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Isolation and Properties of Gamma Protein, the Major Transmembrane Sialoglycoprotein of the HeLa Cell

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The surface of the HeLa cell is composed of a heterogeneous population of sialoglycoproteins which undergo lectin-mediated endocytosis (Kramer and Canellakis, Biochim Biophys Acta 551:328, 1979). One such sialoglycoprotein, gamma protein, is the major periodate-Schiff-reactive and [^{3}H]-glucosamine-labeled component of the plasma membrane; it has an apparent molecular weight of 165,000. Gamma protein is also the major [^{125}I]-wheat germ agglutinin-binding component in sodium dodecyl sulfate gels. Neuraminidase digestion of HeLa cells abolishes binding of [^{125}I]-wheat germ agglutinin to gamma protein, and pretreatment of cells with wheat germ agglutinin protects gamma protein from desialation by neuraminidase, suggesting that wheat germ agglutinin binds to the sialic acid residues of gamma protein at the cell surface. Gamma protein can be extracted with various detergents but not with high-salt, chelating, or chaotropic agents. Intact inside-out plasma membrane vesicles have been prepared from HeLa cells that had phagocytosed latex particles. Treatment of these isolated vesicles with trypsin reduces the molecular weight of gamma protein. These results suggest that gamma protein is an integral membrane protein that spans the plasma membrane. Gamma protein can be purified to homogeneity by sequential lithium diiodosalicylate-phenol extraction, wheat germ agglutinin-agarose affinity chromatography, and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Key words: **N-acetyl neuraminic acid, neuraminidase (Vibrio cholerae), sialic acid, wheat germ agglutinin receptor, membrane glycoprotein**

Cell surface glycoproteins have been implicated in such cellular functions as adhesion [1, 2], cell-cell recognition [3, 4], and differentiation [5]. Characterizing the composition, orientation, and dynamics of these surface membrane glycoproteins is necessary for understanding their functional properties. Only for a few specific glycoproteins have such studies been completed. The major glycoprotein species of the human erythrocyte plasma membrane, glycophorin, is one such glycoprotein for which the ultrastructural organization and transmembrane disposition, as well as the complete biochemical analyses, have been determined [6].

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The isolation and characterization of surface glycoproteins from nucleated cells have been hampered by the low yields of purified plasma membrane used as the initial source of starting material. Furthermore, the presence of a wide variety of glycoprotein species in the plasma membrane has complicated the task of isolating an individual glycoprotein.

One surface glycoprotein which has been successfully purified to homogeneity from a number of different cell types is a 230,000 dalton component designated large external transformation-sensitive (LETS) glycoprotein, or fibronectin [7, 8]. Fibronectin has been implicated in cellular adhesion [2, 9] and has been found to be depleted or absent in tumor cells [10–12]. This component appears to be a peripheral membrane protein which may bind to cell surface glycosaminoglycans [13].

We have been studying the cell surface glycoproteins of HeLa cells and their interactions with lectins. We reported that wheat germ agglutinin induces a massive internalization of gamma protein, the major HeLa membrane glycoprotein [14] that has been isolated and partially characterized. Gamma protein is the predominant wheat germ agglutinin (WGA) binding component of the plasma membrane. Evidence is presented here which demonstrates that this is an integral membrane protein which spans the plasma membrane.

MATERIALS AND METHODS

The materials were from the following sources: Joklik modified minimum essential medium and Eagle's minimum essential medium (MEM) from Gibco (Grand Island, New York); fetal calf serum (FCS) from Flow Laboratories (Rockville, Maryland); cell culture dishes from Falcon Plastics (Oxnard, California); lactoperoxidase (80 units/mg), glucose-oxidase, type V (200 units/mg), papain (2 × crystallized) from Sigma Chemicals (St. Louis, Missouri); Na[^{125}I] (10–20 Ci/mg) and [^{3}H]-glucosamine (30 Ci/mmo) from New England Nuclear (Boston, Massachussetts); WGA, WGA-agarose, concanavalin A, ricin, and chymotrypsin (3 × crystallized) were from Miles Laboratories (Elkhart, Indiana); Sepharose 4B from Pharmacia (Piccataway, New York); Vibrio cholerae neuraminidase from Calbiochem-Behring (La Jolla, California); trypsin (3 × crystallized) from Worthington Biochemicals (Freehold, New Jersey); latex particles (1.1 μ) from Dow Diagnostics (Indianapolis, Indiana); type RC-5 X-ray film from Eastman Kodak Co. (Rochester, New York).

Cell Culture

HeLa-S_3 cells were grown as suspension cultures in spinner flasks as previously described [14]. The cells were maintained at (2–6) × 10^5/ml in Joklik modified medium supplemented with 10% heat-inactivated FCS (56°C for 30 min).

Cell Labeling

HeLa cells were labeled with [^{125}I] using lactoperoxidase as previously described [14]. Cellular glycoproteins were labeled by culturing cells for 48 h in medium containing 5 μCi/ml of [^{3}H]-glucosamine.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Slab gel electrophoresis was performed as previously described [14]. Separation gels were 1.5 mm thick and ranged from 10 to 18 cm in length. Gels were dried onto filter paper and exposed to X-ray film. The method of Fairbanks et al [15] was used for

staining gels with periodic acid-Schiff's (PAS) reagent. Detection of [^{3}H]-labeled polypeptides in slab gels was achieved by the fluorographic method of Bonner and Laskey [16]. For the detection of [^{125}I]-WGA-reactive glycoproteins in SDS gels, the procedure of Burridge [17] was followed.

Lectin Treatment and Neuraminidase Digestion of HeLa Cells

Cells were [^{125}I]-iodinated with lactoperoxidase and allowed to attach to culture dishes prior to treatment with lectins and subsequent neuraminidase digestion. This was performed by suspending [^{125}I]-iodinated cells at 5×10^5/ml in MEM containing 10% FCS and adding 1 ml of this cell suspension to 35-mm-diameter culture dishes. After incubation at 37°C for 1.5 h, more than 90% of the cells had attached. The cells were treated with the appropriate lectin at 4°C for 30 min, washed three times with cold Dulbecco's phosphate buffered saline (DPBS) containing 0.1% bovine serum albumin (BSA), and then exposed to 10 units/ml of neuraminidase at 4°C for 30 min in DPBS, pH 7.0. The reaction was terminated by rapidly washing the dishes twice with cold DPBS and solubilizing the monolayer with gel sample buffer [14].

The procedures for preparation of [^{125}I]-WGA and binding to HeLa cells have been described [14].

Proteolytic Digestion of [^{125}I]-Labeled Cell Surface Membrane Proteins

Stock solutions of trypsin and chymotrypsin were prepared in Ca^{++}-Mg^{++}-free Dulbecco's phosphate buffered saline immediately before use. Papain, at a concentration of 500 μg/ml, was activated in 5 mM dithiothreitol/1 mM EDTA in PBS prior to use. HeLa cells were [^{125}I]-iodinated, suspended in PBS at a concentration of 1×10^6 cells per milliliter, and treated with different concentrations of each protease for 10 min at 37°C. The reactions were terminated with five volumes of cold 10% FCS-MEM, and the cells were washed twice with the same medium at 4°C. The cells were then suspended in 0.2 ml of PBS at 4°C and 0.5 ml of cold 10% TCA was added. After centrifugation, the pellets were extracted twice with cold absolute ethanol and dried under a stream of N_2. The residue was then solubilized for SDS-PAGE.

Extraction of Purified HeLa Cell Membranes

Purified plasma membranes were prepared from [^{125}I]-labeled HeLa cells using our established procedure [14]. Approximately 100 μg of membrane protein were mixed with 2.5 ml of extraction medium at 4°C with stirring. After 40 min the samples were centrifuged at 4°C at 105,000 g for 1 h. The supernatant fractions were carefully removed, a small sample taken for analysis of radioactivity, and the remaining material was dialyzed overnight and freeze-dried. This fraction as well as the membrane pellet were solubilized in SDS and electrophoresed in SDS gels.

Preparation of "Inside-Out" Plasma Membrane Vesicles

Phagocytic membrane vesicles were prepared from HeLa cells according to the procedure of Heine and Schnaitman [18] with modifications. HeLa cells were found to slowly endocytose latex particles in the presence of serum. Therefore, to increase the uptake of particles, the following procedure was devised. HeLa cells, either labeled with [^{3}H]-glucosamine or [^{125}I], were suspended in 0.25 M sucrose, 10 mM sodium phosphate (pH 7.4, 4°C) at a density of $(2–4) \times 10^5$ cells per milliliter. Latex particles (1.1 μ diameter) were washed twice with distilled water and dispersed by sonication. The

particles were then added to the cell suspension in a spinner flask at a concentration of approximately 1–2 mg/ml. After 5–10 min the cells were found to be covered with the latex particles and were collected by centrifugation. The cells were then suspended in 10% FCS in MEM at a concentration of (2–4) $\times 10^5$ cells per milliliter and were incubated for 30–40 min in a spinner flask until the majority of cells had endocytosed the particles, as judged by phase-contrast microscopy. The cells were then washed three times with PBS. To remove the residual, nonendocytosed particles, the cells were suspended in 5 ml of PBS and overlaid on 40 ml of 50% FCS in PBS and centrifuged at 1,200 rpm for 15 min. After two more washings in PBS, the cells were suspended in 5 ml of PBS and disrupted by 5–10 strokes in a tight Dounce homogenizer. The homogenate was adjusted to 2 mM EDTA and an equal volume of 60% sucrose, 10 mM sodium phosphate, pH 7.2, was added. The homogenate was then divided among three cellulose nitrate tubes and overlaid with 7.5 ml of 25% sucrose, 10 ml of 20% sucrose, and 8 ml of 10% sucrose, all in 10 mM sodium phosphate (pH 7.2) and 1 mM EDTA. The gradients were then centrifuged at 25,000 rpm in a Beckman SW 25.1 rotor at 4°C for 90 min. The latex particles banded at the 10%:25% interface and were collected and washed twice in PBS.

Isolation of Gamma Protein

A total of 1×10^9 cells were washed twice with PBS and suspended in 300 ml of 0.25 M sucrose, 10 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 7.5) at 4°C. The suspension was placed in a cavitation bomb (Parr Instruments) and equilibrated with N_2 for 20 min at 800 psi at 4°C. One hundred percent disruption of the cells was achieved by the drop-by-drop release of the suspension from the bomb. Nuclei were removed from the homogenate by centrifugation at 1,400 rpm/5 min at 4°C. All other operations were performed at 2–4°C. The supernatant was adjusted to 5 mM EDTA, and the cellular membranes were collected at 80,000 g for 1.5 h. The membranes were suspended in 200 ml of 1 mM Tris-HCl (pH 8) for 20 min, at which time the suspension was adjusted to 30 mM lithium diiodosalicylate (LIS) and 50 mM Tris-HCl (pH 7.5) and stirred for 30 min. The "LIS-treated membranes" were centrifuged at 80,000 g for 1.5 h, and the supernatant was discarded. The LIS-treated membranes were dispersed in 300 mM LIS, 50 mM Tris-HCl (pH 7.5) and stirred at room temperature for 30 min. After the addition of two volumes of cold distilled water and centrifugation at 100,000 g for 1 h, the LIS extract was then processed as previously described [19].

The LIS-phenol extract was suspended in 5 ml of 1% SDS, 0.25 M NaCl, 10 mM sodium phosphate (pH 7.2) and incubated at 37°C for 30 min. After centrifugation at 48,000 g, the supernatant was diluted with buffer SP (10 mM sodium phosphate, 0.25 M NaCl, pH 7.2) in order to reduce the concentration of SDS to 0.05%. This solution was slowly applied to a 5-ml column of WGA-agarose as previously described [14] and washed with approximately 20 ml of buffer SP containing 0.05% SDS. Elution of the bound material was achieved with the addition of 100 mM N-acetyl glucosamine (GlcNAc) in the same buffer solution. The GlcNAc-released fraction was exhaustively dialyzed against distilled water and freeze-dried.

The GlcNAc-eluted glycoproteins were solubilized in SDS-PAGE buffer at 37°C for 30 min and applied to a 3 mm thick 6% polyacrylamide-SDS-slab gel. Electrophoresis was performed at 50 mA for 5 h until the tracking dye had migrated 10 cm. Reference strips were cut from both sides as well as from the middle of the gel (1 cm wide) and were stained for protein with Coomassie blue. Gamma protein, which appeared as a weak and diffusely stained band with an Rf of about 0.31, was cut from that region of the gel

corresponding to its location in the reference strips. The gel strips (excluding the reference strips) were minced by forcing the gel twice through an 18-gauge needle. The fine suspension of gel particles was then extracted with 25 ml of 0.1% SDS, 5 mM sodium phosphate (pH 7.2) with shaking at room temperature for 12 h. The gel particles were washed once with the same buffer after centrifugation at 4,000 g. The pooled extract was centrifuged at 10,000 rpm for 20 min and concentrated to 1 ml in an Amicon concentrator using a PM 10 membrane.

RESULTS

Gamma Protein is a Cell Surface Sialoglycoprotein

Lactoperoxidase-mediated iodination labels at least 14 major proteins when analyzed by SDS-PAGE gel electrophoresis (Fig. 1). One heavily iodinated glycoprotein, band 6 or gamma protein, with an apparent molecular weight of 165,000 is characterized by a rather diffuse and broad electrophoretic pattern. [^{125}I]-iodinated FCS proteins were electrophoresed in parallel with the labeled plasma membranes, and there is no major labeled component in the region of the gel which corresponds to gamma protein; gamma protein therefore does no appear to be an adsorbed serum protein. Gamma protein displays anomalous migration in SDS gels which is dependent on the gel polyacrylamide concentration. As the polyacrylamide concentration is decreased, the apparent molecular weight increases. At 10% polyacrylamide the molecular weight of gamma protein is 140,000, and at 5% polyacrylamide the apparent molecular weight is 190,000

The cell surface disposition and protein nature of gamma protein was confirmed by the sensitivity of gamma protein to protease digestion. [^{125}I]-iodinated HeLa cells were subjected to a battery of proteases that included trypsin and papain (Fig. 2). Gamma protein was moderately sensitive to digestion with trypsin and completely digested with papain, as indicated by the loss of [^{125}I] label at the 165,000 dalton region. However, this protein appeared to be resistant to chymotrypsin digestion, and even at 50 μg/ml was not appreciably degraded (unpublished observations).

Gamma protein is the major PAS-stained and [^{3}H]-glucosamine-labeled component of the plasma membrane (Fig. 3). In addition, gamma protein is shown to be the predominant [^{125}I]-WGA-binding glycoprotein of the HeLa cell when [^{125}I]-WGA is used to label SDS gels of solubilized HeLa cells (Fig. 4). This indicates that gamma protein carries GlcNAc-like and/or sialic acid-like determinants [21]. There appears to be a correlation between the extent of PAS staining, [^{3}H]-glucosamine labeling, and [^{125}I]-WGA binding of gamma protein (compare Figs. 3 and 4). This seems also to be true of α_1 and α_2 glycoproteins. The binding of [^{125}I]-labeled WGA to gamma protein and to a majority of the other WGA-binding glycoproteins in SDS gels is almost completely lost after neuraminidase digestion of the HeLa cells (Fig. 4).

The presence of sialic acid residues in gamma protein is further shown by the effect of neuraminidase digestion on the electrophoretic migration of gamma protein in SDS gels. When intact HeLa cells are digested with increasing amounts of Vibrio cholerae neuraminidase, and electrophoresed in SDS gels, gamma protein undergoes a decrease in electrophoretic mobility that correlates with the amount of neuraminidase present in the incubation medium (Fig. 5). Digestion of HeLa cells with high concentrations of neuraminidase (10 units/ml) shifts the apparent molecular weight of gamma protein from 165,000 to more than 200,000 (Fig. 6). Gamma protein is unique in this behavior, since no other surface membrane glycoprotein of the HeLa cell demonstrates a decrease in

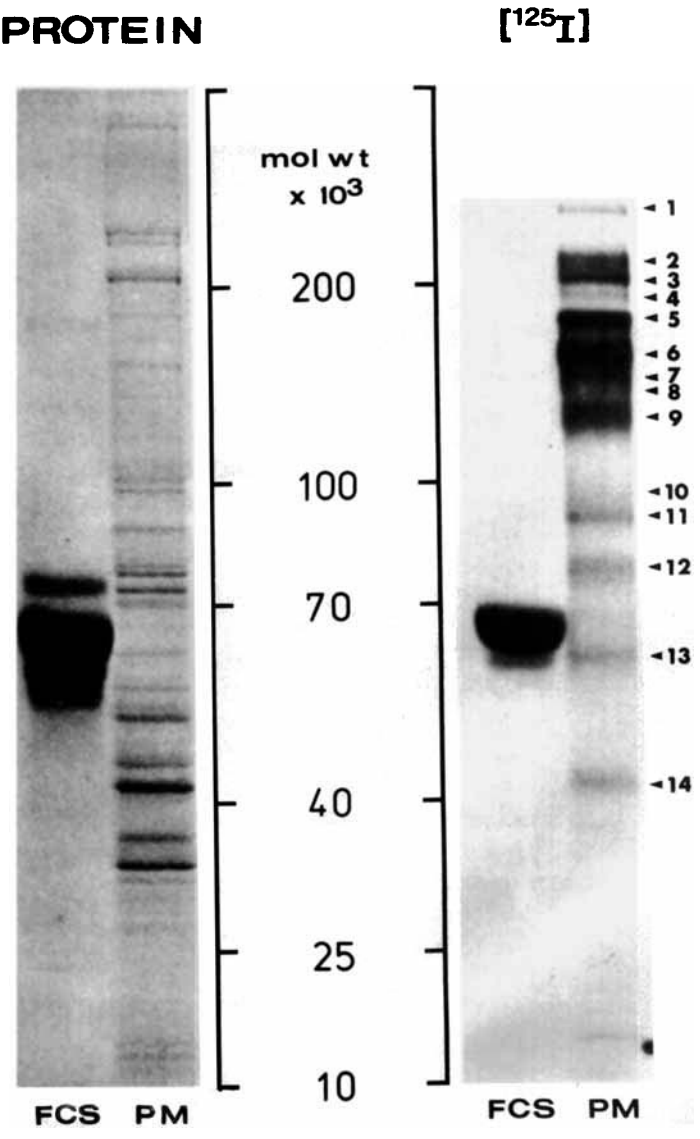


Fig. 1. Analysis of plasma membranes isolated from [^{125}I]-iodinated HeLa cells and [^{125}I]-iodinated fetal calf serum. FCS, fetal calf serum; PM, plasma membrane. Gel system: 5–12% polyacrylamide linear gradient SDS-slab gel. Protein patterns were visualized by staining with Coomassie blue while [^{125}I]-labeled bands were detected by autoradiography. Gamma protein corresponds to [^{125}I]-labeled band 6.

Fig. 3. Comparison of periodate-Schiff-positve and [^{3}H]-glucosamine-labeled components of HeLa cell plasma membranes. HeLa cells were labeled with 5 μCi/ml of [^{3}H]-glucosamine for 48 h, and the plasma membranes were isolated. Approximately 100 μg protein of purified plasma membranes were electrophoresed in a 6% polyacrylamide SDS-slab gel and either scanned at 560 nm after the periodate-Schiff reaction (top) or processed for fluorography (bottom) for [^{3}H]-glucosamine-labeled components.

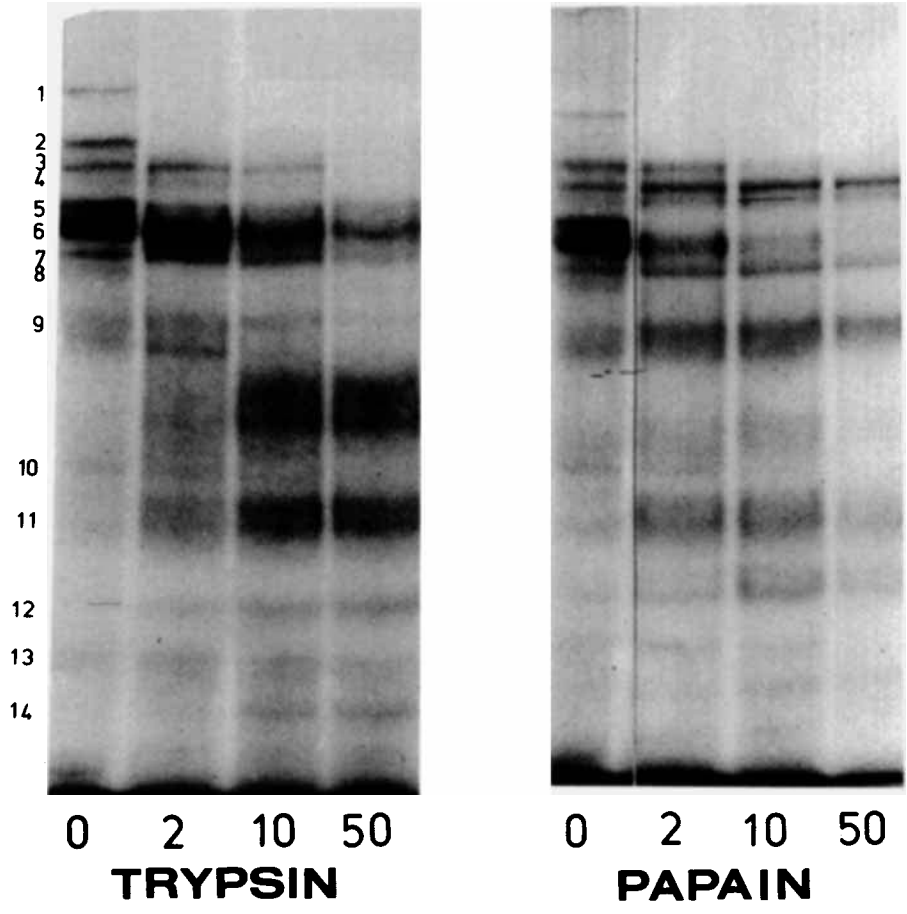
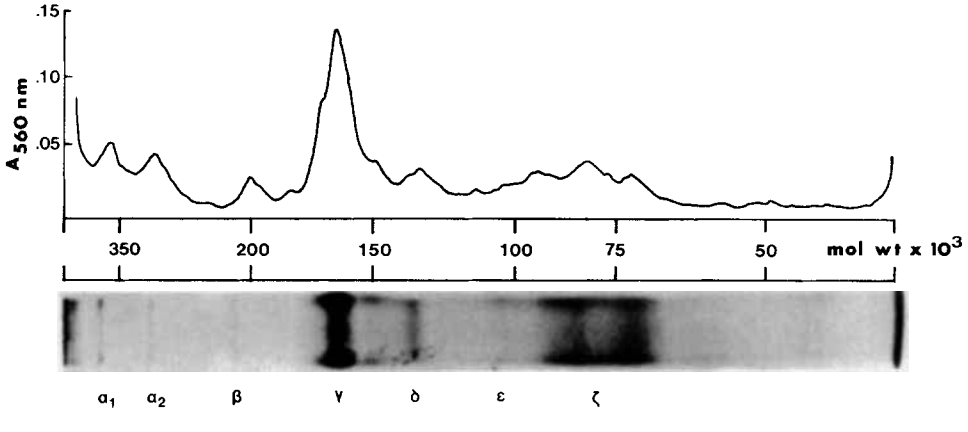


Fig. 2. Proteolytic digestion of [^{125}I]-labeled cell surface membrane proteins. After iodination, HeLa cells were treated with trypsin or papain as described in Methods. The concentration (μg/ml) of enzyme is given below each gel lane; 6% polyacrylamide-SDS slab gels. Gamma protein corresponds to [^{125}I]-labeled band 6.



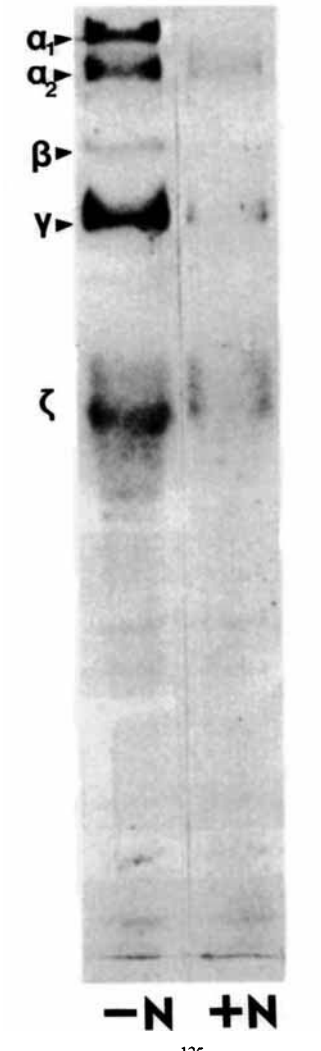


Fig. 4. Effect of neuraminidase treatment on the binding of [^{125}I]-WGA to gamma protein in SDS-polyacrylamide gels. Control HeLa cells or HeLa cells pretreated with neuraminidase (10 units/ml/5 × 10^5 cells) for 10 min at 37°C were solubilized in SDS and electrophoresed in a 6% polyacrylamide SDS gel which was subsequently reacted with [^{125}I]-WGA as described in Methods. The gel was dehydrated and autoradiographed. (−N), no treatment; (+N), neuraminidase treated.

electrophoretic mobility. However, [^{125}I]-iodinated bands 1, 2, 4, 5, 8, and 9 demonstrate a slight increase in their electrophoretic mobilities subsequent to extensive treatment with neuraminidase (Fig. 6).

WGA interacts directly with gamma protein at the surface of intact HeLa cells. Pretreatment of [^{125}I]-labeled HeLa cells with as little as 25 μg WGA/5 × 10^5 cells prevents the neuraminidase-induced shift in the electrophoretic mobility of gamma protein (Fig. 6). From the [^{125}I]-WGA binding curve presented in Figure 7, this amount of WGA is calculated to bind to approximately 20–30% of the total cell surface WGA-binding sites. The

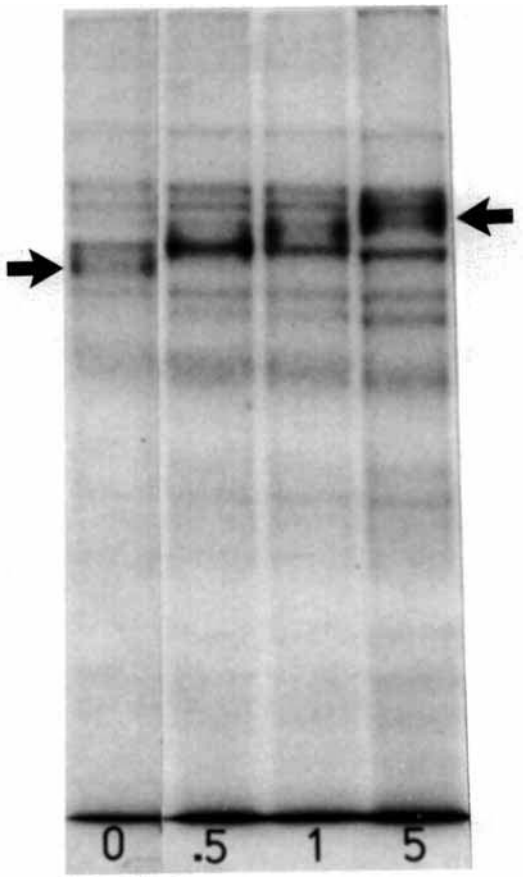


Fig. 5. Effect of neuraminidase treatment on the electrophoretic mobility of gamma protein. HeLa cells were iodinated and treated with a range of neuraminidase concentrations for 10 min at 37°C (cell concentration: 1×10^6/ml). The cells were solubilized in SDS and analyzed on a 6% polyacrylamide SDS slab gel. The concentration of neuraminidase (units/ml) is indicated below each gel lane. Gamma protein corresponds to [^{125}I]-labeled band 6 (Fig. 2) and is indicated here by arrows.

WGA-mediated protection is completely prevented if cells are incubated with WGA in the presence of 0.1 M GlcNAc, and in addition, it can be entirely reversed by extensively washing the WGA-treated cells with 0.1 M GlcNAc at 4°C prior to neuraminidase exposure.

Gamma Protein is an Integral Membrane Protein

The nature of the association of gamma protein with the plasma membrane was investigated by extracting purified plasma membrane isolated from [^{125}I]-labeled HeLa cells with a variety of ionic and detergent solutions. Incubation of the isolated plasma membrane with low-ionic-strength buffer (10 mM Tris-HCl), high-ionic-strength buffer (0.5 M NaCl), or divalent cation chelating agents (10 mM EDTA) elutes less than 6% of the incorporated [^{125}I]-label (Table I). Extraction with solutions containing the chaotropic reagent LIS solubilizes appreciable amounts of [^{125}I]-radioactivity which are related to the

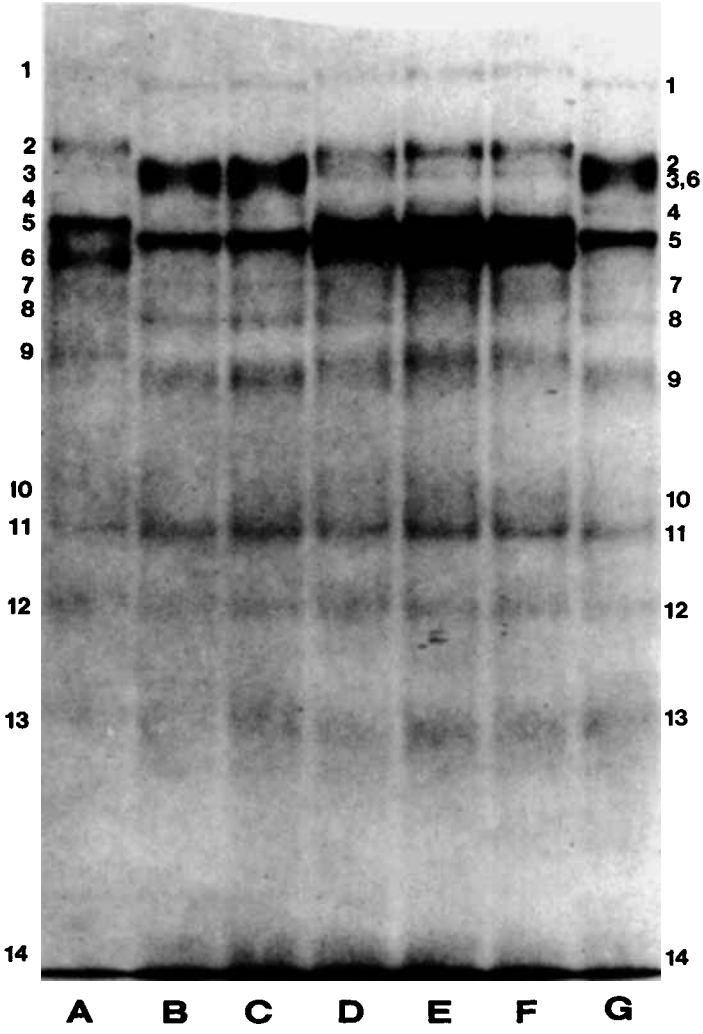


Fig. 6. Interaction of WGA with the surface glycoproteins of HeLa cells as revealed by the inhibition of the neuraminidase reaction. [^{125}I]-iodinated HeLa cells were allowed to attach to tissue culture dishes, pretreated with WGA at 4°C for 30 min, washed, and then reacted with neuraminidase (10 units/ml) for 30 min at 4°C (see Methods). The cells were then solubilized in SDS and approximately equivalent amounts of [^{125}I]-label were loaded onto each gel lane and electrophoresed. The 6% polyacrylamide slab gel was subsequently dehydrated and exposed to X-ray film. Gel lane A, control cells without WGA or neuraminidase treatment; gel lanes B–F, cells were pretreated with 1, 5, 25, 50, and 100 μg of WGA and then digested with neuraminidase; gel lane G, control cells without WGA treatment but digested with neuraminidase. Gamma protein corresponds to [^{125}I]-labeled band 6.

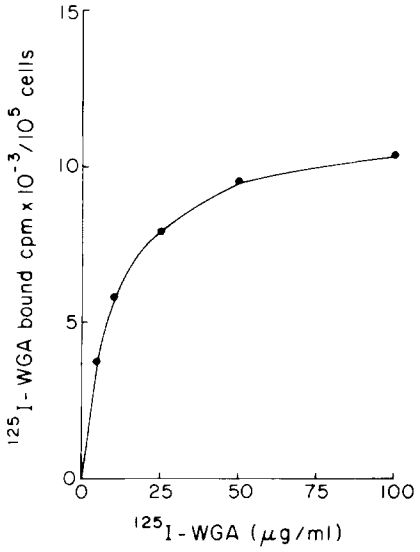


Fig. 7. Binding of [^{125}I]-WGA to HeLa cells. The indicated concentrations of [^{125}I]-WGA (specific activity: 2.3×10^4 cpm/μg) were incubated with 1×10^5 cells at 4°C for 30 min, and the specific binding was determined.

TABLE I. Solubilization of Iodinated Membrane Proteins

Extraction medium	[^{125}I]-soluble cpm ($\times 10^4$)	% Soluble
10 mM Tris-HCl, pH 8.0	1.35	3.0
0.5 M NaCl	1.31	2.9
10 mM EDTA	1.05	2.3
0.5 M NaCl, 10 mM EDTA	2.84	6.3
10 mM LIS	3.15	6.9
30 mM LIS	6.89	15.3
100 mM LIS	15.8	35.0
300 mM LIS	29.8	66.2
0.5% Triton X-100	31.9	70.8

HeLa cells were [^{125}I]-iodinated and the plasma membranes were isolated. Then 130 μg of the labeled plasma membranes, containing 4.5×10^5 TCA-insoluble cpm, were extracted with 2.5 ml of the indicated extraction medium (buffered with 10 mM Tris-HCl, pH 8) at 4°C for 40 min. After centrifugation (6×10^6 g-min), each supernatant solution and pellet was assayed for TCA-insoluble counts.

concentration of LIS present in the extraction medium: 30 mM LIS releases 15.3% while 300 mM LIS elutes nearly 70% of the [^{125}I]-label. The non-ionic detergent Triton X-100 at a concentration of 0.5% solubilizes 70% of the label.

The supernatant and corresponding pellet fraction, after treatment with various extraction media, were analyzed by electrophoresis in SDS gels (Fig. 8). Low or high salt, with or without EDTA, produces essentially the same radiolabeled profiles; small amounts

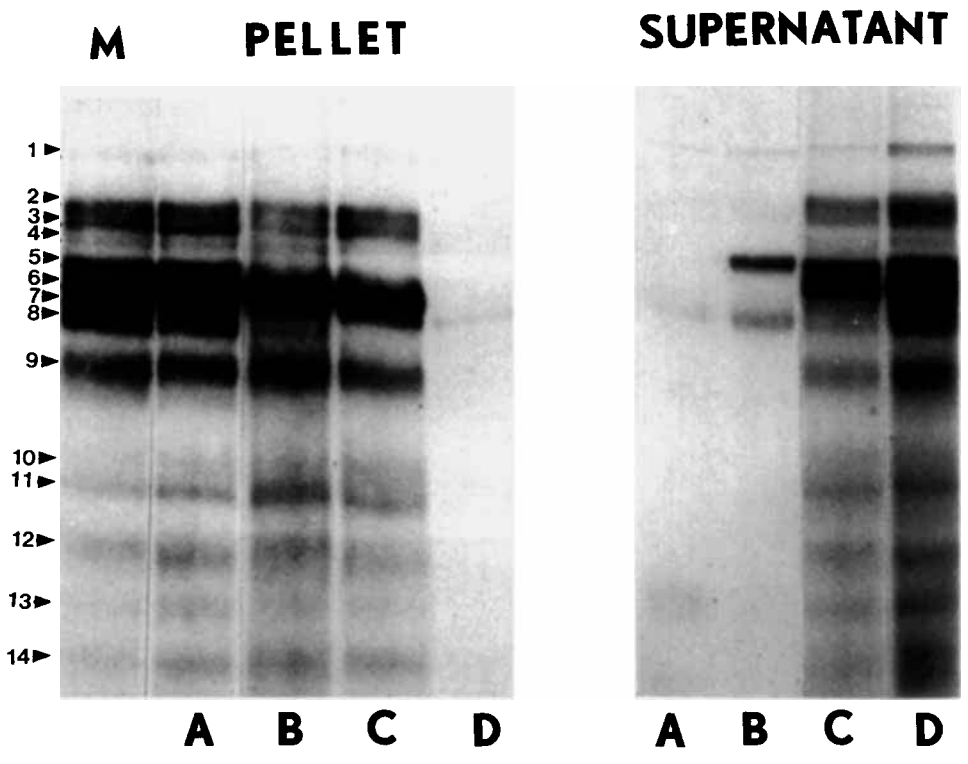


Fig. 8. Analysis of [^{125}I]-iodinated surface polypeptides released after treatment with various extraction media. Purified plasma membranes were prepared from [^{125}I]-iodinated HeLa cells and extracted as described in Table I. Each pellet and supernatant fraction was electrophoresed in a 6% polyacrylamide SDS slab gel and autoradiographed. Untreated plasma membranes are presented in lane M. The pellet fractions (left panel) and supernatant fractions (right panel) were generated by the following extraction media, which in addition contained 10 mM Tris-HCl, pH 8. A, 0.5 M NaCl/10 mM EDTA; B, 10 mM LIS; C, 100 mM LIS; D, 0.5% Triton X-100. Gamma protein corresponds to [^{125}I]-iodinated band 6.

of bands 1, 2, 8, and 12 are present in the supernatant fraction. Gamma protein is not detectable in the supernatant fractions after such treatments. Low concentrations of LIS (5–30 mM) result in the selective elution of a fraction of band 5 along with small quantities of bands 1, 2, 8, and 12; 100 mM LIS produces an extensive solubilization of all [^{125}I]-labeled bands. However, much of gamma protein is still membrane-associated, while essentially all of band 5 is found in the supernatant. Triton X-100 effectively elutes all [^{125}I]-labeled bands including gamma protein.

To examine the possibility that gamma protein is a transmembrane protein, the procedure devised by Hunt and Brown [22] was applied to HeLa cells. Inside-out plasma membrane vesicles are isolated after HeLa cells have phagocytosed latex particles (Fig. 9). Isolated phagosome membrane vesicles prepared from [^{125}I]-iodinated or [^{3}H]-glucosamine-labeled HeLa cells are briefly treated with trypsin. The effect of such proteolysis on the cell surface membrane proteins is assessed by SDS-PAGE. Figure 10

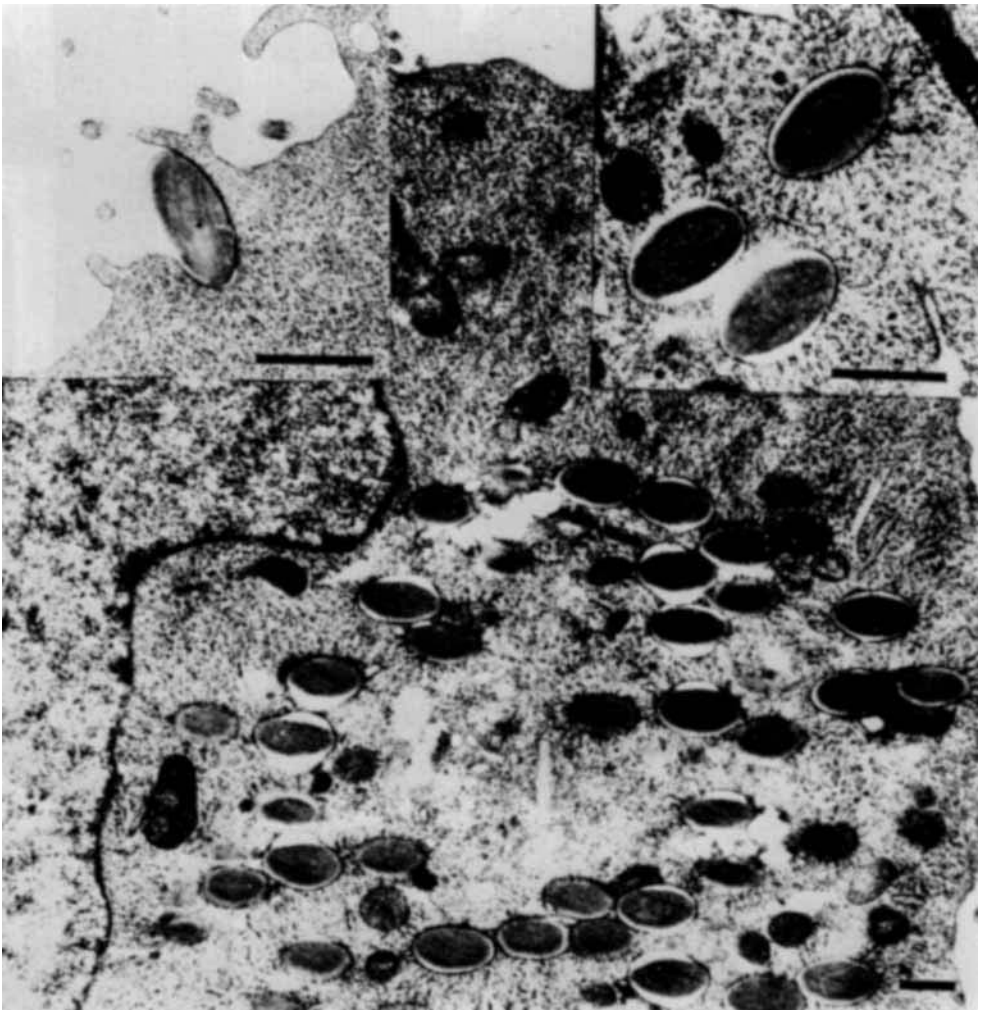


Fig. 9. Electron micrograph of HeLa cell containing internalized latex particles. Bar equals 1 μ.

represents an experiment in which inside-out membrane vesicles prepared from [^{125}I]-labeled cells were treated with either 5 or 50 μg trypsin per milliliter. After such treatment gamma protein is found to undergo a shift in apparent molecular weight from 165,000 to 150,000. When inside-out vesicles prepared from [^{3}H]-glucosamine-labeled cells are similarly treated with 50 μg/ml of trypsin (Fig. 11), an identical decrease in the molecular weight of gamma protein is obtained. Of five such experiments, using either [^{3}H]-glucosamine or [^{125}I]-labeled cells, the result is the same: Gamma protein is degraded to the 150,000 fragment after treatment with trypsin. On the other hand, when the vesicles are disrupted by sonication and then treated with trypsin, gamma protein and most other iodinated surface proteins are extensively degraded. In particular,

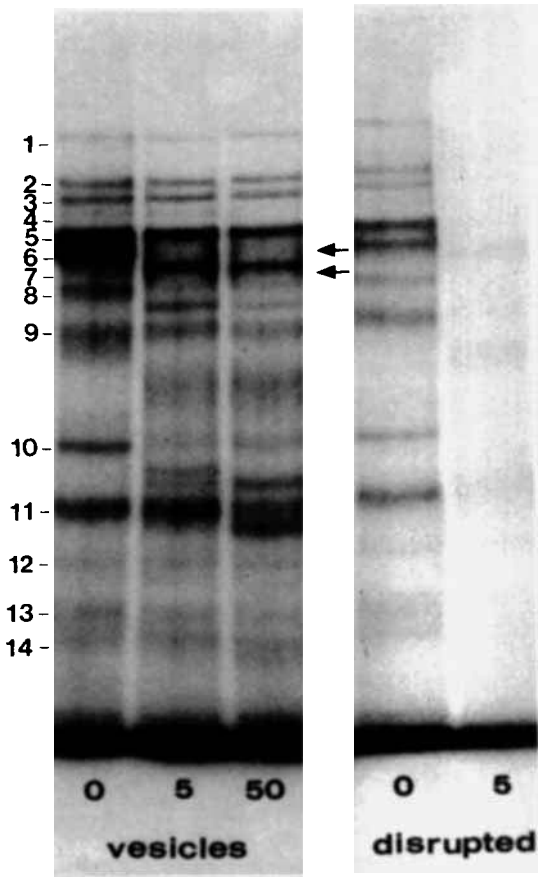


Fig. 10. Analysis of trypsinized inside-out membrane vesicles prepared from [^{125}I]-labeled HeLa cells. Cells were iodinated and allowed to endocytose latex particles. The phagocytic vesicles were isolated and were treated with 5 or 50 μg trypsin per milliliter or were sonicated and then treated with 5 μg trypsin per milliliter. Gel system: 6% polyacrylamide SDS slab gel. Arrows indicate position of gamma protein (band 6) before and after trypsin treatment.

iodinated bands 1, 2, 3, and 5, which are not detectably degraded with trypsin in the intact inside-out vesicles, are completely lost when disrupted vesicles or intact iodinated cells (Fig. 2) are exposed to trypsin.

Isolation of Gamma Protein

Our previous studies demonstrate that the LIS-phenol partitioning procedure is an effective intial step to use for the purification of surface glycoproteins. We have used this method to isolate gamma protein in a partially purified form from either isolated plasma membranes or from the postnuclear fraction of the cell homogenate [19]. The purification of gamma protein from the homogenate of [^{3}H]-glucosamine-labeled HeLa cells using the LIS-phenol procedure is monitored by electrophoresis of the fractions in SDS gels (Fig. 12). Gamma protein is visible in the radioactive profiles of the cell homogenate, postnuclear supernatant fraction, and purified plasma membrane fraction. Upon phenol partition of the 0.3 M LIS

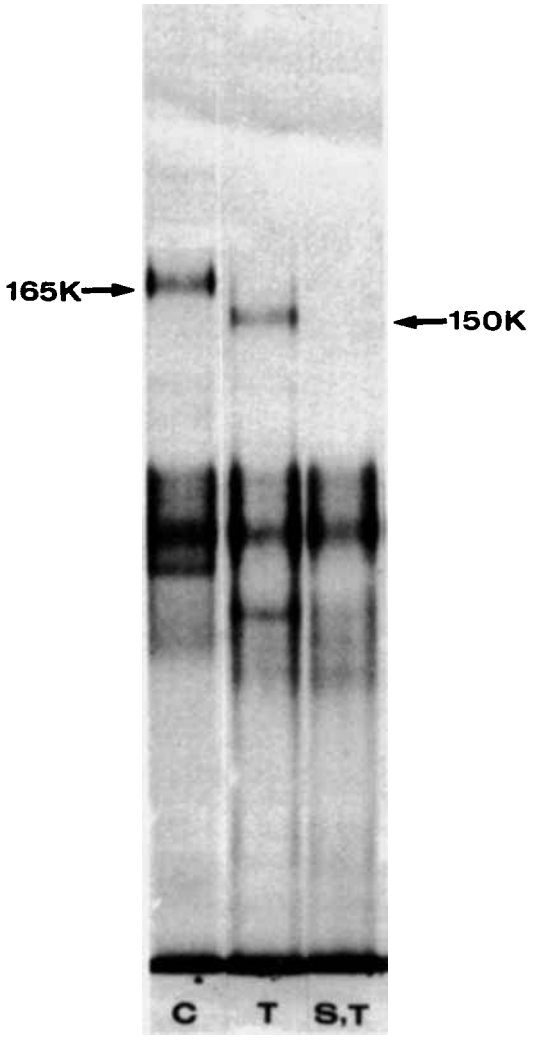


Fig. 11. Analysis of trypsinized inside-out membrane vesicles prepared from [^{3}H]-glucosamine-labeled HeLa cells. Cells were cultured in 5 μCi/ml of [^{3}H]-glucosamine for 48 h and were allowed to endocytose latex particles. The phagocytic vesicles were isolated and were either treated with 50 μg/ml of trypsin (T) or were first sonicated and then exposed to 50 μg/ml of trypsin (S, T). (C) Untreated vesicles. The position of gamma protein in the 6% polyacrylamide gel is indicated by arrows.

extract of the plasma membrane fraction, gamma protein is isolated in the aqueous phase along with several other [^{3}H]-glucosamine labeled components. Nearly all of the major Coomassie blue-positive components partition into the phenol phase [19]. LIS-phenol extraction of the crude cellular membrane fraction yields nearly an identical pattern [19].

Based on the reported ability of low concentrations of LIS to selectively solubilize peripheral plasma membrane-associated proteins and on the resistance of gamma protein to extraction with this concentration of LIS, the initial purification step of gamma protein

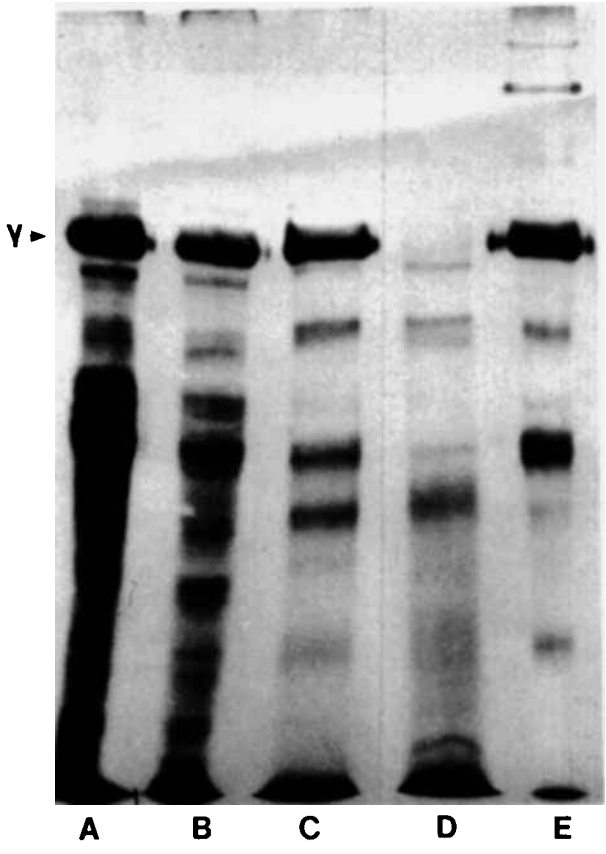


Fig. 12. Purification of gamma protein by the LIS-phenol procedure. HeLa cells were labeled with [^{3}H]-glucosamine (5 μCi/ml) for 48 h. The cells were then homogenized and subcellular fractions were prepared. The purified plasma membranes were extracted by the LIS-phenol procedure. Samples were solubilized in SDS and electrophoresed in a 6% polyacrylamide SDS slab gel. A, Whole cell homogenate; B, postnuclear supernatant from the homogenate; C, purified plasma membranes; D, phenol phase of the LIS-phenol extraction of plasma membranes; E, aqueous phase of the LIS-phenol extraction. Gamma protein is indicated by γ.

from crude cellular membrane fraction involves extraction with 30 mM LIS (Fig. 13). Approximately 10^9 HeLa cells are disrupted by N_2 cavitation and the crude cellular membranes are isolated and incubated with 30 mM LIS at 4°C for 20 min. The LIS-treated-membranes are then recovered by centrifugation. Comparison of the crude membrane fraction with that of the 30 mM LIS membrane pellet demonstrates that the majority of the Coomassie blue-positive bands are solubilized. The LIS-treated membranes are then extracted with 300 mM LIS to solubilize the integral membrane proteins including gamma protein. Subsequent phenol partitioning of the 300 mM LIS supernatant resolves the mixture of weak and diffusely Coomassie blue-stained bands to yield gamma protein in the

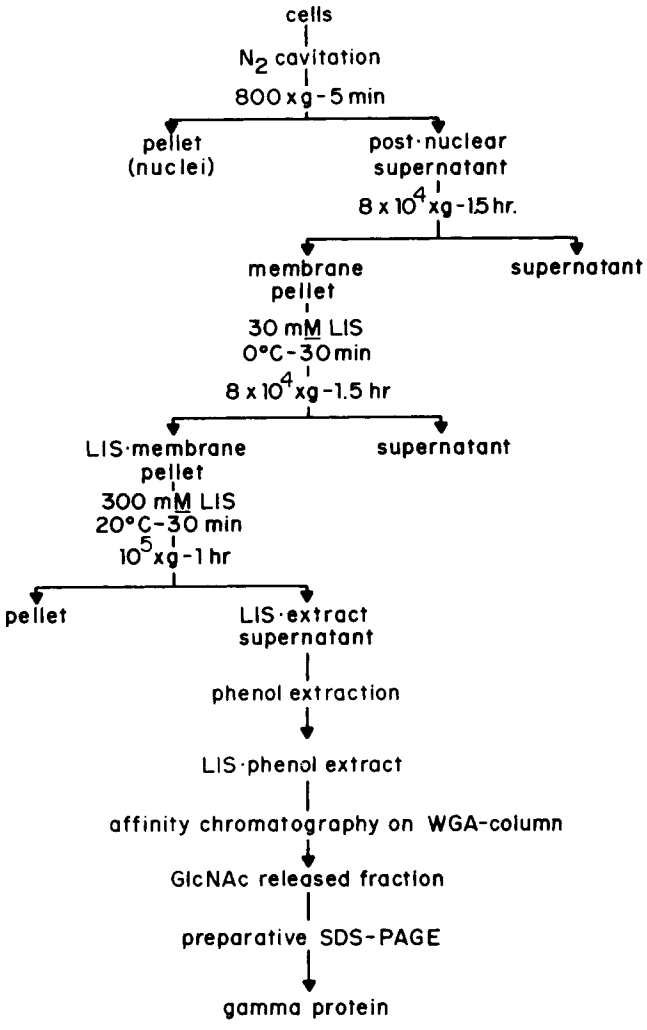


Fig. 13. Gamma protein purification scheme.

aqueous phase. Gamma protein is a WGA-binding glycoprotein and subsequently it is further purified from the LIS-phenol extract by chromatography on a WGA-agarose column. Gamma protein is eluted in the 0.1 M GlcNAc fraction. SDS gels of the GlcNAc-released fraction revealed that gamma protein is well separated from the few contaminating components, which incude bands α_1, α_2, β, and ϵ. Subsequently, the LIS-phenol extract is concentrated and applied to a preparative 6% polyacrylamide SDS gel. After electrophoresis the region of the gel corresponding to gamma protein is removed and extracted to yield the purified gamma protein. Upon re-electrophoresis of gamma protein, the purity of gamma protein is confirmed (Fig. 14). Approximately 50–100 μg of purified gamma protein is obtained from 10^9 cells.

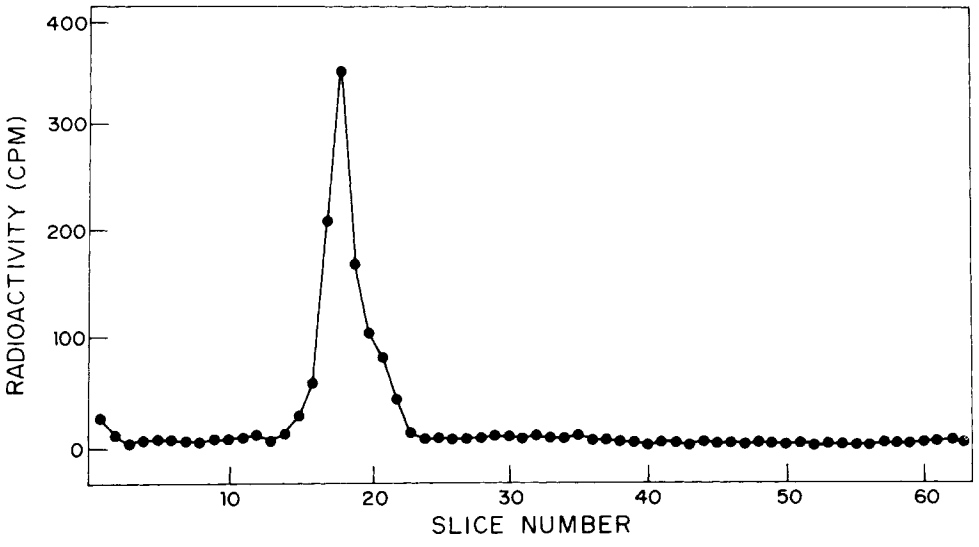


Fig. 14. Pattern of [^{3}H]-glucosamine label in the electrophoretic profile of purified gamma protein. Gamma protein was isolated from HeLa cells cultured in the presence of [^{3}H]-glucosamine according to the purification scheme presented in Figure 13. Purified gamma protein was subjected to SDS-PAGE. The 6% polyacrylamide SDS gel was sectioned into 2-mm slices which were analyzed by liquid scintillation counting.

DISCUSSION

The cell surface disposition of gamma protein has been established by the use of a variety of surface-specific probes. Gamma protein is labeled by lactoperoxidase-mediated [^{125}I]-iodination procedure as well as by sequential galactose oxidase-NaB [^{3}H]$_4$ reduction [23]. Gamma protein is degraded when intact [^{125}I]-labeled cells are treated with proteases. Furthermore, gamma protein is sensitive to neuraminidase treatment, and this sensitivity is lost when cells are pretreated with WGA.

Gamma protein is clearly the major cell surface glycoprotein of the HeLa cell plasma membrane. It is the predominant [^{3}H]-glucosamine-labeled and PAS-stained component of the plasma membrane. The intense PAS reaction generated by gamma protein suggests a high degree of sialation [24]. The effect of neuraminidase upon the electrophoretic mobility of gamma protein in SDS gels further implicates the presence of sialic acid residues. Gamma protein demonstrates anomalous migration in SDS gels, which is a characteristic shared by glycophorin [25]. Like glycophorin [20, 25], gamma protein may bind low amounts of SDS, and its migration in SDS gels may depend to a large degree upon the native molecular charge. Removal of sialic acid would result in the loss of net negative charge, thereby reducing the electrophoretic mobility of the molecule toward the cathode.

Gamma protein is the major WGA receptor on the surface of HeLa cells, as shown by the labeling of gamma protein in polyacrylamide gels with [^{125}I]-WGA and by its isolation from solubilized plasma membranes after chromatography on WGA-agarose columns [14]. Desialation of gamma protein with neuraminidase greatly decreases [^{125}I]-WGA binding to this glycoprotein. Pretreatment of intact HeLa cells with WGA com-

pletely prevents the neuraminidase-induced alteration in the electrophoretic mobility of gamma protein.

The fact that these experiments are performed at 4°C with cells attached to a substratum indicates that the protective effect of WGA is not a result of lectin-induced cell-cell agglutination, or receptor redistribution. Furthermore, this effect is not due to WGA-induced internalization of cell surface receptors. We have previously demonstrated [14] that at 4°C lectin-mediated endocytosis of HeLa cell surface glycoproteins does not occur. It is most likely that WGA binds to cell surface sialic acid residues and thereby provides protection from subsequent neuraminidase digestion. Such an interpretation is consistent with the finding that WGA binds sialic acid as well as GlcNAc [26]. This finding correlates with other studies indicating that neuraminidase digestion of HeLa cells [27] and a variety of other cell types [25–30] will prevent WGA-induced cell-cell agglutination.

Neuraminidase treatment has also been shown to inhibit the binding of WGA to cell surfaces [29–31]. We have also found that neuraminidase pretreatment of HeLa cells dramatically reduces the binding of [^{125}I]-WGA to these cells (unpublished observations). The nearly complete loss of [^{125}I]-WGA binding to gamma protein as well as α_1, α_2, and β components in SDS gels of neuraminidase-treated HeLa cells further supports the contention that WGA binds sialic residues on cell surface glycoproteins. Furthermore, the work of Bhavanandan et al [32] and Bhavanandan and Katlic [33] have demonstrated the existence of a sialoglycopeptide isolated from B16 melanoma cell surface, that is devoid of GlcNAc residues yet is specifically bound by WGA-agarose columns; desialation abolished this binding. In another study Cruz and Gurd [34] have reported that preincubation of WGA with isolated synaptic membranes resulted in an 80% decrease in the hydrolysis of glycoprotein sialic acid by neuraminidase. Neuraminidase pretreatment of membranes also diminished [^{125}I]-WGA binding to glycoproteins in SDS gels.

The protection of neuraminidase-labile surface glycoproteins from desialation by neuraminidase appears to be unique to WGA. The addition of the lectins concanavalin A or ricin, which possess different sugar-binding specificities than WGA [21], did not affect the degradation of these glycoproteins when tested at 0.69 nmoles of lectin per 5×10^5 cells (unpublished observations). The ability of WGA to inhibit neuraminidase-mediated desialation of surface glycoproteins may be a general method for the identification of those WGA-reactive sialoglycoproteins that interact with the lectin in situ on the surface of intact cells.

Selective extraction studies suggest that gamma protein is an integral membrane protein. Gamma protein could not be eluted from the plasma membrane in low or high salt buffers or after treatment with 30 mM LIS, a procedure known to elute peripheral membrane proteins [35]. In addition, brief exposure of the membrane to 0.1 N NaOH failed to release gamma protein (unpublished observations), again indicating its tight association with the lipid bilayer [35]. In contrast, gamma protein is readily solubilized if the plasma membrane is treated with the appropriate concentration of ionic (SDS) or non-ionic (Triton X-100) detergents.

Trypsin treatment of isolated phagocytic membrane vesicles prepared from [^{125}I]-iodinated or [^{3}H]-glucosamine-labeled cells results in a shift in the apparent molecular weight of gamma protein from 165,000 to 150,000. Such a result implies that a fragment of gamma protein is exposed on the cytoplasmic face of the plasma membrane and is accessible to trypsin digestion. A polypeptide that is labeled at the cell surface and is degraded when inside-out vesicles are treated with a protease must be exposed on both the

outer and inner membrane surfaces and therefore must span the membrane.

The validity of this experimental approach depends critically upon the intactness of the inverted vesicles. Proof of this should be provided by the inability of the trypsin to degrade [^{125}I]-label-associated with surface components of the membrane proteins. Treatment of iodinated cells with trypsin (10 μg/ml) results in the nearly complete loss of iodinated bands 1, 2, and 3. When inside-out membrane vesicles are treated with even higher concentrations of trypsin (50 μg/ml), bands 1, 2, and 3 are not detectably degraded. Moreover, if the vesicles are first disrupted by sonication and then exposed to 5 μg/ml of trypsin, these components are completely digested. This indicates that the majority of the vesicles are sealed and inside-out.

Other evidence supports the proposal that gamma protein is a transmembrane protein. The formation of the 150,000 dalton degradation product of gamma protein is observed only when inside-out vesicle preparations are treated with proteases. Exposure of intact cells with trypsin leads to a diminished or complete loss of the label ([^{125}I] or [^{3}H]-glucosamine) from the gamma protein. At no concentration of trypsin tested on intact cells was a degradation fragment formed that corresponds to the 150,000 molecular weight species found in similarly trypsin-digested inside-out vesicles. In addition, the 150,000 molecular weight species was found to remain associated with the membrane vesicles during centrifugation.

The use of the LIS-phenol extraction procedure followed by affinity chromatography on WGA-agarose columns is an efficient method for the purification of gamma protein. The total cellular membranes isolated from the postnuclear supernatants yielded a substantial amount of starting material, and it was not necessary to use highly purified plasma membranes. This purification scheme, if scaled up, would provide a suitable method for the isolation of gamma protein in sufficient quantities for detailed chemical and molecular analysis. Work is now in progress to prepare monoclonal antibody against purified gamma protein. We hope to use this antibody to determine the cell surface distribution of gamma protein and its possible involvement in certain cellular processes such as cell adhesion.

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