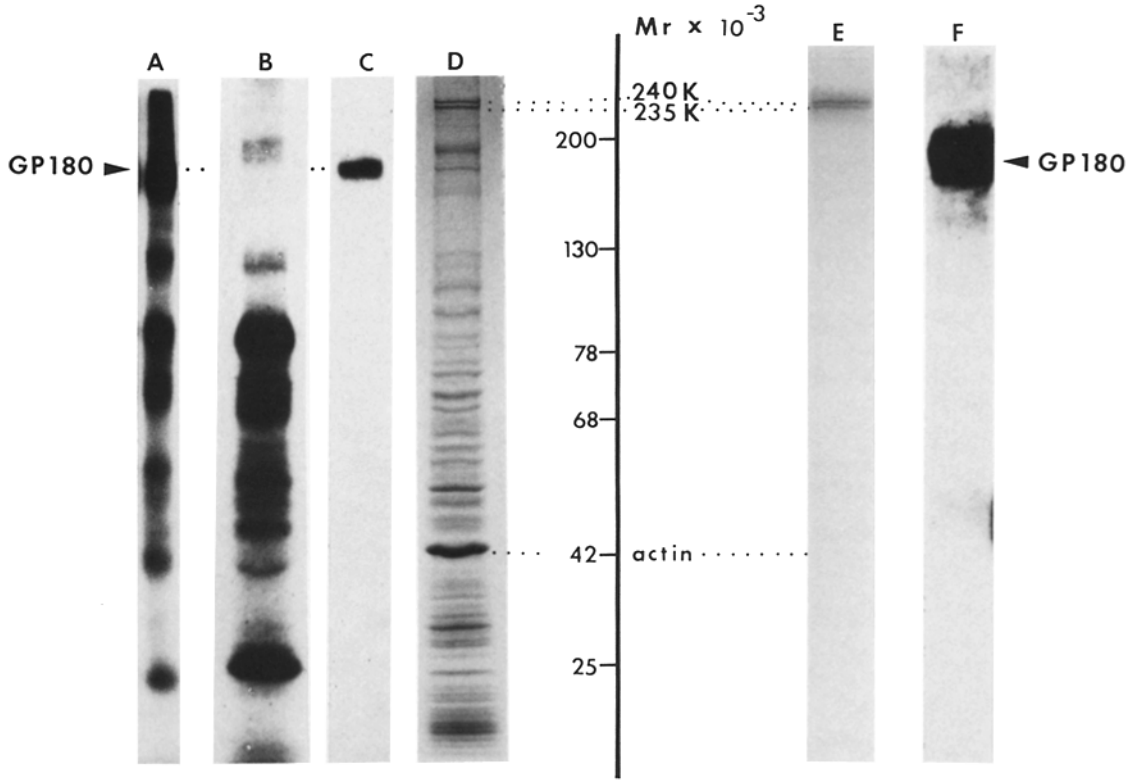

FIGURE 5 SDS PAGE analysis of Triton X-114-extracted PM. (*A*) Autoradiogram of Triton X-114-extracted ^{125}I-labeled total PM prior to partitioning into aqueous and detergent phases. (*B*) Autoradiogram of ^{125}I-labeled surface proteins that partition into the detergent phase. (*C–D*) Autoradiogram of ^{125}I-labeled surface proteins that partition into the aqueous phase (*C*), and Coomassie Blue staining pattern of the same gel lane showing the total protein composition of the aqueous phase (*D*). Note the presence of both gp180 and fodrin in the Triton X-114 aqueous phase. (*E–F*) SDS PAGE analysis of the ^{125}I-gp180 peak (shown in Fig. 7) that is obtained after sucrose gradient centrifugation. Coomassie Blue staining which demonstrates the presence of fodrin in the peak fractions (*E*); autoradiogram of the same lane confirming the presence of ^{125}I-labeled gp180 (*F*). Note the absence of actin in these fractions.

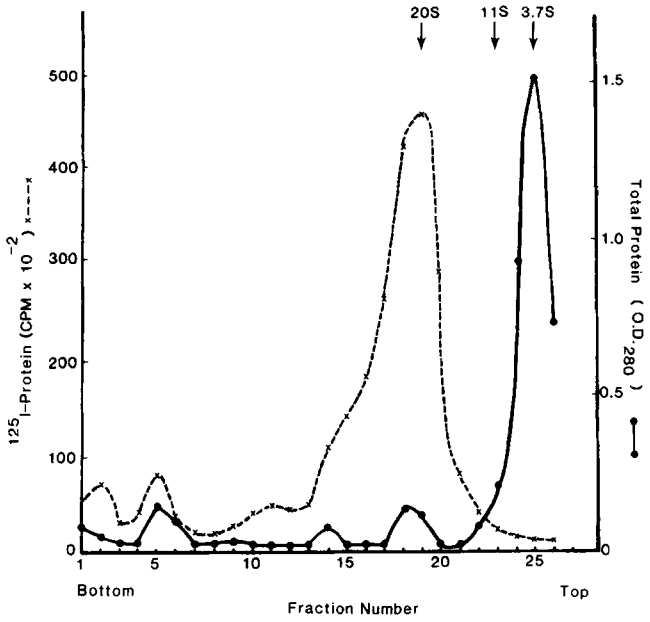

FIGURE 6 Sucrose gradient centrifugation of the aqueous phase derived from Triton X-114-extracted ^{125}I-labeled lymphoma PM's. ●, the total protein profile as determined by turbidity measurements at 280 nm. ×, ^{125}I-labeled protein peaks as determined by counting the fractions on a gamma counter. It has been demonstrated (Fig. 5) that the majority of the ^{125}I-labeled protein in the aqueous phase is gp180. Protein standards of G-actin and terameric

or anti-fodrin-induced immunoprecipitates, we do not consider this a meaningful variation. The antibody might select one form of the complex over another as a result of availability of antigenic sites, therefore giving a slightly different molar ratio (1:0.7 for anti-gp180; 1:1.37 for anti-fodrin). It is assumed that, in all cases, the stoichiometry obtained for gp180:fodrin is an average value. The fact that the 20S complex also displays a 1:1 ratio for these proteins further strengthens the evidence that gp180 and fodrin are specifically associated in mouse T-lymphoma cells.

The initial characterization of the two proteins indicates that under denaturing conditions, fodrin exhibits a pI that is quite distinct from that of gp180 (Fig. 2). However, the analysis of this 20S complex by nondenaturing IEF demonstrates that both gp180 and fodrin remain associated as a complex which exhibits a pI in the range of 5.2–5.7 pH units. The fact that gp180 and fodrin remain in a tight association after sucrose density centrifugation followed by IEF further supports our contention that they exist as a stable complex.

The functional significance of the gp180–fodrin complex is implied by the results of experiments in which mouse T-

spectrin or fodrin with respective S values of 3.7 (reference 7) and 11 (reference 15) were used to determine the S value of the ^{125}I peak. This peak actually represents the gp180–fodrin-containing complex as demonstrated in Figs. 5*E* and 5*F*.

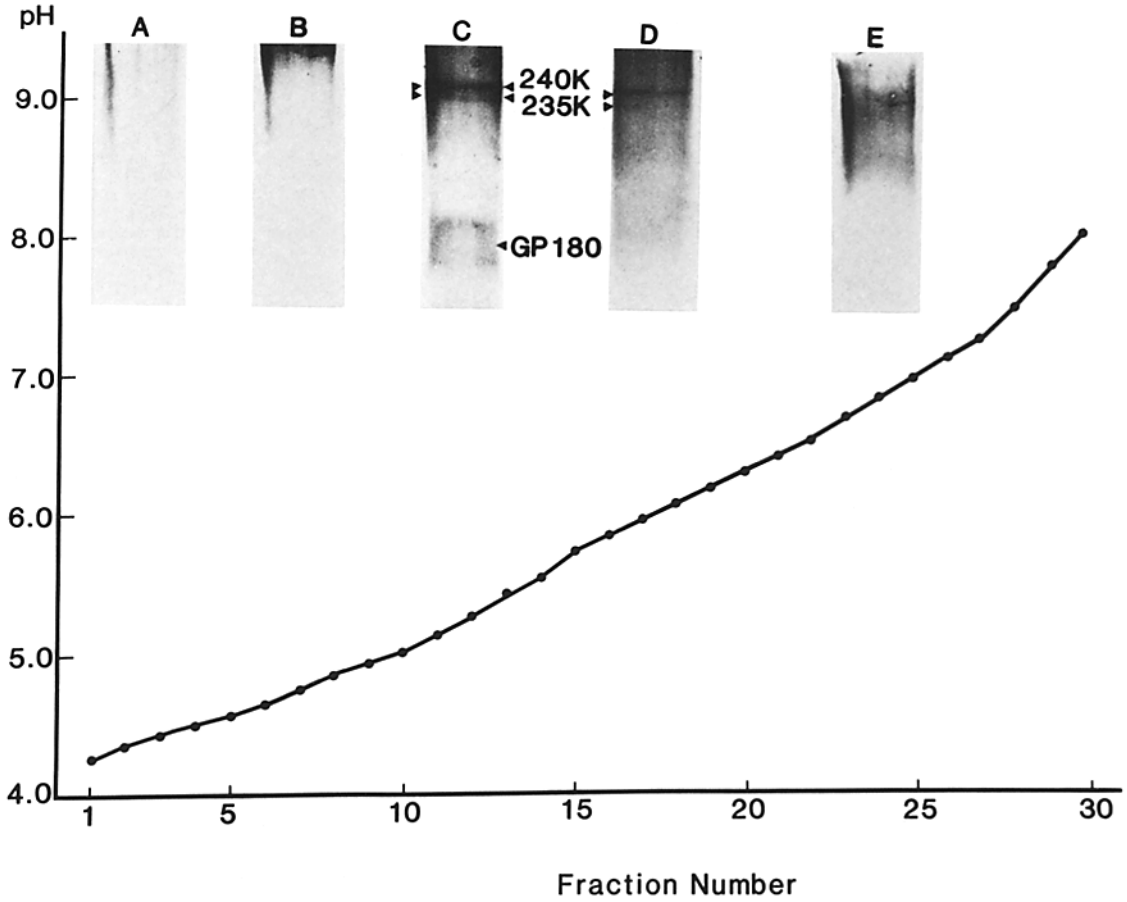

FIGURE 7 IEF of the gp180–fodrin complex obtained by sucrose density centrifugation. The co-migration of gp180 and fodrin was determined by analyzing pooled fractions with SDS PAGE followed by silver staining. gp180 and fodrin were only detected together in lane C which represents pooled fractions 11–15. As indicated by the pH of these fractions, the gp180–fodrin complex has a pI in the range of 5.2–5.7. The remaining gel lanes represent the pooled fractions as follows: *A*, 1–5; *B*, 6–10; *D*, 16–20; *E*, 21–25.

lymphoma cells were induced to patch and cap. When cells are induced to cap, the time course of accumulation of gp180 in the membrane-associated cytoskeletal fraction closely parallels the time course of the increase in numbers of capped cells. Furthermore, after anti-T-200 induction of receptor redistribution, immunocytochemistry reveals fodrin accumulation directly beneath surface receptor patches and caps. These results confirm the previous observations that fodrin (or α-spectrin) co-localizes with receptor aggregates (52, 53). Moreover, parallel biochemical analysis reveals that, following patch/cap induction by any of a variety of different ligands, there is a dramatic increase in gp180 and a concomitant increase in fodrin and actin in the membrane-associated cytoskeleton fraction. These observations are consistent with the hypothesis that lymphoma fodrin is intimately associated with actin and myosin and is thereby involved in surface receptor aggregation. These correlations are also consistent with the suggestion that the gp180–fodrin complex may be involved in the formation of receptor patches and caps.

Biochemical detection of cell surface receptor–cytoskeleton complexes in lymphoid cells has recently been reported by several research groups (8, 9, 35, 36). In addition, myosin light chain phosphorylation, known to activate actomyosin machinery during muscle contraction, was detected in isolated PM from capped cells (30–32, 54). These findings are consistent with the transmembrane interaction hypothesis proposed previously as a possible mechanism for surface receptor capping (5). In this study, we have taken advantage of the differential extraction properties of two non-ionic detergents (i.e., Triton X-114 and NP-40) to identify a membrane protein–cytoskeleton complex that meets some of the criteria of this model. Using Triton X-114, we are able to selectively isolate a gp180–fodrin complex from uncapped (unchallenged) cells that is free from actin (Fig. 5). More importantly, we have observed that during capping this complex becomes associated with the filamentous actin-containing cytoskeletal material (operationally defined as the NP-40–insoluble fraction; Fig. 1), and that the amount of the gp180–fodrin complex associated with the cytoskeleton is directly proportional to the percentage of capped cells (Fig. 4). Furthermore, the fact that gp180 (a membrane glycoprotein) and fodrin (an actin-binding protein) exist as a stable complex allows us to speculate that such an association might represent the bridge between actin and the PM proteins required for receptor capping to occur. In the absence of ligands (in unchallenged cells), some of the gp180 is already bound to the cytoskeleton, apparently via its tight association with the actin-binding protein, fodrin. It is possible that, after ligand binding to surface receptors, the aggregated receptors then bind effectively to the gp180–fodrin complex. Through the cross-linking effect of fodrin to actin filaments and a subsequent actin/myosin sliding filament mechanism (28, 30–32, 54), the ag-

TABLE I. *The Relative Molar Ratios of gp180 and Fodrin*

Fractions	Unchallenged (uncapped)	Patched/ Capped
NP-40 insoluble material	1:1.05 (± 0.05)	1:1.07 (± 0.14)
Anti-180 immunoprecipitates	1:0.70 (± 0.04)	1:0.70 (± 0.06)
Anti-fodrin immunoprecipitates	1:1.37 (± 0.10)	1:1.37 (± 0.08)
20 S complex	1:105 (± 0.03)	—

The values are expressed as mean ± standard deviation of at least five experiments.

These values were estimated from scanning densitometry of [^{35}S]methionine-labeled autoradiograms, assuming molecular weights of 180,000 for monomeric gp180 and 950,000 for tetrameric fodrin, and assuming that these proteins label equivalently with [^{35}S]methionine. The same relative proportions of gp180 and fodrin are obtained with both ^{125}I-labeling and silver staining.

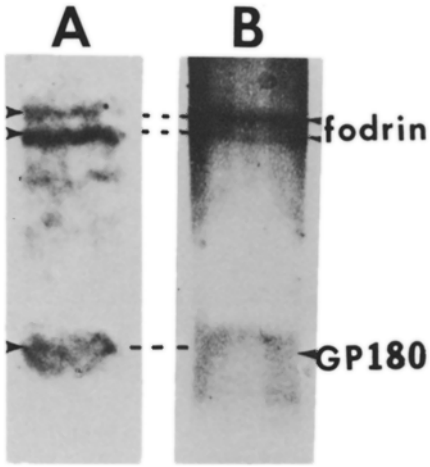

FIGURE 8 Representative [^{35}S]methionine-labeled autoradiogram and silver staining of two different preparations have been enlarged to show the region scanned for quantitation of these two proteins. (*A*) [^{35}S]Methionine-labeled fodrin and gp180 from anti-180 immuno-ppt and (*B*) silver-stained protein pattern of fodrin and gp180 from the 20 S complex. A similar pattern is obtained with ^{125}I-labeled material.

gregates of ligand-receptor complexes are collected into a cap (5, 6, 36). Although an ankyrin-like protein (a spectrin-binding protein) has been shown to accumulate under lymphocyte cap structures by the immunofluorescence technique (28), the relationship between this ankyrin-like protein and the gp180–fodrin complex is presently unresolved. Of course, at this time we can not preclude the possibility that other lymphoma membrane proteins and different actin-binding proteins can and do function as transmembrane linkers. A detailed analysis of various types of transmembrane complexes is currently under investigation.

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