A Cell Surface Integral Membrane Glycoprotein of 85,000 mol wt (gp85) Associated with Triton X-100–insoluble Cell Skeleton

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ABSTRACT The Triton X-100–insoluble skeleton of baby hamster kidney BHK cells consists of the nucleus, intermediate-size filaments, and actin fibers. By transmission electron microscopy, membrane fragments were found to be associated with these insoluble structures. When radioiodinated or [^{3}H]glucosamine-labeled cells were extracted with 0.5% Triton, most plasma membrane glycoproteins were solubilized except for a glycoprotein with a molecular weight of 85,000 (gp85) that remained associated with the insoluble skeletons. Immunoprecipitation with a specific antiserum indicated that the gp85 is not a proteolytic degradation product of fibronectin, an extracellular matrix glycoprotein insoluble in detergent.

A monoclonal antibody of BHK cells specific for gp85 was produced. Immunofluorescence analysis with this monoclonal antibody indicated that gp85 is not associated with the extracellular matrix, but is confined to the cell membrane. Both in fixed and unfixed intact cells, fluorescence was concentrated in dots preferentially aligned in streaks on the cell surface. Gp85 was found to behave as an integral membrane protein interacting with the hydrophobic core of the lipid bilayer since it was extracted from membrane preparations by ionic detergents such as SDS, but not by 0.1 N NaOH (pH 12) in the absence of detergents, a condition known to release peripheral molecules. Association of gp85 with the cell skeleton was unaffected by increasing the Triton concentration up to 5%, but it was affected when actin filaments were dissociated or when a protein-denaturing agent (6 M urea) was used in the presence of Triton, suggesting that protein–protein interactions are involved in the association of gp85 with the cell skeleton. We conclude that gp85 is an integral plasma membrane glycoprotein that might have a role in cell surface–cytoskeleton interaction.

When nucleated cells in culture are extracted with non-ionic detergents, most cellular components, including membrane-bound organelles, are solubilized. However, intermediate filaments and actin fibers are left as an insoluble residue that retains the three-dimensional organization of the intact cell cytoskeleton (1–3). Moreover, cytoskeleton-associated molecules, known to play a role in actin–plasma membrane interaction, have been detected in the detergent-insoluble cell fraction. These include the two subunits of spectrin, ankyrin, a polypeptide termed band 4.1 in erythrocytes (4–7), fimbrin (8), villin (9), vinculin (10), c actinin (11), and spectrin-like molecules (12, 13) in nucleated cells. In addition, one integral membrane glycoprotein, band III, has been reported to remain associated to the erythrocyte submembranous cytoskeleton after detergent extraction (14). This glycoprotein represents the membrane attachment protein for spectrin–ankyrin complexes.

In this paper, we identified, by means of a monoclonal antibody, a cell surface integral membrane glycoprotein of 85,000 mol wt (gp85) associated to the Triton-insoluble skeleton. The detergent insolubility and possible function of this molecule are discussed.

MATERIALS AND METHODS

Cell Culture and Metabolic Labeling: Early passage baby hamster kidney (BHK) 21/C13 cells,[1] obtained from the stock of Dr. Macpherson

[1] *Abbreviations used in this paper:* BHK, baby hamster kidney; FITC, fluorescein isothiocyanate.

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(Imperial Cancer Research Fund, London), were routinely grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, and antibiotics. Metabolic labeling of glycoproteins was achieved by incubating cell monolayers for 15 h in complete medium containing 30 μCi/ml of D-[6-^{3}H]glucosamine (20 Ci/mmol, Amersham Corp., Arlington Heights, IL). Whole cellular proteins were labeled by incubating cell monolayers for 6 h with 20 μCi/ml of [^{35}S]methionine (800 Ci/mmol, Amersham Corp.) in methionine-free medium containing 10% fetal calf serum.

Radioiodination of Surface Proteins: The lactoperoxidase-glucose oxidase–catalyzed radioiodination procedure was used (15). Cell monolayers were rinsed briefly with PBS and incubated with 2 ml of PBS containing 2.5 mM glucose, 40 μg of lactoperoxidase, and 250 μCi of carrier-free [^{125}I]sodium iodide. The reaction, started by adding 0.4 U of glucose oxidase, was carried out for 5 min at room temperature. After repeated washings with cold serum-free medium, labeled cells were extracted with detergent as described below.

Detergent Extraction: Adherent cell monolayers were rinsed twice with medium lacking serum and incubated at 0°C for 20 min with the Triton buffer (20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1 mM calcium chloride, 1 mM magnesium chloride, 0.5% Triton X-100 and 2 mM phenylmethylsulphonyl fluoride as protease inhibitor). Cell layers were then rinsed twice with fresh Triton buffer and the insoluble material was solubilized with 2% SDS and 0.2% mercaptoethanol. After boiling for 2 min and 20-s sonication to decrease viscosity, samples were analyzed by electrophoresis.

Preparation of Antisera and Monoclonal Antibodies: The antiserum to fibronectin was prepared as described below. Human plasma fibronectin was purified by affinity chromatography on denatured collagen (16) as previously described (17). 300 μg of the purified protein was emulsified in complete Freund's adjuvant and injected intramuscularly in rabbits. After 25 d, rabbits were boosted with the same amount of protein, and 7 d later, blood was collected. The antiserum reacted selectively with fibronectin as shown by immunoprecipitation analysis (18).

We obtained monoclonal antibodies using the technique of Koehler and Milstein (19) by fusing mouse myeloma cells P3/X63-Ag8 with splenocytes of mice immunized with BHK cells, as previously described (20). Antibodies to surface antigens were isolated by measuring the binding activity of hydridoma culture supernatants on fixed BHK cells (20). The monoclonal antibody to gp85 (K-3) was identified by immunoprecipitation of [^{3}H]glucosamine-labeled Triton-insoluble cell skeletons using the procedure described below.

Immunofluorescence Microscopy: Cells were grown to subconfluent monolayers on 12-mm glass coverslips, rinsed with PBS, and fixed with 3.5% formaldehyde for 20 min at room temperature. After washing, fixed cells were incubated for 60 min with mouse ascitic fluid containing the monoclonal antibody diluted 1/100. In some experiments, cells were rinsed in serum-free medium and incubated with the monoclonal antibody, at either 4° or 25°C, before fixation. Affinity-purified rabbit antibodies to mouse immunoglobulins were used as a second antibody layer, followed by fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin (Miles Laboratories Inc., Elkhart, IN). The three antibody layers were necessary to amplify the fluorescent signal that was otherwise too weak for photography. An identical procedure was followed with Triton-extracted cells except that formaldehyde fixation was performed after detergent extraction. Coverslips were then mounted with 50% glycerol in PBS and examined in a Leitz Dialux microscope equipped with an epiillumination system. Control experiments, performed using ascitic fluids containing unrelated monoclonal antibodies, were constantly negative.

Staining for F-actin was obtained by incubating detergent-extracted cells with FITC-labeled phalloidin (kindly provided by Dr. T. Wieland, Max Planck Institute for Experimental Medicine, Heidelberg). Intermediate filaments were decorated by using affinity chromatography–purified guinea pig antibodies to vimentin (a generous gift of Dr. W. Franke, German Institute for Cancer Research, Heidelberg) and FITC-labeled antiserum to guinea pig immunoglobulins (Miles Laboratories Inc.).

Immunoprecipitation Analysis: Selective immunoprecipitation of labeled glycoproteins was performed as previously described (18). To identify components of the Triton-insoluble cell residues, we solubilized this material with the Triton-deoxycholate-SDS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, and 2 mM phenylmethylsulphonyl fluoride as protease inhibitor). The extracts were sonicated to reduce viscosity and centrifuged in an Eppendorf microfuge for 15 min. The solubilized material was incubated with the appropriate amount of antiserum (10 μl of antifibronectin serum or 20 μl of mouse ascitic fluid containing 40 μg of specific monoclonal antibody) for 1 h at 0°C, and immunocomplexes were recovered by adsorption on Protein A-Sepharose beads (Pharmacia Inc., Piscataway, NJ). After washing, we eluted bound material by boiling beads in 1% SDS and analyzed it by SDS PAGE and fluorography (see below). The monoclonal antibody K-3 is of the IgM class and does not react with the Protein A of *Staphylococcus*. For this reason affinity-purified rabbit antibodies to mouse immunoglobulins were bound to Protein A-Sepharose (100 μg of antibodies per 50 μl of packed beads) and the complex was then used to recover the antibody–antigen immunocomplexes. A second immunoprecipitation cycle, performed on the supernatant of the Protein A-Sepharose, gave negative results indicating that all gp85 molecules present in solution were quantitatively recovered in the first immunoprecipitation.

Membrane Extraction: A crude plasma membrane fraction was prepared from [^{3}H]glucosamine-labeled BHK by Dounce homogenization in isotonic buffer (Dulbecco's modified Eagle's medium). Nuclei were removed with low gravity centrifugation for 10 min, and membrane fragments were collected by sedimentation at 100,000 *g* for 1 h. 1 vol of packed membranes was extracted with 10 vol of 0.1 N sodium hydroxide in ice and immediately centrifuged at 100,000 *g* (21). The supernatant was neutralized with the addition of diluted hydrochloric acid and the pellet was solubilized in the Triton-deoxycholate-SDS buffer with brief sonication. In parallel experiments membrane pellets were extracted with the standard Triton buffer and after centrifugation the insoluble residue was solubilized with Triton-deoxycholate-SDS buffer as above. All fractions were subjected to immunoprecipitation with the monoclonal antibody K-3 to identify gp85.

Electrophoresis and Fluorography: SDS PAGE was carried out on 5–15% acrylamide gradient slab gels using the procedure described by Laemmli (22). Gels were processed for fluorography as described by Laskey and Mills (23), dried, and placed in contact with a Kodak X Omat R film. The following ^{14}C-labeled molecular weight markers (Amersham Corp.) were used: myosin heavy chain (200,000 mol wt), phosphorylase *b* (92,000), BSA (68,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,000).

Transmission Electron Microscopy: Both intact and Triton-extracted cell monolayers were fixed in 1% glutaraldehyde, and postfixed in 1% osmium tetroxide according to Goldman et al. (24). Sections of Epon-Araldite–embedded material were cut perpendicular to the cell monolayer.

RESULTS

Morphological and Biochemical Analysis of Triton-extracted BHK Cells

Extraction of adherent BHK cells with 0.5% Triton X-100 in an isotonic buffer did not detach the cells from the substratum. As viewed by phase-contrast microscopy, detergent-treated cells appeared as ghosts that retained the original morphology of the cell and contained the nucleus surrounded by a barely visible meshwork of fibrils (Fig. 1, *B* and *C*). Cytoplasmic organelles, which were clearly visible in intact cells (Fig. 1*A*), were no longer detectable after detergent extraction. Immunofluorescence staining with FITC-labeled phalloidin or with antivimentin antibodies (Fig. 1, *D* and *E*) indicated that actin fibers and intermediate-size filaments were present in the insoluble cell residues. No staining was observed with antitubulin antibodies, indicating that microtubules had been extracted under these conditions.

Transmission electron microscope analysis of the Triton-insoluble cell residues revealed the presence of filamentous material and electron-dense particles that may represent residual ribosomes (Fig. 2). In addition, a considerable number of membrane vesicles of heterogeneous size and discontinuous plasma membrane fragments delimiting the outer boundary of the skeletons were constantly observed (Fig. 2, *B–D*). These Triton-insoluble cell residues will be referred to as cell "skeletons."

About 20% of total [^{35}S]methionine-labeled cellular proteins were left in the cell skeletons (Table I). This material was resolved by SDS PAGE into two major components with apparent molecular weights of 43,000 and 58,000 (Fig. 3*A*), which co-migrated with purified actin and vimentin, respectively (not shown), and are likely to represent the cytoskeletal proteins of the micro- and intermediate-size filaments detected by immunofluorescence experiments. Weakly stained

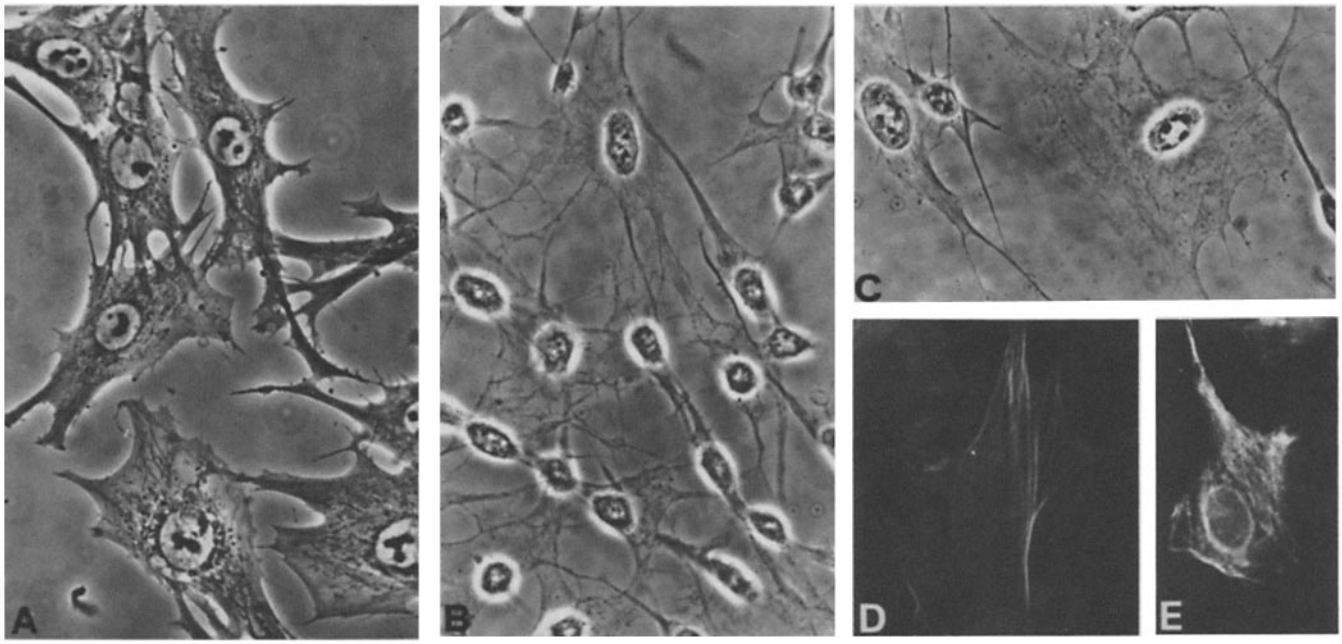

FIGURE 1 Phase-contrast and immunofluorescence microscopy of intact and Triton-extracted BHK cells. Subconfluent monolayers of BHK cells grown on glass coverslips were extracted with 0.5% Triton in isotonic buffer for 20 min at 0°C as described in Materials and Methods and fixed with 3.7% formaldehyde. Coverslips were mounted in PBS containing 50% glycerol on microscope slides. (*A*) Unextracted cells; (*B* and *C*) Triton-extracted cells; (*D*) Triton-extracted cells stained with FITC-phalloidin. (*E*) Triton-extracted cells stained with affinity-purified antivimentin antibodies (20 μg/ml) followed by FITC-labeled anti-guinea pig immunoglobulins. × 1,000.

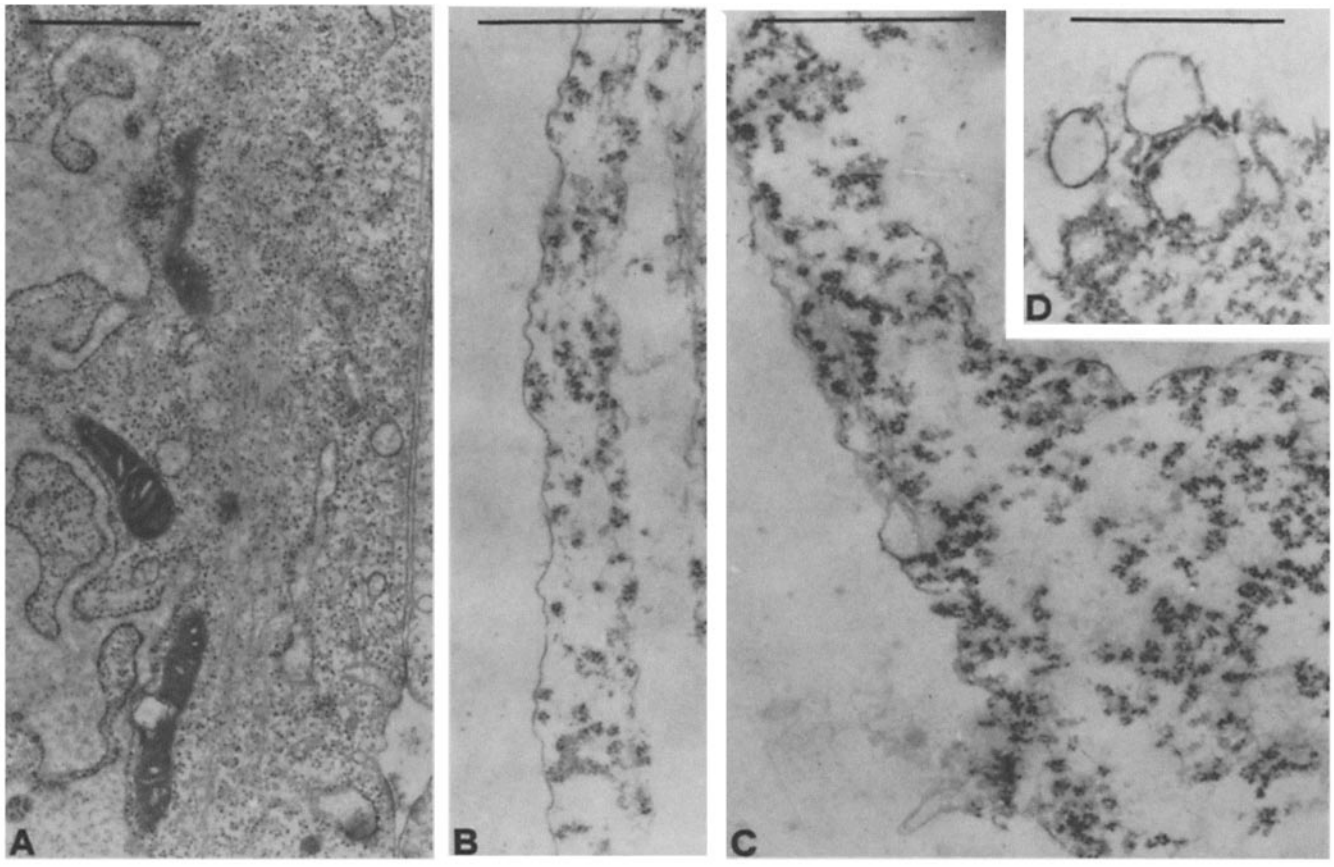

FIGURE 2 Electron micrographs of thin-sectioned intact and Triton-extracted BHK cells. Monolayers of either intact or 0.5% Triton-extracted BHK cells were fixed in 1% glutaraldehyde and processed for Epon-Araldite embedding as described in Materials and Methods. (*A*) intact cell; (*B–D*) Triton-extracted cells. Bars, 1 μm.

bands with molecular weights of 180,000, 110,000–130,000, 80,000, and 35,000, representing unidentified minor components, were also detected.

Identification of Cell Surface Molecules Associated with the Triton-insoluble Cell Skeletons

When subconfluent cultures of surface-radioiodinated BHK cells were extracted with Triton, most labeled material was solubilized, while ~10% of the radioactivity remained associated with the insoluble cell layer (Table I). This material was resolved by SDS PAGE into a major labeled component with a molecular weight of 85,000 (Fig. 3*C*). This component was also labeled with [^{3}H]glucosamine (Fig. 3*E*), indicating that this is a surface glycoprotein (gp85) synthesized by the cell. Gp85 migrates in SDS PAGE as a diffuse band.

In addition to the gp85, a surface-labeled component with a molecular weight of 220,000 was found in the Triton-insoluble residue (Fig. 3, *D* and *E*). This protein was identified as the reduced subunit of fibronectin, as it was selectively immunoprecipitated by specific antibodies (Fig. 3*F*). The amount of fibronectin in the insoluble residue varied in different preparations, being higher when confluent cells were used. This is in agreement with previous reports showing that fibronectin is deposited in a detergent-insoluble extracellular matrix in large amounts only by confluent cell cultures (25). In contrast, the amount of gp85 in cell skeleton preparations was rather constant and did not vary significantly when the cell monolayer became confluent. The antifibronectin serum did not recognize the gp85 (Fig. 3*F*), indicating that the latter is immunologically distinct from fibronectin and does not represent a proteolytic fragment of this molecule.

In addition to the gp85 and fibronectin, material that poorly penetrated the gel was observed in [^{3}H]glucosamine-labeled cells (Fig. 3*E*). This component might represent glycosaminoglycans of the extracellular matrix in that it was significantly reduced after digestion with chondroitinase (not shown).

The insolubility of gp85 was not due to an unfavorable detergent–protein ratio. In fact, the amount of gp85 associated with the skeleton did not vary when the cells were extracted for longer times (up to 60 min) or when the Triton concentration was increased 10 times (Table II). On the other hand, gp85 was extracted by 6 M urea in 0.5% Triton (Table II), indicating that protein-denaturing agents, in association with the detergent, are required to achieve solubilization. This behavior suggests that association of gp85 with the cell skele-

TABLE I

Extraction of Labeled Cellular Proteins with Triton X-100

Label	Triton soluble	Triton insoluble
	%	
[^{35}S]Methionine	78	22
^{125}I Surface radioiodination	90	10
[^{3}H]Glucosamine	85	15

Subconfluent monolayers of labeled BHK cells were extracted for 20 min at 0°C with 0.5% Triton X-100 in isotonic buffer at pH 7.4 and rinsed two times with fresh Triton buffer. The counts per minute in the soluble and insoluble fractions are expressed as percentage of total radioactivity incorporated by the cells. Numbers indicate the mean of three independent determinations.

TABLE II

Amount of GP85 Associated with Cell Skeletons Prepared under Different Extraction Conditions

Extraction buffer	Time of extraction	gp85*
	min	
0.5% Triton	20	1.29
0.5% Triton	60	1.25
5.0% Triton	20	1.32
0.5% Triton + 6 M urea	20	0.01

Subconfluent cell monolayers, labeled with [^{3}H]glucosamine, were extracted at 0°C with the standard buffer solution (20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1 mM calcium chloride, 1 mM magnesium chloride, and 2 mM phenylmethylsulphonyl fluoride) containing the appropriate concentration of detergent or urea. Extraction was performed for the indicated time with two buffer changes. Insoluble material was desolved in SDS and analyzed by SDS PAGE as described in Materials and Methods. After fluorography and exposure to the x-ray film, the electrophoretic band corresponding to gp85 was excised from the gel and the associated radioactivity was determined by liquid scintillation counting.

* The amount of gp85 associated with the skeletons is expressed as percentage of total radioactivity incorporated by the cells. Numbers indicate the mean of three independent determinations.

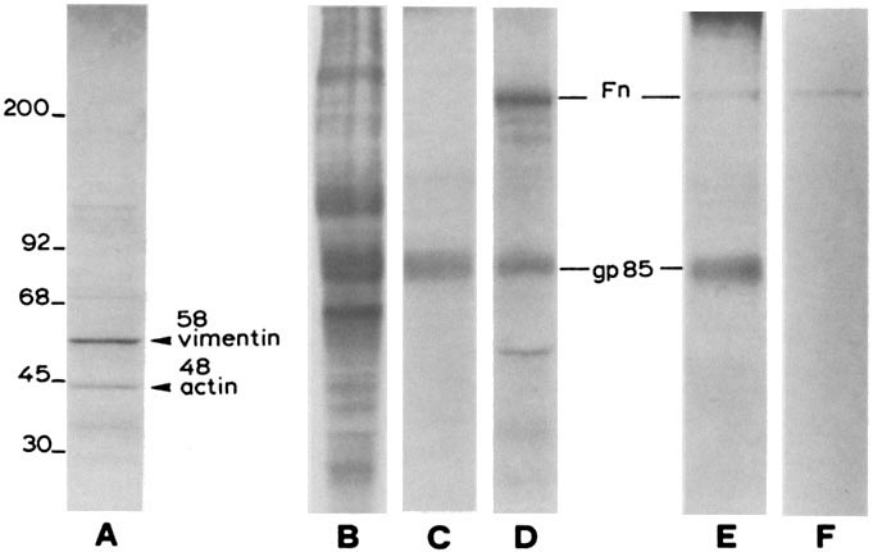

FIGURE 3 Cellular polypeptides and surface glycoproteins of the Triton-insoluble skeletons. BHK cells, either unlabeled or labeled with lactoperoxidase-catalyzed surface radioiodination or with [^{3}H]glucosamine, were extracted with 0.5% Triton for 20 min at 0°C and rinsed two times with fresh Triton buffer. The insoluble skeletons were solubilized with SDS and analyzed by SDS PAGE. Labeled material was detected by fluorography. (*A*) Coomassie Blue-stained pattern of the Triton-insoluble skeletons. (*B–D*) Surface radioiodinated cells: Triton-soluble fraction (*B*); Triton-insoluble skeletons prepared from sparse cell culture (*C*); Triton-insoluble skeletons prepared from confluent culture (*D*). (*E–F*) [^{3}H]-Glucosamine-labeled cells: Triton-insoluble skeletons from confluent culture (*E*); material immunoprecipitated with antibodies to fibronectin from the Triton-insoluble fraction (*F*). Material corresponding to 5 × 10 cells was loaded on each lane. The mobility of proteins with known molecular weights (× 10^{-3}) is indicated on the left. Arrowheads point to polypeptides with molecular weights identical to those of actin and vimentin. *Fn*, fibronectin.

ton depends upon interactions with other protein components. To test this possibility, we performed the following experiments. The cell surface was subjected to mild proteolytic treatment with trypsin (20 μg/ml for 10 min at 20°C) to digest the extracellular matrix. After this treatment, fibronectin was no longer detectable in the skeletons. The gp85, in contrast, was unaffected by the low trypsin concentration used and remained associated to the Triton-insoluble material (not shown). We performed another set of experiments by extracting the cells with Triton X-100 under different ionic conditions that allow selective extraction of cytoskeletal elements. Virtually all vimentin, but only small amounts of actin, were extracted at low ionic strength in the absence of divalent cations (26) (Table III). However, at high ionic strength (600 mM potassium chloride) and in the presence of divalent cations (27), a significant fraction of actin was extracted without affecting vimentin (Table III). Gp85 was extracted more effectively under the high ionic strength conditions that caused actin solubilization.

TABLE III

Extraction of Cytoskeletal Proteins and gp85 with High or Low Ionic Strength Buffers

Ionic strength of buffer	Mg++	Percent of total Triton-insoluble proteins		
		43K (actin)	58K (vimentin)	gp85
Low‡	+	100*	100	100
Low§	–	80	5	70
High‖	+	40	98	40

[^{3}H]glucosamine-labeled BHK cells were extracted for 20 min at 0°C with buffers indicated below and rinsed two times with the same buffer, and the insoluble cell skeletons were analyzed by SDS PAGE. The amount of each protein was determined by densitometry of the gel stained with Coomassie Blue (actin and vimentin) or by cutting the electrophoretic band and counting the associated radioactivity (gp85). (*43K* and *58K*, 43,000- and 58,000-mol-wt proteins, respectively.)

* Extraction in low ionic strength buffer in the presence (+) of Mg resulted in maximal recovery of actin, vimentin, and gp85 in the insoluble residues. These values were then arbitrarily considered as 100% and the amount of proteins left in the residues after extraction with other buffers is expressed as percentage referred to this sample. Values represent the means of three independent experiments.

‡ 4 mM Mg-acetate, 20 mM Tris-acetate, pH 7.4, 1 mM EGTA, 0.5% Triton.

§ 20 mM Tris-acetate pH, 7.4, 1 mM EGTA, 0.5% Triton.

‖ 600 mM KCl, 4 mM Mg-acetate, 40 mM imidazole, 1 mM cysteine, 1 mM EGTA, 10 mM ATP, 0.5% Triton, pH 7.0.

These results indicate that protein–protein bonds, disrupted by high ionic strength or by denaturing agents, rather than hydrophobic interactions are likely to be involved in the association of gp85 with the cell skeleton. Moreover, the results of extraction experiments are compatible with an interaction of gp85 with actin filaments.

Immunoprecipitation of gp85 with a Specific Monoclonal Antibody

A monoclonal antibody to gp85 was isolated by screening, in immunoprecipitation assay, a battery of monoclonal antibodies directed to membrane antigens of BHK cells. The skeleton fraction, prepared from surface-radioiodinated BHK cells, was solubilized by sonication in a buffer containing 0.1% SDS as described in Materials and Methods. Under these conditions, ~80% of the protein-bound ^{125}I present in this fraction was solubilized. As shown in Fig. 4*A*, the monoclonal antibody K-3 selectively precipitated the gp85 molecule from the cell skeleton fraction. None of the membrane proteins identified by other isolated monoclonal antibodies (reacting with glycoproteins of 140,000, 67,000, and 65,000 mol wt) were found to be associated with the insoluble skeleton (not shown).

Immunoprecipitation with the monoclonal antibody showed that only a fraction of the total gp85 molecules was associated with the insoluble skeleton (Fig. 4, *A* and *B*). To evaluate the relative amount of Triton-soluble and skeleton-associated gp85, we performed quantitative immunoprecipitation experiments as described in Materials and Methods. In lactoperoxidase-radioiodinated cells, ~50% of the recovered surface gp85 molecules was solubilized by Triton, while the remaining 50% was associated to the cell skeleton and was not extracted even after repeated treatments with detergent. In glucosamine-labeled cells the fraction of gp85 associated to the cell skeleton ranged from 20 to 40% of the total molecules (Fig. 4, *D* and *E*). This difference might be explained by the fact that glucosamine also labels the intracellular pool of gp85.

To test whether the association of gp85 with the cell skeletons is an artifact of detergent treatment, extracted cells with

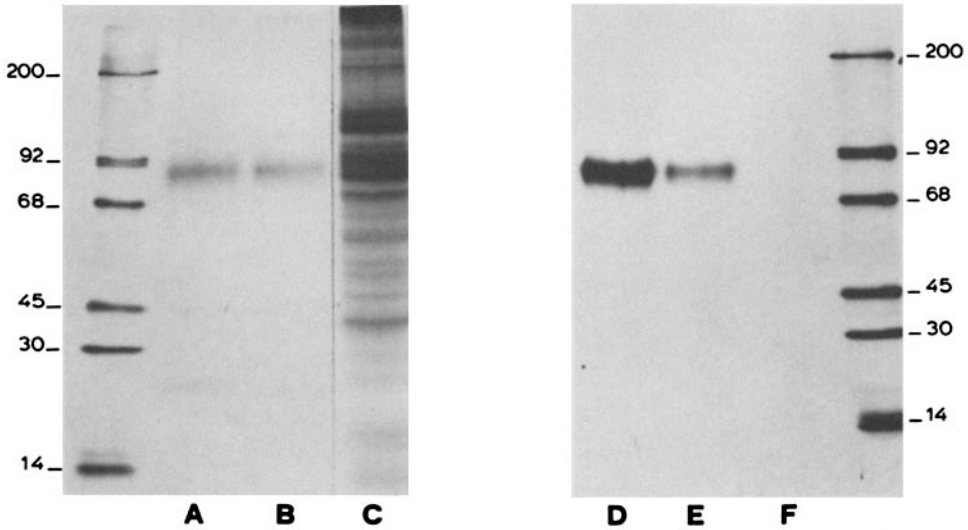

FIGURE 4 Immunoprecipitation of gp85 with monoclonal antibody K-3. BHK cells labeled with lactoperoxidase-catalyzed surface radioiodination or with [^{3}H]-glucosamine were extracted with Triton under standard conditions. Both detergent-soluble and cell skeleton fractions were subjected to immunoprecipitation with the monoclonal antibody K-3 specific for gp85 following the procedure described in Materials and Methods. (*Left*) Surface-radioiodinated cells: Material specifically immunoprecipitated from the Triton-soluble fraction (*A*) and insoluble skeletons (*B*); total radiolabeled cell surface proteins (*C*). (*Right*) [^{3}H]glucosamine-labeled cells: Material specifically immunoprecipitated from the Triton-soluble fraction (*D*) and insoluble skeletons (*E*); control immunoprecipitation with nonimmune mouse ascitic fluid (*F*). The two lanes at the extreme left and right are ^{14}C-labeled molecular weight (× 10^{-3}) markers.

Triton in the presence of exogenously added, radioactive gp85. A Triton-soluble fraction was prepared from a [^{3}H]-glucosamine-labeled BHK culture and used to extract a monolayer of unlabeled cells. Only trace amounts of the exogenous radiolabeled gp85 were recovered in the cell skeleton fraction, indicating that this glycoprotein neither becomes adsorbed onto the cell skeleton nor exchanges with the bound fraction.

Extraction of gp85 from Membrane Preparation

To establish the nature of the interaction of gp85 with the plasma membrane, we tested different extraction conditions. It has been shown that protein-perturbing reagents elute polar polypeptides (peripheral) from the human erythrocyte membrane leaving hydrophobic glycoproteins bound to the lipid core (4, 21). The latter class of molecules can only be solubilized by detergents that disrupt hydrophobic interactions (4). Membrane fractions, prepared from [^{3}H]glucosamine-labeled BHK cells, were incubated either with low ionic strength solution at pH 12 (0.1 N NaOH) or with 0.5% Triton in the standard buffer, and the soluble fractions were tested for the presence of gp85 by immunoprecipitation with the monoclonal antibody K-3. As shown in Fig. 5, gp85 was extracted by detergent but not by NaOH, indicating that gp85 is tightly bound to the lipid core and behaves as an integral plasma membrane glycoprotein.

Triton extraction of the membrane fraction further confirmed the results obtained with whole cells. In this case, ~40% of the gp85 molecules was associated to the detergent-insoluble membranous pellet. This fraction could be solubilized only by addition of SDS, a ionic detergent that causes protein denaturation (Fig. 5 *B*).

Localization of gp85 by Immunofluorescence

When cells were incubated with the monoclonal antibody K-3 and FITC-labeled antibodies, a number of small fluorescent dots distributed over the entire cell surface were observed (Fig. 6 *A*). The tiny fluorescent dots appeared in linear arrays

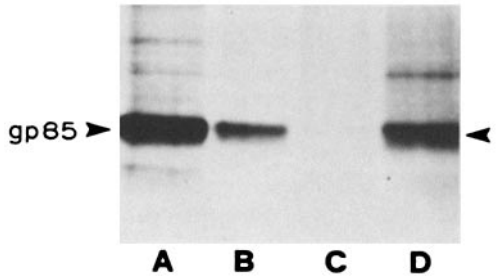

FIGURE 5 Differential release of gp85 from membrane preparation under different extraction conditions. A crude membrane fraction was prepared from [^{3}H]glucosamine-labeled BHK cells by Dounce homogenization as described in Materials and Methods. The membrane pellet was extracted either with ice-cold 0.1 N NaOH or with 0.5% Triton under standard conditions. The insoluble membrane residues were solubilized with sonication in a buffer containing 0.1% SDS (see Materials and Methods). Labeled gp85 molecules were identified by immunoprecipitation with the monoclonal antibody K-3 and SDS PAGE. Triton-soluble (*A*) and insoluble (*B*) fractions; sodium hydroxide-soluble (*C*) and insoluble (*D*) fractions. Only the relevant portion of the acrylamide gel is shown.

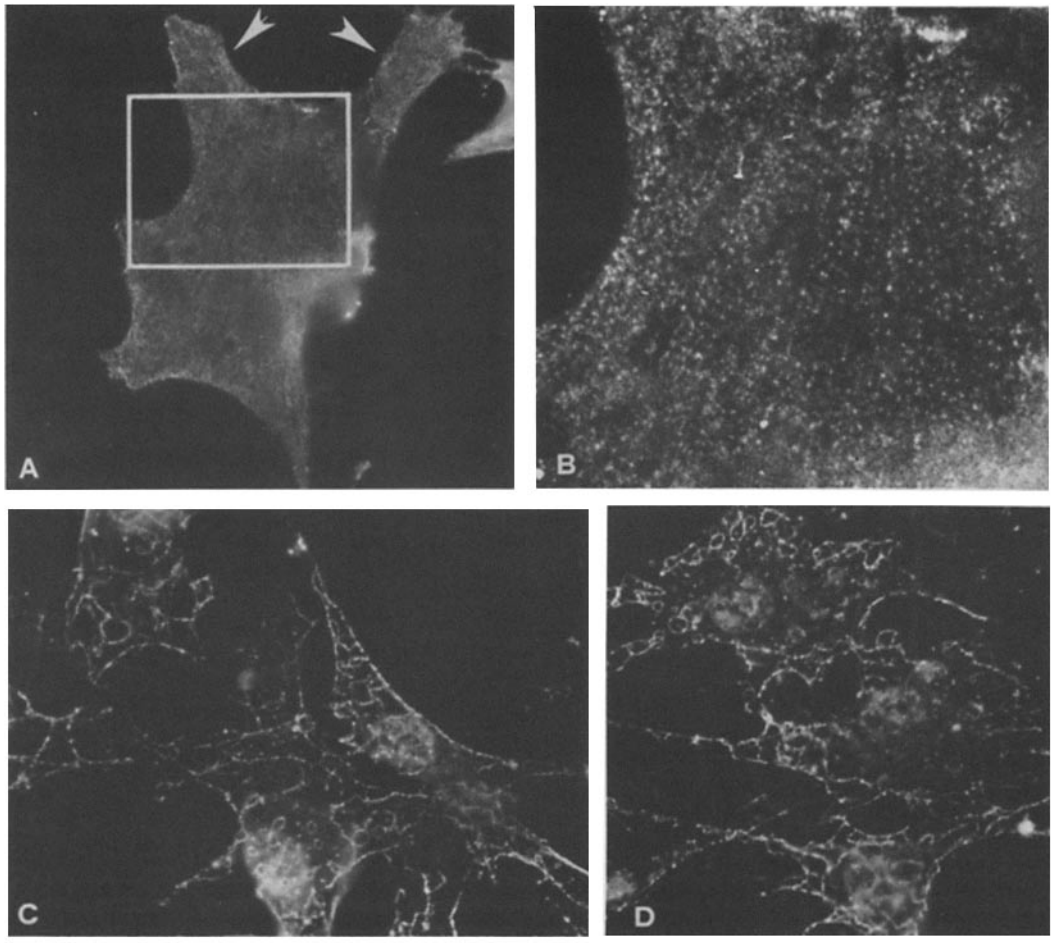

FIGURE 6 Immunofluorescence staining of gp85 with monoclonal antibody K-3 in intact and Triton-extracted BHK cells. BHK cells or detergent-insoluble cell skeletons on glass coverslips were fixed with 3.7% formaldehyde for 20 min at room temperature. Gp85 was localized by incubation with monoclonal antibody K-3 followed by rabbit antibodies to mouse immunoglobulins and FITC-labeled goat anti-rabbit antibodies as described in Materials and Methods. (*A*) intact BHK cell; (*B*) higher magnification of the boxed area in *A*; (*C* and *D*) Triton-insoluble cell skeletons. Arrowheads point to areas of the cell surface where fluorescent dots are randomly distributed.

parallel to the major cell axis. This peculiar pattern was particularly evident on the dorsal surface of the cell over the nucleus (Fig. 6 *B*). However, gp85 was not found to be distributed exclusively in such linear arrays, since in other region of the cell surface the tiny dots appeared to be randomly distributed (Fig. 6 *A*, arrowheads). This pattern was reproducibly observed with fixed or unfixed cells and did not depend on the temperature of incubation (0 or 37°C) with the antibodies, indicating that gp85 is arranged in small clusters at the cell surface.

When Triton-extracted cells were stained with the monoclonal antibody, fluorescence was localized in thin, discontinuous lines at the edges of the cell skeletons and in vesicular structures of heterogeneous size (Fig. 6, *C* and *D*). This pattern did not correspond to that of the detergent-insoluble filaments (Fig. 1, *D* and *E*), indicating that gp85 is not merely adsorbed onto the filamentous cytoskeletal elements but rather is organized in discrete structures.

DISCUSSION

In this paper we describe a membrane glycoprotein, gp85, that is associated with the Triton X-100–insoluble skeleton of BHK cells. Gp85 is not immunologically related to fibronectin, which was also found to be insoluble in detergent. Moreover, in contrast to fibronectin, gp85 is a cell surface molecule, not associated with the extracellular matrix but localized exclusively within the plasma membrane. The association of gp85 with the insoluble skeletons does not seem to be an artifact of detergent treatment. In fact (*a*) radioactive gp85 added during extraction did not become bound to insoluble structures; and (*b*) immunofluorescence on cell skeletons indicated that this molecule is not unspecifically adsorbed onto filamentous material.

It has been shown that actin, spectrin, ankyrin, and band 4.1 of the erythrocyte cytoskeleton are insoluble in detergents (4–7). All these proteins are exposed at the cytoplasmic face of the plasma membrane and behave as peripheral membrane molecules readily extractable with protein-perturbing agents such as 0.1 N sodium hydroxide. This is not the case with gp85 which (*a*) is exposed at the outer cell surface, as shown by lactoperoxidase-catalyzed radioiodination of intact cells and immunofluorescence experiments, and (*b*) behaves as an integral component of the plasma membrane, in that it cannot be extracted by 0.1 N sodium hydroxide.

Using a specific monoclonal antibody, we showed that part of gp85 is solubilized by Triton—consistent with its integral nature—but ~50% of the cell surface gp85 is associated with the skeletal structures. This fraction is not solubilized by increasing the Triton concentration to 5% but is effectively released by protein-denaturing agents (6 M urea) in association with Triton or by selective dissociation of actin filaments. We conclude that this fraction of cell surface gp85 is associated with the skeletal structures via protein–protein interactions.

The distribution of gp85 at the cell surface indicates that this glycoprotein may be organized in some sort of supramolecular complex. Gp85 is not uniformly distributed but rather is clustered in dots that in some regions of the cell surface are disposed in linear arrays. Whether this arrangement is due to interaction with cytoskeletal elements underlying the cell membrane, or with other molecules in the plane of the membrane, remains to be investigated.

The localization of gp85 in the Triton-insoluble skeletons is peculiar and does not correspond to the localization of other cytoskeleton-associated molecules previously described (8, 10, 13, 28). Immunofluorescence experiments showed that gp85 is localized in discontinuous lines at the edges of the cell skeletons and in vesicle-like structures of heterogeneous size. The simplest explanation of this pattern comes from morphological analysis. Electron micrographs of the Triton-insoluble skeletons revealed the presence of membrane fragments and vesicles that are similar in shape and distribution to the structures observed by immunofluorescence. "Detergent-resistant" plasma membrane fragments, anchored to residual cytoskeletal material, have been described in a number of different systems (4, 29–32). This membranous material probably consists of sphingolipids and glycolipids (4). Considering the integral nature of gp85, we suggest that the detergent-resistant membrane fragments are the most likely localization for this molecule. Perhaps gp85 may interact with specific glycosphingolipids generating a highly organized and rigid membrane structure that might have a role in the interaction with cytoskeletal components.

A cell surface glycoprotein of 140,000 mol wt (gp140) has been reported to remain associated to the detergent-insoluble residues of human fibroblasts (33–36). This molecule seems to be in close association with vimentin and can be released from cytoskeletons by buffers that disrupt intermediate-size filaments (37). This behavior is opposite to that of gp85, which is released only by buffers that depolymerize microfilaments. Analyzing different mammalian fibroblasts in culture, we found that gp85 is present in mouse, rat, hamster, and human cells (results not shown), but we detected a 140,000-mol-wt component only in the skeletons of normal rat kidney and HT-1080 (human) cells. We thus conclude that in BHK cells, gp140 is either not expressed or not incorporated into the detergent-insoluble skeletons.

It has been shown that antibodies and lectins could induce linkage of specific plasma membrane proteins to intracellular actomyosin filaments; these include histocompatibility antigens (38–40), surface immunoglobulins (41), aminopeptidase and Na+,K+-ATPase (39), and platelet (42) and leukocyte (43) surface glycoproteins. The resulting complexes are stable in detergent suggesting that protein–protein interactions exist between cytoskeletal and membrane glycoproteins (40–43). To explain how a large number of different membrane glycoproteins that are normally free in the membrane could be induced to interact with the submembranous cytoskeleton, Bourguignon and Singer (38) postulated the existence of an integral cross-linker "protein X" that is stably associated to actomyosin filaments. When any membrane glycoprotein is clustered by its specific extracellular ligand, it would become bound to the putative X protein and, hence, to the cytoskeleton. Gp85 described in this paper is itself associated to the cell skeleton in the absence of ligand-induced clustering. The possibility that gp85 behaves as an X protein is now under investigation. Membrane glycoproteins, which have characteristics and sizes similar to those of gp85, have also been described in neutrophil leukocytes (43), in isolated microvilli of a mammary adenocarcinoma cells (44, 45), and in chicken sympathetic neurons (46).

In conclusion, gp85 is a cell surface integral glycoprotein that is stably associated with the detergent-insoluble cell skeletons. The precise mechanisms of the interaction of this molecule with the insoluble structures and its possible role in cytoskeleton–plasma membrane interaction remain to be determined.

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