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Further characterization of a fodrin-containing transmembrane complex from mouse T-lymphoma cells

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A transmembrane complex containing fodrin (an actin-binding protein) and a major surface glycoprotein (GP 180) was previously isolated from mouse T-lymphoma cells by the complementary techniques of non-ionic detergent extraction and sucrose gradient centrifugation (Bourguignon et al. (1985) J. Cell Biol. 101, 477–487). The analysis of this complex has been extended to verify the structural association and further define the interaction between fodrin and GP 180. The association between fodrin and GP 180 has been confirmed by the following evidence: (1) co-sedimentation of fodrin and GP 180 in a single peak on a sucrose gradient with a sedimentation coefficient of 20 S; (2) a constant ratio of fodrin and GP 180 across the 20 S peak; (3) the specific co-precipitation of GP 180 with fodrin from the 20 S peak using anti-fodrin antibody; and (4) the colocalization of fodrin and GP 180 from the 20 S peak on actin filaments using an immuno-electron microscopic technique. Furthermore, this fodrin-GP 180 complex can be readily dissociated and reassembled in the presence and absence of 0.6 M NaCl, respectively. The fact that this fodrin-GP 180 complex displays actin-binding ability indicates that this transmembrane complex may play an important role in the linking event between receptors and the cytoskeleton during lymphocyte patching and capping.

Introduction

A direct connection between transmembrane cell surface proteins and the underlying cytoskeleton may be essential for processes such as cell motility, cell-cell recognition, phagocytosis, endocytosis etc. (for a general review see Ref. 2) that require the lateral movement of cell surface receptors. Plasma membrane-cytoskeleton interactions have been demonstrated in a number of differing cell types. Such interactions may be relatively static as in erythrocytes [3,4], the microvilli of intestinal brush-border cells [5,6] or ascites tumor cells [7]. On the other hand, plasma membrane-cytoskeleton interactions may be the result of an inducible event such as receptor redistribution in lymphocytes and fibroblasts [8–11].

Currently, the most comprehensive view of the protein composition and structural arrangement of plasma membrane–cytoskeleton interactions is from the more structured systems of erythrocytes and intestinal brush-border cells. In erythrocytes, binding of cytoskeletal spectrin to the transmembrane anion-channel protein, band 3, is mediated by the membrane attachment protein, ankyrin [3,12]. The binding of spectrin to actin, in turn, appears to be mediated by band 4.1 [4]. Band 4.1 may also serve as a membrane attachment protein, since it can also bind to the erythrocyte membrane through its direct interaction with the glycophorins and band 3 [13,14]. In brush-border cells,

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recent evidence indicates that the 110 kDa actin-binding protein may be an integral membrane protein [6]. The direct attachment of this protein to cytoskeletal actin would represent a simpler system of membrane–actin interaction and an alternative model to that represented by the erythrocyte system.

In lymphocytes, the initial implication of an interaction between membrane and cytoskeletal components was provided by immunofluorescent experiments. It was demonstrated that during ligand-induced cell-surface receptor redistribution there was a parallel accumulation of actin, myosin, and α-actinin directly beneath receptor patches and caps [8,15,16]. More direct evidence of an association between specific surface receptors and the underlying cytoskeleton was suggested by non-ionic detergent extraction of isolated plasma membranes to obtain a detergent insoluble, membrane-associated cytoskeletal residue [17]. For example, when surface immunoglobulin molecules are induced to patch and/or cap, they become tightly linked to the detergent insoluble cytoskeleton [10,11].

Because so little is known about the detailed molecular organization of transmembrane-cytoskeletal protein associations in lymphocytes, we have sought to draw analogies to the well-defined erythrocyte system. The existence of cytoskeletal proteins common to both lymphocytes and erythrocytes has provided insight into how such cytoskeletal elements may regulate lymphocyte receptor mobility. For example, the co-localization of actin and myosin with receptor patches and caps influenced Bourguignon and Singer [8] to propose that surface receptors were clustered into caps via a sliding filament mechanism analogous to that occurring during muscle contraction. Subsequent experiments demonstrated that (i) myosin light chain phosphorylation occurs during lymphocyte capping [18], (ii) a myosin light-chain kinase is associated with the lymphocyte cytoskeleton [19], and (iii) a Ca^{2+}-activated myosin light-chain kinase is responsible for the regulation of receptor capping [20].

Even as we begin to understand the regulation of the movement of cell surface molecules following their linkage to the cytoskeleton, we still know very little about the protein or proteins that mediate the interactions between transmembrane proteins and the cytoskeleton. Recently, several laboratories have investigated a group of non-erythroid spectrins (fodrin, TW 260/240) that have the ability to cross-link actin and bind to spectrin-depleted erythrocyte membranes [21–25]. The widespread distribution of fodrin has led Glenney and Glenney [26] to suggest that fodrin could provide the general linkage system between microfilaments and the membrane in nonerythroid and nonmuscle cells. Fodrin has been identified in both plasma membrane and membrane-associated cytoskeleton fractions obtained from lymphocytes [1,27]. Indirect evidence from immunofluorescent studies demonstrated that fodrin localizes beneath concanavalin A caps in fibroblasts and concanavalin A, immunoglobulin, and H2 caps in lymphocytes [27–29], suggesting a possible role in receptor reorganization. Moreover, the amount of fodrin associated with the Nonidet P-40-insoluble cytoskeleton increases significantly during lymphocyte patching and capping [1,27]. In addition to fodrin, a major cell-surface transmembrane glycoprotein with a molecular weight of 180 000 is also recruited to the lymphocyte cytoskeletal fraction during patching and capping [1,27]. Interestingly, when this 180 kDa glycoprotein (GP 180) is immunoprecipitated by anti-GP 180 antibody from Nonidet P-40-solubilized plasma membrane, fodrin is also selectively co-precipitated [1]. Bourguignon et al. [1] have also demonstrated that GP 180 can be preferentially separated from other cell-surface proteins following Triton X-114 extraction and partitioning. This GP 180-enriched fraction also contained fodrin, and preliminary analysis of this material, coupled with the co-recruitment and immunoprecipitation data, led these authors to suggest that GP 180 and fodrin are closely associated in vivo.

In the present paper, we have conducted a variety of experiments including stoichiometric measurements, dissociation and reassembly assays, immunoprecipitation, immunoelectron microscopic localization, and actin-binding analyses to demonstrate further the close interaction between fodrin and GP 180 as a transmembrane complex. We believe that this fodrin-GP 180 complex may be very important for the transmembrane interactions occurring between membrane proteins and the cytoskeleton.

Materials and Methods

Cell culture and plasma membrane isolation. Mouse T lymphoma cells (BW5147), provided by R. Hyman (The Salk Institute), were grown and plasma membranes isolated as previously described [1].

Radioactive labeling of cellular proteins. Surface proteins were labeled with ^{125}I using the iodogen method of Fraker and Speck [30]. To label proteins metabolically with [^{35}S]methionine or [^{3}H]glucosamine, approx. $1 \cdot 10^5$ cells/ml were incubated with either [^{35}S]methionine (1000 Ci/mmol) or [^{3}H]glucosamine (100 Ci/mmol) (ICN, Irvine, CA) in Dulbecco's modified Eagle's medium under standard culture conditions for 16 h.

Concanavalin A blotting. Isolated plasma membrane was electrophoresed as described below. The polypeptides were then transferred to nitrocellulose sheets [31], incubated with 30 μg of ^{125}I-labeled concanavalin A ([30]; Calbiochem, La Jolla, CA), dried and analyzed by autoradiography.

Con A-Sepharose affinity chromatography. A Con A-Sepharose (Pharmacia, Piscataway, NJ) column (6.0×0.5 cm) was washed with 0.15 M NaCl/10 mM Tris-HCl (pH 7.3) containing 0.5% Triton X-114. Plasma membrane isolated from ^{125}I-labeled cells was extracted in 1% Triton X-114 as described below. The material was passed over the column at least ten times to allow for sufficient binding. The column was then washed until no further radioactivity was removed. Wash buffer containing 1 M methyl α-D-mannoside was then used to elute the column. Fractions containing unbound and specifically eluted radioactive proteins were separately pooled and analyzed by SDS-gel electrophoresis.

Non-ionic detergent extraction. ^{125}I- or [^{35}S]methionine-labeled lymphocyte plasma membrane was isolated from intact cells, washed in 0.15 M NaCl/10 mM Tris-HCl (pH 7.3) and then resuspended in 200 μl of 1.0% (v/v) Triton X-114 in the same buffer. The protein concentration in all samples was kept at about 2.5 mg/ml. Extraction and partitioning was carried out as previously described [1]. Since GP 180 can be separated from other cell surface proteins by its preferential partitioning into the upper, aqueous phase, we used the Triton X-114 aqueous phase as the starting material for all subsequent experiments.

Isolation of a native 20 S transmembrane complex. ^{125}I- or [^{35}S]methionine-labeled plasma membrane was isolated from intact cells, extracted with Triton X-114 and partitioned as described above. The aqueous phase was then loaded onto a linear sucrose gradient (7–28%) with a 0.5 ml cushion of 65% sucrose and centrifuged at 70000 $\times g_{av}$ for 22 h. Fractions were counted on a LKB MiniGamma gamma counter (LKB Instruments, Rockville, MD) to determine the distribution of surface-iodinated protein within the gradient. Total protein concentration in each fraction was determined by absorbance at 280 nm. Fractions that corresponded with either the 280 nm protein peak or ^{125}I-labeled peak were pooled, precipitated with 10% trichloroacetic acid, and subsequently analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The [^{35}S]-methionine-labeled fractions that corresponded to the ^{125}I-labeled peak were precipitated with trichloroacetic acid and the individual fractions were analyzed by SDS-polyacrylamide gel electrophoresis autoradiography, and scanning densitometry. The ^{125}I-labeled peak fractions corresponded to a sedimentation value of 20 S as calculated using protein standards of tetrameric fodrin, tetrameric spectrin (11 S), and G-actin (3.7 S) as described previously [1]. In order to iodinate the proteins in the 20 S peak fractions (i.e., fodrin and GP 180), the Triton X-114 aqueous fraction was iodinated prior to centrifugation on sucrose gradients.

SDS-polyacrylamide gel electrophoretic 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 [32]. Gels containing samples labeled with either ^{125}I or [^{35}S]methionine were stained, fluorographed [33], vacuum dried, and exposed to Kodak X-ray (X-Omat, AR-5) film at $-70°C$. In order to quantitate the relative amounts of GP 180 and fodrin in gel lanes, silver-stained gels and autoradiograms of [^{35}S]methionine-labeled or ^{125}I-labeled proteins were scanned on a Quick Scan R & D (Helena Laboratories, Beaumont, TX) scanning densitometer.

Isolation of 9 S, GP 180 by high salt extraction and sucrose gradient centrifugation. The ^{125}I-labeled 20 S material was isolated as described above, diluted with 0.15 M NaCl/10 mM Tris-HCl (pH 7.3) containing 0.01% Triton X-114, and concentrated using a 3 ml Amicon stirred filtration cell. This material was then divided into an experimental sample exposed to high salt and a control. The experimental sample was adjusted to 0.6 M NaCl/10 mM Tris-HCl (pH 8.0)/0.5% Triton X-114; the control sample to 0.15 M NaCl/10 mM Tris-HCl (pH 7.2)/0.5% Triton X-114. The extraction buffer was modeled after ones used to extract cytoskeletal proteins [21,22,34]. 9 S, GP 180 was obtained by an overnight incubation of 20 S material with high salt (0.6 M NaCl) at 4°C followed by centrifugation on a linear sucrose gradient (7–28%) for 22 h at $70000 \times g_{av}$. 20 S control material, incubated overnight in physiological strength salt (0.15 M), was run on a parallel 7–28% sucrose gradient. The high-salt sample was centrifuged on a sucrose gradient containing 0.6 M NaCl, whereas the control sample was run on a gradient containing 0.15 M NaCl. Parallel high-salt and control gradients containing ^{125}I-labeled human transferrin and spectrin were included as internal controls to demonstrate that high salt had no inherent effect on S values.

In vitro reassembly of a transmembrane complex from 9 S, GP 180 and fodrin. (1) Preparation of a 9 S, GP 180-Con A-Sepharose column. Isolated 9 S, GP 180 material was first dialyzed overnight against physiological strength salt (0.15 M NaCl/10 mM Tris-HCl (pH 7.3)/0.1% Triton X-114) to reduce the salt concentration and remove sucrose, and then passed over a Con A-Sepharose column. This 9 S, GP 180 bound column was used for the following in vitro reassembly assay. (2) In vitro reassembly assay. After washing the 9 S, GP 180-Con A-Sepharose column with physiological strength salt (0.15 M NaCl buffer), 3–5 μg of ^{125}I-labeled [30] pig brain fodrin was passed over the unlabeled 9 S, GP 180-Con A-Sepharose column at least ten times to insure maximum fodrin binding, and the column was then washed until no more radioactivity was removed. A parallel Con A-Sepharose column with no bound GP 180 was run as a control for nonspecific binding and/or elution of ^{125}I-labeled fodrin. Following washing, columns were eluted with 1 M methyl α-D-mannoside and 0.5 ml fractions collected. To demonstrate specific sugar elution profiles, 9 S, GP 180-bound and 20 S-bound Con A-Sepharose columns were also prepared as positive controls. Fractions from all four columns were then counted and plotted vs. fraction number to establish an elution profile for each column. (3) Characterization of in vitro assembled 9 S, GP 180-fodrin transmembrane complex. The newly formed 9 S, GP 180-fodrin transmembrane complex was preferentially eluted by methyl α-D-mannoside from a Con A-Sepharose column and analyzed by sucrose gradient (7–28%) centrifugation for 22 h at $70000 \times g_{av}$. 9 S, GP 180 alone, sugar-eluted from a Con A-Sepharose column, was run on a parallel gradient as a control.

Immunoprecipitation procedure. The 20 S, GP 180-containing peak fraction (isolated by sucrose density centrifugation as described above) was incubated with a polyclonal rabbit anti-fodrin antibody (kindly provided by Dr. Shin Lin, Johns Hopkins University) or pre-immune serum at 4°C for 30 min. This step was followed by an overnight incubation with goat anti-rabbit immunoglobulin [35]. The resulting immunoprecipitates were pelleted by centrifugation at $700 \times g_{av}$ for 5 min, washed three times with 0.1% Triton X-114 in 0.15 M NaCl/10 mM Tris-HCl (pH 7.3) solubilized in sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis autoradiography.

Actin-binding analysis. The ^{125}I-labeled 20 S, GP 180-containing peak was isolated and concentrated as described above. F-actin was prepared according to the procedure of Pardee and Spudich [36]. For these analyses, the peak material was incubated with actin under two different sets of conditions. Peak material was incubated with 40 μg of F-actin, an estimated 50 molar excess, in a solution comprising 0.1 M NaCl/2 mM $MgCl_2$/1 mM EGTA/10 mM imidazole-Cl (pH 7.0) [37]. In the second type of experiment, the peak material was incubated with equimolar concentrations of F-and G-actin (20 μg of each) in 0.1 M NaCl/2 mM Tris-HCl (pH 8.0)/1.2 mM $MgCl_2$/1.2 mM $CaCl_2$/ and 0.5 mM 2-mercaptoethanol, a buffer favoring actin polymerization [36]. In parallel controls, peak material

was incubated in the absence of actin. After an incubation period of 4–12 h at 4°C, the material was loaded onto a standard linear sucrose gradient (7–28%) and centrifugated at $70000 \times g_{av}$ for 22 h. The gradients were then fractionated and the ^{125}I-labeled peak was determined by gamma counting.

Immunoelectron microscopy. The isolated ^{125}I-labeled 20 S, GP 180-containing peak material was dialyzed overnight against G-actin buffer comprising 5 mM Tris-HCl (pH 8.0)/0.2 mM ATP/0.2 mM $CaCl_2$/0.5 mM 2-mercaptoethanol. This dialyzed material was then added to G-actin and polymerization was initiated by the addition of 50 mM KCl/1 mM ATP/1 mM $MgCl_2$ [36]. Polymerization was carried out for 1 h at room temperature or overnight at 4°C. Following polymerization, anti-GP 180 or anti-fodrin was added and the material incubated at 4°C for 30 min. This step was followed by the addition of protein A-gold [38]. Control samples, which were either incubated with antisera preabsorbed with GP 180 or fodrin or in which the primary antibody addition step was omitted, were run in parallel with the experimental samples. Following 30 min incubation with protein A-gold, aliquots of material were removed, applied to formvar-coated grids, washed extensively with buffer, and negative stained [39]. Grids were then examined in a Philips 300 electron microscope operating at 60 kV and photographed.

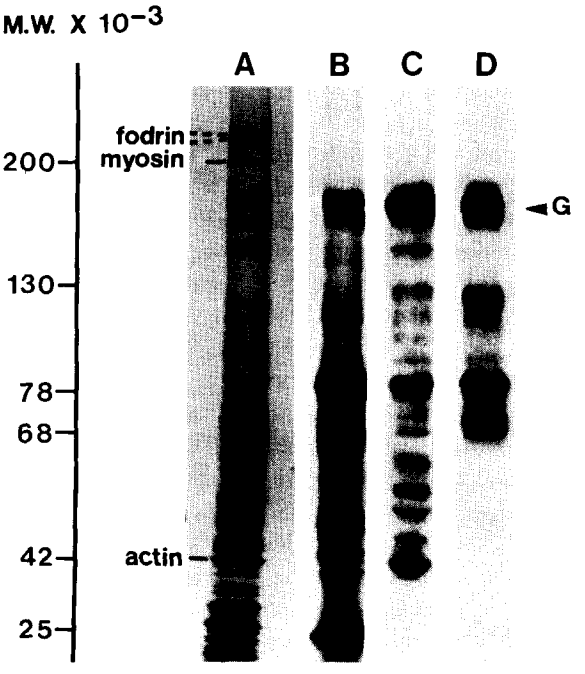


Fig. 1. Characterization of mouse T-lymphoma plasma membrane proteins. (A) Coomassie blue staining of total plasma membrane. (B) Autoradiogram of ^{125}I-surface-labeled plasma membrane. (C) Autoradiogram of total plasma membranes that are processed by SDS-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose sheets, and then incubated with ^{125}I-labeled concanavalin A. G indicates the M_r 180000 (GP 180) band. (D) Autoradiogram of detergent solubilized, ^{125}I-labeled plasma membrane proteins that were bound to a Con A-Sepharose column and specifically eluted with methyl α-D-mannoside. (Molecular weight markers: myosin, 200000; β-galactosidase, 130000; lactoperoxidase, 78000; bovine serum albumin, 68000; actin, 42000; concanavalin A, 25000).

Results

GP 180 as a concanavalin-A-binding glycoprotein

The Coomassie blue staining pattern after SDS-polyacrylamide gel electrophoresis of isolated plasma membrane from ^{125}I-labeled mouse T-lymphoma cells revealed many membrane-associated polypeptides with molecular weights ranging from about 300000 to 17000 (Fig. 1A). We have previously identified the high-molecular-weight doublet above myosin in lane 1A as the actin-binding protein, fodrin [1]. The corresponding autoradiogram shows several ^{125}I-labeled cell surface proteins with molecular weights ranging from 25000 to 250000 (Fig. 1B). An M_r 180000 protein appears to be one of the major iodinated proteins (Fig. 1B). The results of blotting and binding of plasma membrane with ^{125}I-labeled concanavalin (Fig. 1C) and Con A-Sepharose columns (Fig. 1D) indicate that the 180000 protein is a major concanavalin A-binding glycoprotein previously identified as GP 180 (also known as T200) [1,40]. The binding specificity of GP 180 for concanavalin A was confirmed by its specific binding and elution from Con A-Sepharose columns [Fig. 1D). In addition to GP 180, at least three other cell-surface proteins, with molecular weights ranging from 130000 to 70000, were specifically bound and eluted from Con A-Sepharose.

Characterization of the fodrin-GP-180-containing peak material

A fodrin-GP 180 complex can be routinely isolated by a selective Triton X-114 extraction procedure [1,41] followed by sucrose gradient centrifugation as described previously (Ref. 1; Fig. 2a, 2a-A; 2a-B). To characterize the fodrin-GP-180-containing peak material, we determined the sedimentation coefficient (S value) of the peak and the [^{35}S]methionine ratio of GP 180 to fodrin across the peak. Using 3.7 S G-actin [42] and 11 S tetrameric spectrin or fodrin [24] as protein standards, we were able to confirm that the sedimentation coefficient of the fodrin-GP 180 peak is 20 S (Fig. 2a). The relative ratio of fodrin to GP 180 in the individual gradient fractions corresponding to the 20 S peak was examined using plasma membrane isolated from cells metabolically labeled with [^{35}S]methionine. (Previously [1], we had pooled the 20 S peak fractions to estimate a 1 : 1 ratio of fodrin to GP 180.) The analysis was done on [^{35}S]methionine autoradiograms instead of Coomassie-blue-stained gel lanes because GP 180, like many other glycoproteins, does not stain well with Coomassie. The ratio of fodrin to GP 180 was determined by integration of the area under the respective peaks generated by scanning densitometry of SDS-electrophoresis autoradiograms. For our ratio calculations we compared relative arbitrary units of fodrin and GP 180. The relative ratio of fodrin to GP 180 is constant across the 20 S peak (compare Figs. 2a and 2b), suggestive of a specific association between fodrin and GP 180. Similar results were also obtained following the analysis of total ^{125}I-labeled and silver-stained 20 S material (data not shown).

Next, we wanted to examine the effect of high salt on the components of the 20 S peak, since similar treatments have been shown to dissociate fodrin from membranes [21,22] and spectrin from the band 3-ankyrin complex of erythrocytes [34]. Following incubation in 0.6 M NaCl, the ^{125}I-labeled GP 180 exhibits a lower sedimentation coefficient (Fig. 3b) than the control (Fig. 3a). When compared to ^{125}I-labeled transferrin with an average S value of 5 [43], the high-salt-treated peak has an apparent S value of 9. The control peak retains an S value of 20, identical to the value calculated during the original isolation procedure (Fig. 2). Parallel high-salt and control gradients containing ^{125}I-labeled transferrin were run to demonstrate that the high salt gradient has no apparent effect on S value. Note that the ^{125}I-labeled 9 S peak obtained under high-salt conditions displayed a significant shoulder, with an S value of more than 10. When all the 9 S peak material is collected (including the shoulder), and dialyzed against physiological strength buffer (0.15 M NaCl), the 20 S peak is partially reformed (data not shown). Previous controls demonstrated that tetrameric fodrin, with an S value of 11, would be included in the shoulder of the 9 S peak.

Further analysis indicates that both native fodrin-GP 180 (20 S) complex (Fig. 2a and 3a) and GP 180 (9 S) (Fig. 3b), but not fodrin (Fig. 4b) bind specifically to Con A-Sepharose and can be eluted by the sugar, methyl α-D-mannoside (Fig. 4a). In order to demonstrate the direct binding between fodrin and GP 180, in the absence of high salt, an in vitro reassembly assay that involves the addition of ^{125}I-labeled fodrin to 9 S, GP 180 material bound to a Con A-Sepharose column was performed. Our data reveals that ^{125}I-labeled fodrin can associate with GP 180 as shown by the following two pieces of evidence: (1) the ^{125}I-labeled fodrin-GP 180-Con A-Sepharose bound material was eluted by the specific sugar, methyl α-D-mannoside, suggesting the co-elution of ^{125}I-labeled fodrin and GP 180; and (2) the sugar eluted material containing the newly formed ^{125}I-labeled fodrin-GP 180 complex displayed a sedimentation coefficient of 20 S (Fig. 5), similar to that of the native fodrin-GP 180 complex (Figs. 2a and 3a). (A parallel gradient of 9 S, GP 180 alone was run as a control (Fig. 5).) Our data demonstrate that fodrin and GP 180 can be dissociated and reassembled in the presence and absence of high salt, respectively, implying that the binding between these two molecules is rather specific.

To provide further evidence of a close association between fodrin and GP 180 in the 20 S peak, we immunoprecipitated fodrin from the ^{125}I-labeled GP 180-containing 20 S peak material using an anti-fodrin antibody. Analysis of the precipitated proteins by SDS-electrophoresis and silver staining [44], demonstrated that fodrin was the major visible protein precipitated from the 20

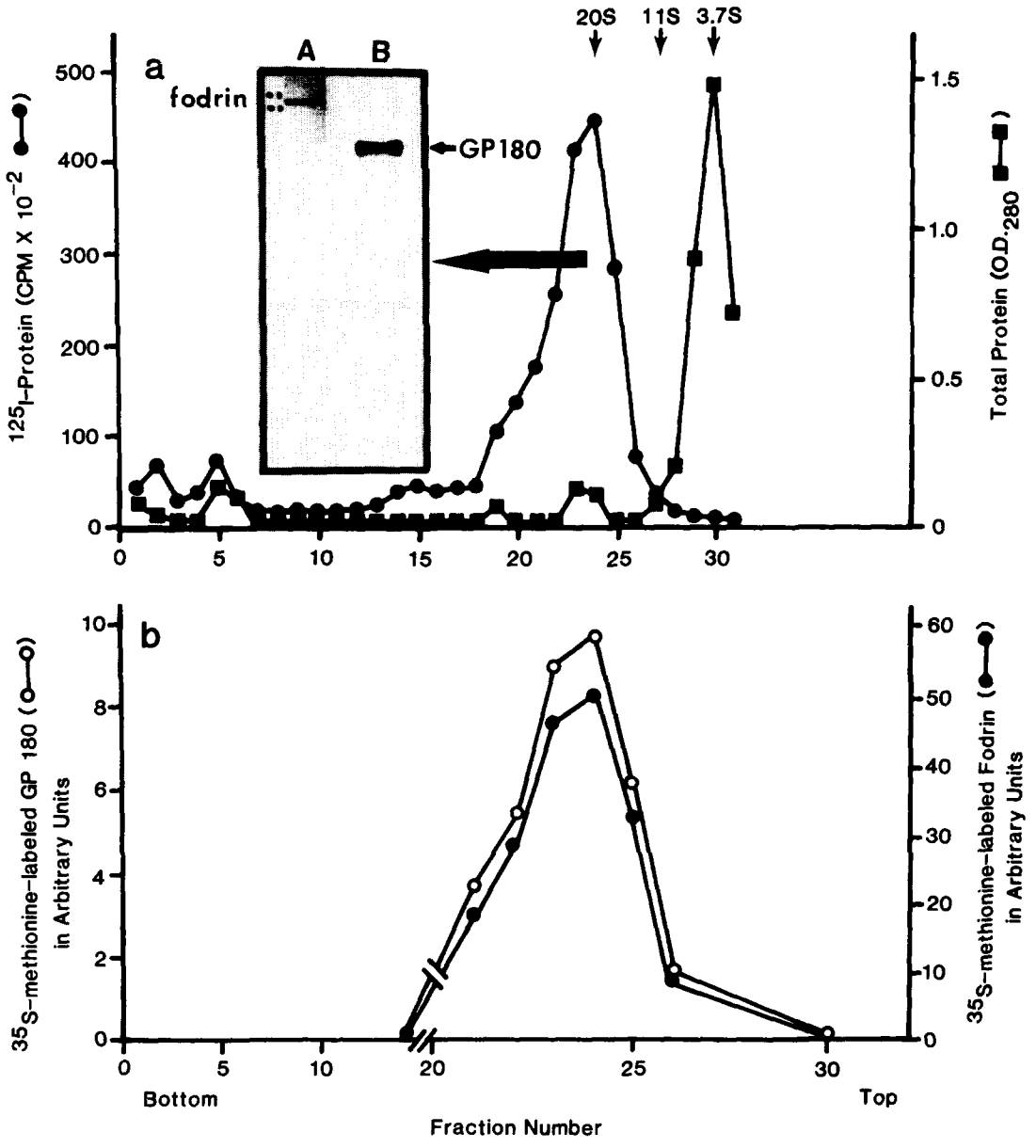


Fig. 2. Analysis of ^{125}I-labeled Triton X-114 aqueous phase by sucrose gradient centrifugation. (a) The total protein profile as determined by absorbance at 280 nm (■) vs. the ^{125}I-labeled proteins (●). Protein standards of G-actin and tetrameric spectrin or fodrin with respective S values of 3.7 and 11 were used to determine the S value of the ^{125}I-labeled peak. (A and B): SDS-polyacrylamide gel electrophores analysis of the pooled ^{125}I-labeled 20 S peak fractions with Coomassie blue staining pattern (A) showing the actin-binding protein, fodrin (240/235) and corresponding autoradiogram (B) demonstrating the presence of ^{125}I-labeled GP 180. (b) Profiles of [^{35}S]methionine-labeled GP 180 and fodrin across the 20 S peak fractions indicated in (a). The relative ratios of fodrin (●) and GP 180 (○) were calculated from densitometry tracings of autoradiograms obtained from individual fractions within the peak. Similar results were also obtained with both ^{125}I-labeled and silver-stained material.

S peak material (Fig. 6A). A band in the range of M_r 180000 suggested that GP 180 was co-precipitated along with fodrin. Autoradiographic analysis of this same gel lane confirmed the presence of GP 180 in the immunoprecipitated material (Fig. 6B). Analysis of this immunoprecipitated material

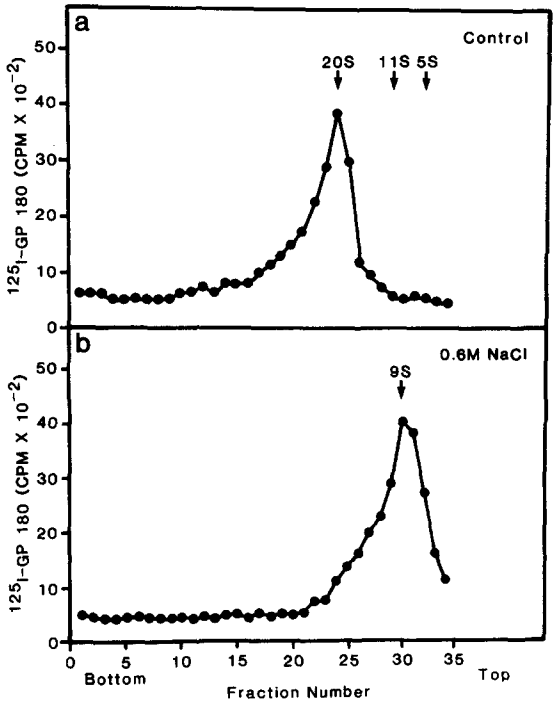
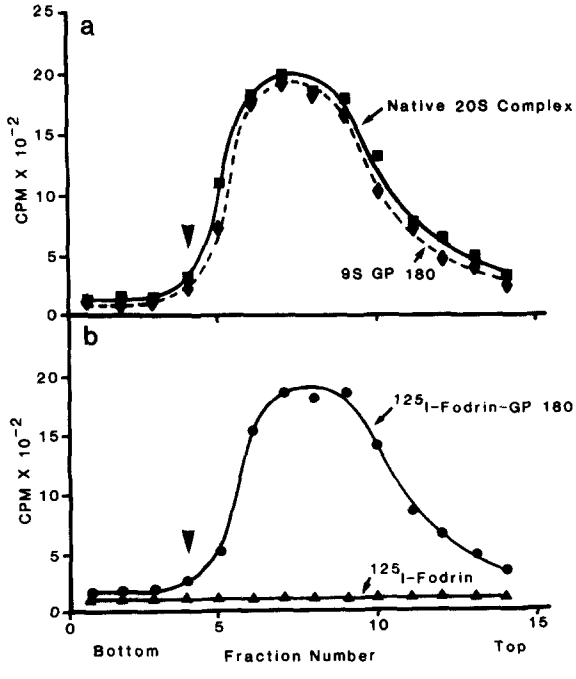


Fig. 3. High-salt treatment of the 20 S, ^{125}I-labeled GP 180-containing material. (a) Sucrose gradient centrifugation of the control 20 S material. (b) Sucrose gradient centrifugation of the 20 S material after exposure to 0.6 M NaCl. Following high-salt treatment the 20 S, GP 180-containing material is reduced to a value of 9 S.



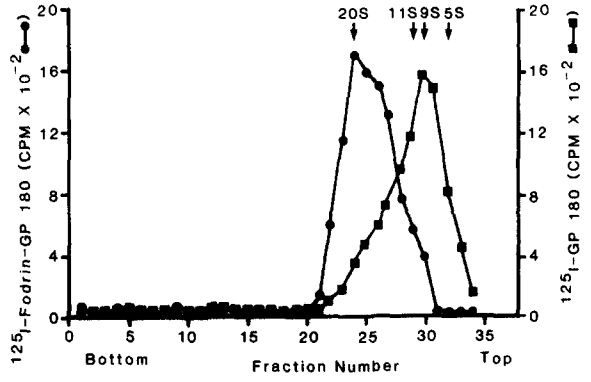


Fig. 5. Sucrose gradient centrifugation of newly formed ^{125}I-labeled fodrin-GP 180 material (prepared according to the procedure outlined in Materials and Methods and shown in Fig. 4b (●)) eluted from Con A-Sepharose by the addition of methyl α-D-mannoside. The ^{125}I-labeled fodrin containing material (●) sediments with a sedimentation coefficient of approx. 20 S, indicating that the fodrin-GP 180 complex can be assembled by the addition of exogenous fodrin. A parallel gradient of 9 S, ^{125}I-labeled GP 180-containing material alone, sugar eluted from a Con A-Sepharose column, was run as a control (■). Protein standards of G-actin (3.7 S), transferrin (5 S), and tetrameric fodrin or spectrin (11 S) were used to determine S value.

indicates that fodrin and GP 180 appear in a ratio similar to that observed in the 20 S peak fractions (Fig. 2) and in our previously published immunoprecipitation experiments [1]. The control precipitate showed neither fodrin (Fig. 6C) nor GP 180 (Fig. 6D). These data provide more evidence that GP 180 and fodrin are specifically associated in the 20 S peak.

Fig. 4. In vitro reassembly of exogenous pig brain fodrin with lymphocyte GP 180. (a) Con A-Sepharose binding properties of native 20 S complex and 9 S, GP 180. Native 20 S, fodrin-^{125}I-labeled GP 180 complex (■) is first bound to a Con A-Sepharose column and then specifically eluted by addition of the sugar, methyl α-D-mannoside (arrowhead). This parallels the pattern of binding and elution of 9 S, GP 180 (◆). b. Binding of ^{125}I-labeled fodrin to a GP 180-Con A-Sepharose column and specific elution with the sugar, methyl α-D-mannoside. ^{125}I-labeled fodrin is bound to GP 180 (9 S)-Con A-Sepharose (●) and then specifically eluted following the addition of the sugar, methyl α-D-mannoside (arrowhead). ^{125}I-labeled fodrin that is nonspecifically bound to Con A-Sepharose in the absence of GP 180 (▲) is not eluted by the addition of sugar. The elution of ^{125}I-fodrin from the GP 180-Con A-Sepharose column parallels that of both 9 S, GP 180 alone and the native 20 S, fodrin-GP 180 complex.

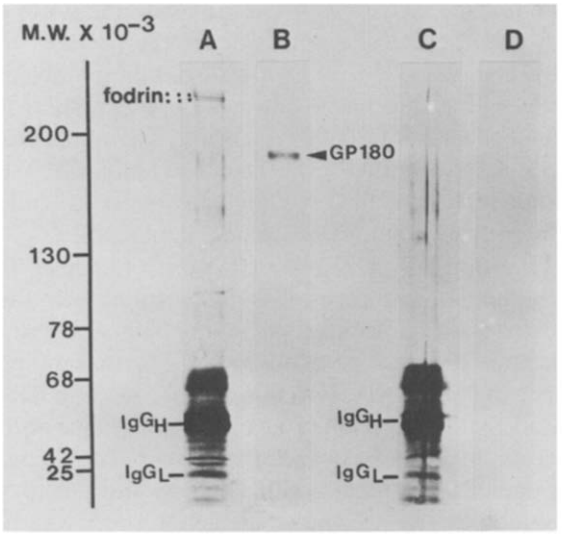


Fig. 6. Immunoprecipitation of the 20 S peak material with rabbit anti-fodrin antibody followed by goat anti-rabbit immunoglobulin (IgG). (A) Silver staining pattern of the immunoprecipitate showing fodrin as the major specific precipitated protein. (B) Corresponding autoradiogram indicating the co-precipitation of GP 180. (C) Control lane showing the nonspecifically precipitated bands and (D) the corresponding control autoradiogram indicating the absence of GP 180 (IgG_H = heavy chain of IgG; IgG_L = light chain of IgG).

Additional evidence that fodrin is associated with GP 180 in the 20 S peak came from examining the localization of these proteins along actin filaments formed in the presence of the 20 S complex material. The localization of fodrin using anti-fodrin antibody followed by protein A-gold showed labeling of small clusters along actin filaments (Fig. 7a and 7b). A very similar labeling pattern was observed when the distribution of GP 180 along actin filaments, using anti-GP 180 antibody followed by protein A-gold, was examined (Fig. 7c and 7d). No labeling was observed on control grids that were either treated with fodrin or GP 180 pre-adsorbed serum or in which the primary antibody step was omitted.

Since the 20 S, fodrin-GP 180-containing peak material was shown to be associated with F-actin filaments by cytochemical procedures, we decided to examine biochemically its ability to bind actin. When isolated ^{125}I-labeled 20 S peak material is incubated in the presence of actin, it moves farther into the sucrose gradient than control material (Fig. 8). Protein standards demonstrated that the actin-incubated material had sedimentation coefficient of 34 S. The increased S value indicated that

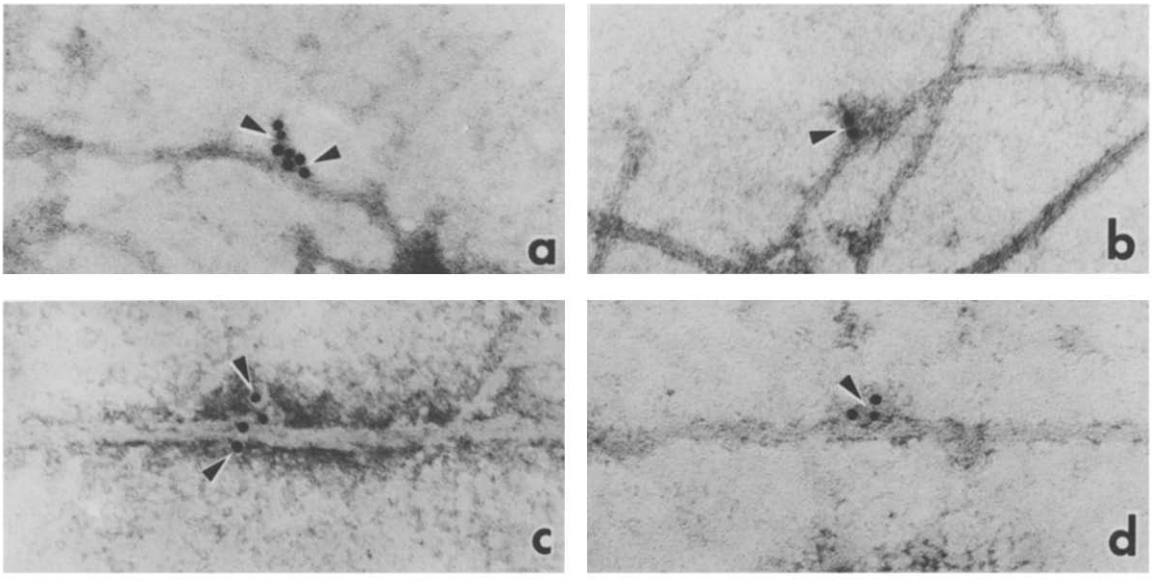


Fig. 7. Immuno-gold localization of both GP 180 and fodrin on actin filaments that were formed in the presence of the 20 S, GP 180-fodrin containing material. (a and b) Negative-stain image of actin filaments showing the association of fodrin (arrowheads; 108000). (c and d) Negative-stain image of actin filaments showing the association of GP 180 (arrowheads; 108000).

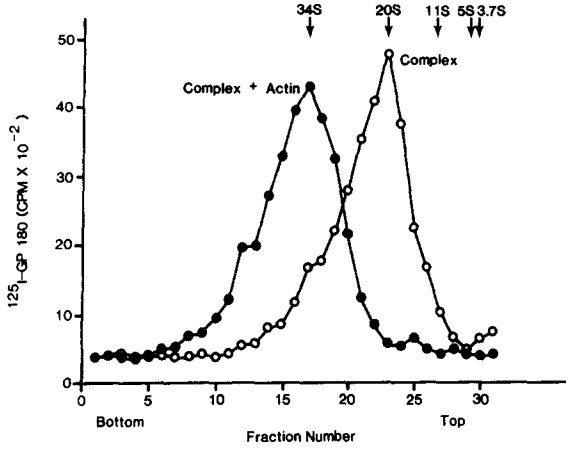


Fig. 8. The ^{125}I-labeled 20 S peak material was incubated with G-actin under polymerizing conditions and then analyzed by sucrose gradient centrifugation. Following incubation with actin, the S value of the complex shifted from 20 S to 34 S, indicating that the complex has the ability to bind to actin. Fodrin (11 S), transferrin (5 S) and G-actin (3.7 S) were used as protein standards of known S values.

the isolated 20 S peak could bind actin. Actin binding with the fodrin and GP 180 complex was verified by SDS-electrophoretic analysis (data not shown). The immuno-gold localization of fodrin and GP 180 along the actin filaments in the 34 S peak fractions was very similar to that observed in Fig. 7 (data not shown). The results from these aforementioned experiments, examined as a whole, suggest that lymphoma fodrin and GP 180 are closely associated as a stable transmembrane complex with the ability to bind actin. This transmembrane complex might thereby mediate or participate in the interaction of cell-surface receptors with the cytoskeleton during patching/capping in mouse T-lymphocytes.

Discussion

Previously, we have identified a transmembrane complex from T-lymphoma plasma membrane that may play a role in linking one or more surface receptors to the cytoskeleton during ligand-induced cell-surface capping or other cytoskeleton-mediated surface events [1]. In this study, we have carried out further structural analysis of this transmembrane complex in order to understand the linkage properties between membrane protein (s) and the cytoskeleton. One of the proteins in this complex, GP 180, is a transmembrane glycoprotein that has been shown to be very similar if not equivalent to T-200 (a major T-lymphocyte-specific glycoprotein) [1]. Like T200 [40], GP 180 binds concanavalin A and can be specifically eluted from columns of Con A-Sepharose.

During Triton X-114 extraction of mouse T-lymphoma membranes, GP 180 partitions into the Triton X-114 aqueous phase along with such cytoskeletal proteins as actin and the actin-binding protein, fodrin [1]. For this reason, we hypothesized that GP 180 might be closely associated with certain hydrophilic cytoskeletal components in unstimulated lymphoma cells. Glenney and Glenney [6] used this same type of extraction procedure to isolate the M_r 110000 brush-border protein from other cytoskeletal proteins and characterize it as an integral membrane protein.

Further analysis of the aqueous phase of Triton X-114-extracted ^{125}I-labeled lymphoma membranes enabled us to identify a distinct peak on sucrose density gradients in which ^{125}I-labeled GP 180 and the actin-binding protein, fodrin, are major components (Fig. 2; Ref. 1). The co-sedimentation of fodrin and GP 180 suggests that these two proteins may be closely associated. Carraway et al. [7] have used similar separation procedures to isolate a transmembrane protein-actin complex from detergent-extracted microvillar membranes. Our ability to co-isolate fodrin and GP 180 from this 20 S peak material with anti-fodrin antibody (data not shown) further strengthens the evidence that GP 180 and fodrin are specifically associated.

By running protein standards with known S values during the isolation of the GP 180 containing material on sucrose gradients, we were able to calculate a sedimentation coefficient of 20 S for the associated fodrin-GP 180 material. To determine the fodrin : GP 180 ratio throughout the 20 S, GP 180-containing peak, we isolated this material from cells metabolicity labeled with [^{35}S]methionine. SDS-electrophoresis autoradiography followed by scanning densitometry of the autoradiogram established a constant ratio of fodrin to GP 180 across the 20 S peak. In addition, these two proteins appear to be labeled to

a similar extent by silver staining, ^{125}I-labeling, and [^{35}S]methionine labeling. Therefore, it is possible for us to speculate on the stoichiometry of these proteins based on the following assumptions. The assumptions are: (1) fodrin is present in a tetrameric form (combined M_r 950000); (2) GP 180 is present in a monomeric form (M_r 180000); and (3) both proteins are labeled equivalently. According to the aforementioned assumptions, we can calculate a 1 : 1 molar ratio of fodrin to GP 180 in the pooled fractions of the 20 S peak as described previously [1]. In this study, the results of similar calculations also reveal a 1 : 1 molar ratio of fodrin to GP 180 in the individual fractions across the 20 S peak. Therefore, we believe that a close association exists between these two molecules. The same 1 : 1 molar ratio has been demonstrated for the ankyrin-band 3 transmembrane complex from erythrocytes [34], and the actin-CAG transmembrane complex from the microvillar membranes of ascites tumor cells [7].

Following exposure to the same high salt conditions previously used to remove fodrin from membranes [21,22], the ^{125}I-labeled GP 180 containing material sediments at an S value of 9, less than that estimated for isolated tetrameric fodrin (11 S). This reduced S values suggested that fodrin was separated from GP 180 under high-salt conditions. Similar high-salt conditions have been shown to cause the dissociation of the ankyrin-band 3 complex from spectrin [34]. The shift in S value of the GP 180, 9 S peak material to its original 20 S value can occur following the binding of ^{125}I-labeled fodrin to 9 S, GP 180 in the absence of high salt (Fig. 4). These data suggest that these two proteins are able to reassemble as a transmembrane complex. Most importantly, our reassociated fodrin-GP 180 complex also displays lectin-binding properties similar to those of the native 20 S complex, implying that the in vitro assembly of these two proteins may be similar to that of the native complex. Our ability to high-salt-dissociate the 20 S, fodrin-GP 180-containing material and then reform it by the addition of fodrin to the 9 S, GP 180-containing material in the absence of high salt further corroborates our evidence that there is an interaction between fodrin and GP 180.

Both cytochemical (Fig. 7) and biochemical (Fig. 8) data indicate that the 20 S, fodrin-GP 180 complex is capable of binding F-actin. In addition, the amount of both fodrin and GP 180 in the actin-containing cytoskeleton increases significantly during lymphocyte patching and capping [1,27]. Since fodrin is an actin-binding protein, it is reasonable to assume that, functionally, the interaction of the fodrin-GP 180 complex with actin is through fodrin.

From this study, we have further demonstrated that fodrin is closely associated with a specific transmembrane protein, GP 180, in T-lymphoma plasma membranes. Additionally, this transmembrane complex has the ability to interact with actin, and such an interaction may be responsible for the linkage of the GP 180 receptor to the cytoskeleton. Apparently missing from our fodrin-GP 180 complex is an ankyrin-like or 4.1-like protein, spectrin-binding proteins that appear to mediate the binding of spectrin to erythrocyte membranes [23,45]. Although both an ankyrin-like protein and a 4.1-like protein have been localized in the subcap region of capped lymphocytes [27,46,47], and polypeptides of both proteins have been isolated from lymphocytes [46,47], these proteins have not been successfully identified in our 20 S material. It is possible that these proteins (present in small quantities in the complex) are responsible for linking GP 180 and fodrin. Alternatively, GP 180 may itself have certain ankyrin-like or 4.1-like fodrin-binding properties in its cytoplasmic domain that allows it to bind directly to fodrin in mouse T-lymphoma cells. This possibility is under active investigation in our laboratory.

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