Biochimica et Biophysica Acta, 551 (1979) 328–348

BBA 78303

THE SURFACE GLYCOPROTEINS OF THE HeLa CELL

INTERNALIZATION OF WHEAT GERM AGGLUTININ-RECEPTORS

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(Received July 11th, 1978)

Key words: Sialoglycoprotein; Endocytosis; Agglutinin receptor; Glycoprotein; Neuraminidase; Cell surface; (Wheat germ)

Summary

The sensitivity of ^{125}I-labeled sialoglycoproteins to neuraminidase digestion was used to monitor the loss of specific membrane glycoproteins from the cell surface into the cytoplasmic compartment during lectin-mediated endocytosis. These studies demonstrated that a major portion of the surface glycoproteins had undergone internalization concurrently with wheat germ agglutinin in a time- and temperature-dependent process. The internalized ^{125}I-labeled glycoproteins were associated with the small vesicle fraction and were present in the same relative proportion as they existed in the plasma membrane isolated from control untreated cells. Many of the ^{125}I-labeled membrane proteins were shown to be receptors and were isolated after affinity chromatography of the solubilized plasma membranes on wheat germ agglutinin-agarose columns.

Introduction

The binding of a variety of macromolecular ligands to the cell surface is often followed by the internalization of the ligand-binding site complex within an endocytotic vesicle. Such internalized vesicles, may then fuse with primary or secondary lysosomes; eventually this results in the digestion of the endocytosed material [1]. Macromolecular ligands which have been shown to undergo extensive endocytosis when bound to the cell surface include such diverse

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agents as hormones [2—4], low density lipoproteins [5], antibodies [6], bacterial toxins [7], and lectins [8—13].

Lectins which can bind to specific saccaride determinants of glycoproteins on the cell surface, are well known for their ability to stimulate endocytosis. Most recently, Edelson and Cohn [9] have shown that concanavalin A can increase the overall pinocytotic rate of peritoneal macrophages by 3-fold [9].

We demonstrate that most, but not all, of the enzymatically ^{125}I-iodinated surface polypeptides of HeLa cells can bind wheat germ agglutinin. We also report that wheat germ agglutinin can induce an extensive endocytosis of both receptor and non-receptor surface polypeptides, and that each individual molecular species of surface polypeptide is internalized in proportion to its concentration in the plasma membrane. A summary of these results has been previously communicated [15,16].

Materials and Methods

The materials were purchased from the following commercial sources: the media used for cell culture from Gibco, Grand Island, NY; the fetal bovine serum from Flow Laboratories, Rockville, MD; the cell culture dishes from Falcon Plastics, Oxnard, CA; lactoperoxidase (80 U/mg) and glucose oxidase, Type V, (200 U/mg) from Sigma Chemicals, St. Louis, MO; Na^{125}I (10—20 Ci/mg) and [^{3}H]glucosamine (700 mCi/mol) from New England Nuclear, Boston, MA; Sepharose 4B from Pharmacia, Piscataway, NJ; *Vibrio cholerae* neuraminidase from Calbiochem-Behring, La Jolla, CA; acrylamide, *N,N'*-methylene bisacrylamide, wheat germ agglutinin and fluorescein-conjugated wheat germ agglutinin from Miles Laboratories, Elkhart, IN; horseradish peroxidase from Worthington Bichemicals, Freehold, NJ; Dounce homogenizers from Bellco, Vineland, NJ; type X-Omat X-ray film from Eastman Kodak Co., Rochester, NY. In addition to these, commercial analytical grade reagents were used.

Cell culture. HeLa-S_3 cells were routinely grown as suspension cultures in spinner flasks as previously described [17]. The cells were maintained at $2—6 \cdot 10^5$/ml in Joklik modified minimum essential medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum.

^{125}I-labeled wheat germ agglutinin binding experiments. Wheat germ agglutinin was ^{125}I-iodinated by the chloramine T method as described by Cuatrecasas [18]. More than 95% of the radioactivity in the ^{125}I-labeled lectin preparation could be precipitated with cold 10% trichloroacetic acid.

For binding studies, HeLa cells grown in suspension culture were washed three times in ice-cold phosphate-buffered saline and suspended at a final density of $1 \cdot 10^5$ cells/ml in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.1% glucose. 1 ml of the cell suspension was incubated with the ^{125}I-labeled wheat germ agglutinin at 4 or at 37°C in polypropylene tubes which had been treated previously with 1% bovine serum albumin. After the incubation period, the cells were washed three times with ice-cold phosphate-buffered saline containing 0.1% bovine serum albumin and counted in a Beckman gamma-scintillation counter. Non-specific binding was determined for each concentration of lectin by performing parallel assays containing 0.1 M *N*-acetylglucosamine, the haptenic inhibitor. Specific binding was then calcu-

lated by subtracting the non-specific binding from the total amount bound. Non-specific binding, under the conditions reported here, was never greater than 10% and usually less than 6% of the total binding.

Fluorescent microscopy. Monolayer HeLa cell cultures were usually used for labeling with fluorescein isothiocyanate-conjugated wheat germ agglutinin although in certain experiments suspension culture cells were used. Between 2 and $5 \cdot 10^5$ cells were seeded onto 35 mm culture dishes 24—48 h prior to the labeling. The monolayer cells were exposed to 25 μg/ml of fluorescein isothiocyanate-conjugated wheat germ agglutinin for 2 h at either 4 or 37°C in phosphate-buffered saline/0.1% bovine serum albumin/Eagles minimal essential medium. Incubation was terminated by washing the dishes three times with phosphate-buffered saline. After fixing the cells with 10% formalin in phosphate-buffered saline for 15 min at 25°C, the cells were mounted in 10% glycerol in phosphate-buffered saline and viewed immediately with a Leitz Orthoplan Microscope equipped with fluorescent objectives.

In certain experiments the cells were prefixed with 10% formalin in phosphate-buffered saline for 10 min at 25°C and washed extensively with Eagles minimal essential medium. The cells were then exposed to fluorescein isothiocyanate-conjugated wheat germ agglutinin, washed and post-fixed as described above.

Labeling of cells with horseradish peroxidase-wheat germ agglutinin. Horseradish peroxidase-conjugated wheat germ agglutinin was prepared according to the procedure of Gonatas and Avrameas [19]. HeLa cells, grown in suspension culture, were washed three times in cold phosphate-buffered saline and suspended at a density of $2 \cdot 10^5$ cells/ml in phosphate-buffered saline/0.1% bovine serum albumin. The cells were labeled with 40 μg/ml of the horseradish peroxidase-wheat germ agglutinin reagent for 30 min at 4°C and then washed three times with cold phosphate-buffered saline/0.1% bovine serum albumin. One group of cells was incubated at 37°C for 2 h and another group was incubated at 4°C for 2 h. At the termination of the incubation period, the cells were washed twice with phosphate-buffered saline and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min at room temperature. The cells were reacted with diaminobenzidine as described [19] and post-fixed with 1% O_sO_4 in 0.1 M cacodylate buffer for 1 h at 22°C. The cell pellets were dehydrated, embedded in Epon 812 and gold-to-silver sections were cut with a diamond knife. The sections were stained with 2% uranyl acetate and examined in a Zeiss electron microscope. Peroxidase-diaminobenzidine staining was completely prevented by the addition of 0.1 M *N*-acetylglucosamine during the initial period of exposure to horseradish peroxidase-wheat germ agglutinin.

Labeling procedures. HeLa cells were ^{125}I-iodinated with lactoperoxidase as previously described [17].

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. Maizel's [20] method was used for slab gel electrophoresis. Samples were diluted with three times concentrated solubilization solution which contained the following reagents: 0.18 M Tris-0.09 M H_3PO_4, 10% sodium dodecyl sulfate (w/v), 10% 2-mercaptoethanol (v/v) and 30% glycerol (w/v), pH 6.7. The samples were

heated at 100°C for 3 min immediately prior to electrophoresis.

Gels were stained for protein overnight with Coomassie Blue as described by Fairbanks et al. [21]. Destained gels were dried onto filter paper for autoradiography of ^{125}I-labeled polypeptides and exposed to X-ray film. Detection of ^{3}H-labeled polypeptides in slab gels was achieved by the fluorographic method of Bonner and Laskey [22].

Plasma membrane isolation. The isolation procedure of HeLa cell plasma membranes [23] was modified as follows: Cells were grown in spinner cultures to a density of $4—5 \cdot 10^5$ cells/ml, and collected by centrifugation at $150 \times g$ for 5 min. The cell pellet was washed three times with cold phosphate-buffered saline. After the final wash in a 50 ml polypropylene centrifuge tube, the supernatant solution was thoroughly aspirated. The pellet was suspended in 10 volumes of ice-cold 10 mM Tris-HCl, pH 7.5, and the cells were allowed to swell for approx. 5 min at 0°C, at which time they were homogenized in a tight fitting Dounce homogenizer.

Usually 2—5 strokes were required to lyse 70—80% of the cells. Cell breakage was monitored by phase-contrast microscopy. The membrane ghosts and the nuclei were stabilized by immediately adjusting the homogenate to 0.25 M sucrose with 1.5 M sucrose in 10 mM Tris-HCl, pH 7.5. The homogenate was then divided into 8—10-ml portions which were placed in specially designed flat bottom tubes constructed from 100-ml polymethylpentene Nalgene graduated cylinders. Nuclei were differentially separated from the membrane ghosts by centrifugation at $800 \times g$ for 1 min. The nuclear pellet was suspended in 0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl, pH 7.5, and centrifuged at $800 \times g$ for 1 min. The pooled supernatant solutions were centrifuged at $800 \times g$ for 5 min to collect the plasma membrane ghosts. The resultant supernatant solution was designated the small vesicle fraction (V); it was free of the large sac-like membrane ghosts and contained substantial vesiculated material. The membrane ghost pellet was washed twice in 0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl (pH 7.5) and similarly centrifuged to remove smaller cellular debris and soluble protein. Finally, the membranes were gently suspended in a large volume of 1 mM EDTA/10 mM Tris-HCl (pH 7.5) and pelleted by centrifugation at $1500 \times g$ for 3 min to give the purified plasma membrane ghost fraction (M). The entire procedure generally required less than 1.5 h to complete.

The total yield of incorporated ^{125}I radioactivity in the membrane fraction averaged from 20 to 40% of that present in the initial homogenate [24] indicating a relatively high recovery of membranes by this method. An indication of the purification of this material is provided by a 10—12-fold increase in the specific activity (cpm/μg protein) of the acid-insoluble radioactivity in the membrane fraction over that of the whole cell homogenate [24]. The specific activity of alkaline phosphatase, a membrane enzyme in HeLa cells [25], was enriched also approx. 10-fold compared to that in the homogenate [24]. When the homogenates of iodinated cells were analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis, the ^{125}I radioactivity gel profile was identical to that of the purified plasma membranes [24]. Therefore, no detectable loss of specific ^{125}I-labeled macromolecules occurs during the process of membrane isolation.

Isolation of plasma membrane ghosts from cells treated with wheat germ agglutinin. Approx. $1 \cdot 10^8$ HeLa cells were iodinated according to our established procedure [17] and suspended at a density of $5 \cdot 10^5$ cells/ml in 1% fetal bovine serum/0.1% bovine serum albumin/Joklik-modified minimum essential medium. The cell suspension was placed in a Bellco spinner flask and a solution of wheat germ agglutinin was added to bring the final concentration of wheat germ agglutinin to 25 μg/ml. The cells were stirred at about 30 rev./min for 2 h at 37°C at which time the suspension was adjusted to 0.1 M *N*-acetylglucosamine with a 1 M solution of *N*-acetylglucosamine; the incubation was continued for another 10 min. The cells were then harvested and washed twice with cold 0.1 M *N*-acetylglucosamine/0.1% bovine serum albumin/phosphate-buffered saline and finally washed twice with cold phosphate-buffered saline. At this point, cells were either treated with neuraminidase, extensively washed and the plasma membranes then isolated or the cells were processed directly for plasma membrane isolation. Neuraminidase digestion was performed by incubating $5 \cdot 10^5$ cells/ml in phosphate-buffered saline containing 1 mM $CaCl_2$, pH 7.0, and 10 U *Vibrio cholerae* neuraminidase.

Affinity chromatography of solubilized plasma membranes. HeLa cell surface wheat germ agglutinin receptors were isolated according to a modification of the method described by Kahane et al. [26]. Approx. 500 μg of plasma membranes isolated from ^{125}I-labeled cells was solubilized in 0.5 ml of 1% sodium dodecyl sulfate in 10 mM Tris-HCl, pH 7.5, for 15 min at 37°C. After centrifugation at 100 000 $\times g$ for 1 h, more than 95% of the incorporated ^{125}I radioactivity was recovered in the supernatant solution. The supernatant solution was then diluted with 0.25 M NaCl/15 mM Tris-HCl, pH 7.5, to give a final sodium dodecyl sulfate concentration of 0.05%. Approx. 100 μg of this solubilized plasma membrane protein preparation was loaded onto a 1—2 ml wheat germ agglutinin-agarose column and the column was eluted with the same 0.05% sodium dodecyl sulfate/0.25 M NaCl/15 mM Tris-HCl (pH 7.5) solution until the level of ^{125}I was less than 50 cpm per 0.1 ml fraction. Elution of specifically bound receptors was achieved by washing with 1.5 column volumes of 0.1 M *N*-acetylglucosamine in the chromatography buffer. Based on the radioactivity profiles, peak fractions were pooled, dialyzed against distilled water, and freeze-dried.

Results

^{125}*I-labeled wheat germ agglutinin binding*

The rate as well as the extent of ^{125}I-labeled wheat germ agglutinin binding remained the same whether the binding of ^{125}I-labeled wheat germ agglutinin was performed at 4 or at 37°C. The ^{125}I-labeled wheat germ agglutinin binding to HeLa cells was characterized by a steep increase in the amount of radioiodinated lectin bound to the cells during the first 5 min of incubation. Beyond this period of time, continued incubation showed only a slow increase in the amount of ^{125}I-labeled wheat germ agglutinin associated with the cells.

The concentration dependence of ^{125}I-labeled wheat germ agglutinin binding to HeLa cells indicated that the amount of ^{125}I-labeled wheat germ agglutinin bound to the cells approached saturation at 100 μg wheat germ agglutinin/ml

per $1 \cdot 10^5$ cells; half maximal binding of wheat germ agglutinin occurred at approx. 10 μg wheat germ agglutinin/ml per $1 \cdot 10^5$ cells.

In Fig. 1, the results of an experiment are described where HeLa cells were exposed to ^{125}I-labeled wheat germ agglutinin at 4°C for 30 min and washed. The cells were then either treated with 0.1 M *N*-acetylglucosamine at 4°C or incubated for the indicated times at 37°C and then treated with 0.1 M *N*-acetylglucosamine. More than 95% of the ^{125}I-labeled wheat germ agglutinin bound at 4°C could be dissociated with 0.1 M *N*-acetylglucosamine during a 30 min incubation period at 4°C. This successful removal of the lectin by *N*-acetylglucosamine was observed even if the cells had been previously incubated with the lectin for as long as 2 h at 4°C. In contrast, the ^{125}I-labeled wheat germ agglutinin bound to cells which were subsequently incubated at 37°C was found to become progressively more resistant with time to removal by the hapten sugar. The amount of apparent irreversibly bound wheat germ agglutinin was found to increase more or less at a linear rate and was seen to plateau after 2 h. Prolonged exposure to *N*-acetylglucosamine failed to increase significantly the percent of radioactive lectin that could be eluted.

Distribution of cell-bound wheat germ agglutinin

When HeLa cells, whether grown as monolayer or as suspension cultures, were incubated with fluorescein isothiocyanate-conjugated wheat germ agglutinin at 4°C for 2 h, the cell-bound fluorescein isothiocyanate-conjugated wheat germ agglutinin was found to be diffusely and uniformly distributed over the entire cell surface (Fig. 2A). An identical distribution of fluorescein isothiocyanate-conjugated wheat germ agglutinin was found when formaldehyde-prefixed HeLa cells had been exposed to the labeled lectin. Cells incubated

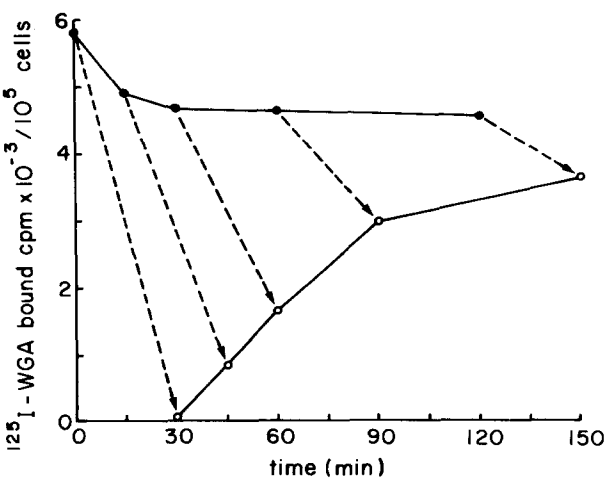

Fig. 1. Displacement of cell-associated ^{125}I-labeled wheat germ agglutinin (^{125}I-WGA) from HeLa cells by *N*-acetylglucosamine. Cells ($1 \cdot 10^5$) were labeled with 25 μg/ml of ^{125}I-labeled wheat germ agglutinin (specific activity: $1.7 \cdot 10^4$ cpm/μg) for 30 min at 4°C and washed with phosphate-buffered saline. The cells were resuspended in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% glucose at 37°C for the indicated time intervals (●). *N*-Acetylglucosamine was added to a final concentration of 0.1 M and the cells were incubated for an additional 30 min at 4°C (○). The specific binding of ^{125}I-labeled wheat germ agglutinin was then determined as described in Materials and Methods.

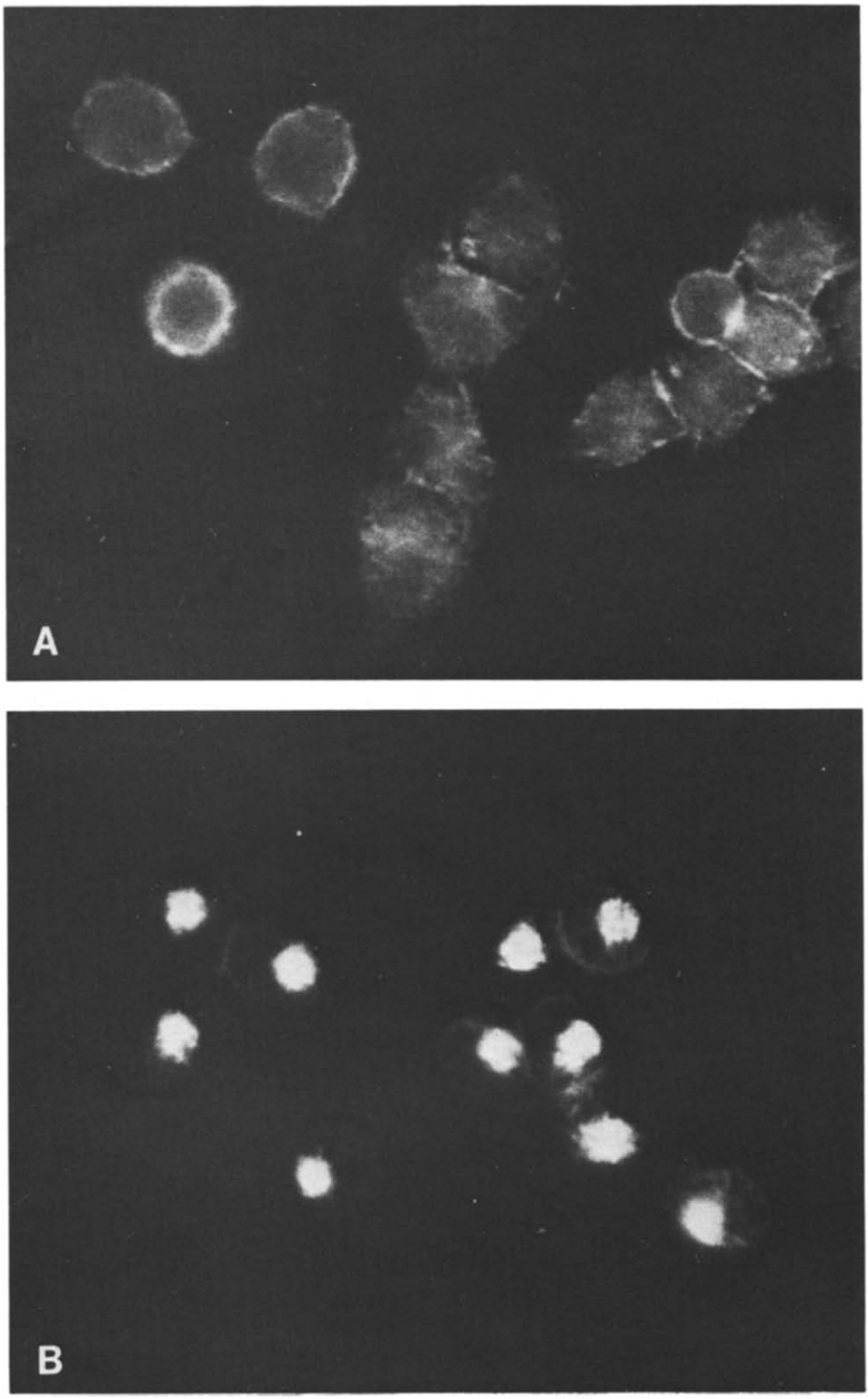

Fig. 2. The distribution of fluorescein-wheat germ agglutinin bound to HeLa cells. Cells were incubated with fluorescein isothiocyanate-conjugated wheat germ agglutinin (25 μg/ml) either at 4°C (A) or at 37°C (B) for 2 h, washed and fixed with formaldehyde (× 500).

with horseradish peroxidase-wheat germ agglutinin under similar conditions at 4°C and reacted with diaminobenzidine showed a correspondingly uniform distribution of the horseradish peroxidase-wheat germ agglutinin, as evidenced by the electron dense deposit, around the cell periphery (Figs. 3A and 3C); no label was detected deep in the cytoplasmic compartment.

If, however, cells are incubated with fluorescein isothiocyanate-conjugated wheat germ agglutinin or horseradish peroxidase-wheat germ agglutinin at 37°C for 2 h before fixation, the distribution of labeled lectin was markedly altered. At the level of the fluorescent microscope, a majority of the bound fluorescein isothiocyanate-conjugated wheat germ agglutinin was found located at the cell center with some amounts of diffuse label visible at the cell periphery Fig. 2B). Depth of field studies revealed that this concentrated region of fluorescent label was not located at the cell surface but rather was located intracellularly.

When cells in suspension were labeled in a similar fashion, and agitated under the microscope, the central cluster of fluorescent label was inevitably found to be at the center of the cell and never situated at a pole. Fluorescent microscopy in parallel with phase-contrast microscopy demonstrated that this central core of fluorescence was associated with phase-dense particles located at the perinuclear region of the cell. Although *N*-acetylglucosamine was effective in dissociating essentially all of the fluorescein isothiocyanate-conjugated wheat germ

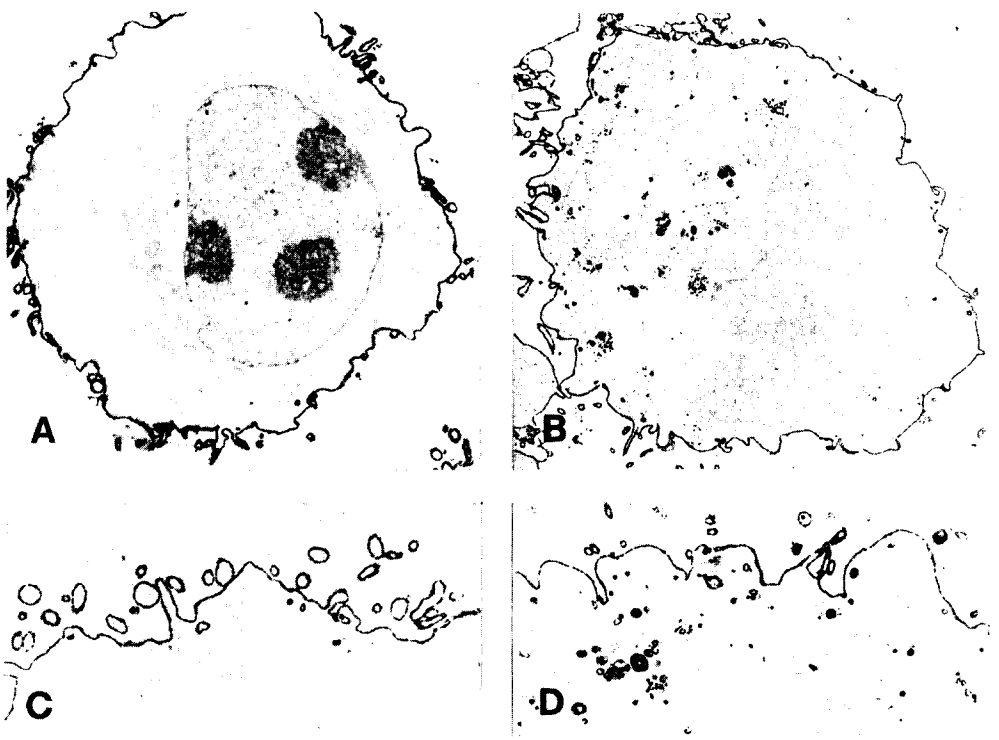

Fig. 3. The ultrastructural distribution of horseradish peroxidase-conjugated wheat germ agglutinin bound to HeLa cells. HeLa cells were incubated with horseradish peroxidase-conjugated wheat germ agglutinin at 4°C for 30 min, washed, and then incubated for 2 h at 4°C (A and C) or at 37°C (B and D). A, × 6000; B, × 6000; C, × 11 000; D, × 9000.

agglutinin bound at 4°C, this intense centralized region of label formed during incubation at 37°C was resistant to disruption even after a 1 h exposure to the hapten sugar. All of these fluorescent microscopic observations suggested that the fluorescein isothiocyanate-conjugated wheat germ agglutinin had undergone extensive internalization.

Examination of electron microscopic sections prepared from HeLa cells incubated with horseradish peroxidase-wheat germ agglutinin at 37°C for 2 h confirmed these observations made by fluorescent microscopy. While some horseradish peroxidase-wheat germ agglutinin label remained localized at the cell surface, substantial amounts of the horseradish peroxidase-wheat germ agglutinin, in the form of small vesicles, were detected throughout the cytoplasm and particularly at the perinuclear region (Figs. 3B and 3C). These intracellular horseradish peroxidase-wheat germ agglutinin-labeled vesicles appeared to be mostly pinosomes although some larger vesicles were present which may represent primary or secondary lysosomes. The horseradish peroxidase-wheat germ agglutinin which was still associated with the cell surface was homogeneously distributed and no areas of discontinuity were visible.

Neuraminidase treatment alters the electrophoretic mobility of surface membrane glycoproteins

^{125}I-labeled HeLa cells were treated with varying concentrations (0—5.0 U/ml) of *V. cholerae* neuraminidase; they were then solubilized and electrophoresed in sodium dodecyl sulfate slab gels. The autoradiogram of such a preparation shows a number of alterations in the apparent molecular weights of the ^{125}I-labeled polypeptides after neuraminidase treatment (Fig. 4). Careful examination of the positions of the iodinated bands from the control cells (extreme left and right lanes of the slab gel) and of the neuraminidase-treated cells, indicates a progressive increase in the electrophoretic mobility of the ^{125}I-labeled bands 1, 2, 4, 5, 8 and 9 as the amounts of neuraminidase were increased in the incubation medium. In contrast, band 6 underwent a progressive decrease in electrophoretic mobility after treatment with increasing concentrations of neuraminidase. Thus, exposure of cells to 5.0 U/ml of neuraminidase, produced an increase in the apparent molecular weight of band 6 (γ-protein) from 165 000 to over 200 000. Higher concentrations of neuraminidase or longer incubation periods only slightly accentuated these effects. Neuraminidase treatment did not appear to modify significantly the electrophoretic mobility of bands 3, 7, 10, 11, 12 and 13.

Alterations in the electrophoretic mobility of [^{3}H]glucosamine-labeled glycoproteins were also noted after neuraminidase treatment (Fig. 5). In particular, a decrease in the electrophoretic mobility of γ-protein, which corresponds to ^{125}I-labeled band 6 [17] was most striking. The α_2, β, and δ bands demonstrated increases in their electrophoretic mobilities after neuraminidase digestion, while α_1 could not be detected after treatment with neuraminidase.

The observed changes in electrophoretic mobility of membrane glycoproteins were not due to trace protease contamination in the neuraminidase preparation because: (a) treatment of ^{125}I-labeled cells with 10 U/ml neuraminidase for 10 min at 37°C did not release any detectable radioactivity; (b) *V. cholerae* neuraminidase requires divalent cations for activity [27] and we found that 2

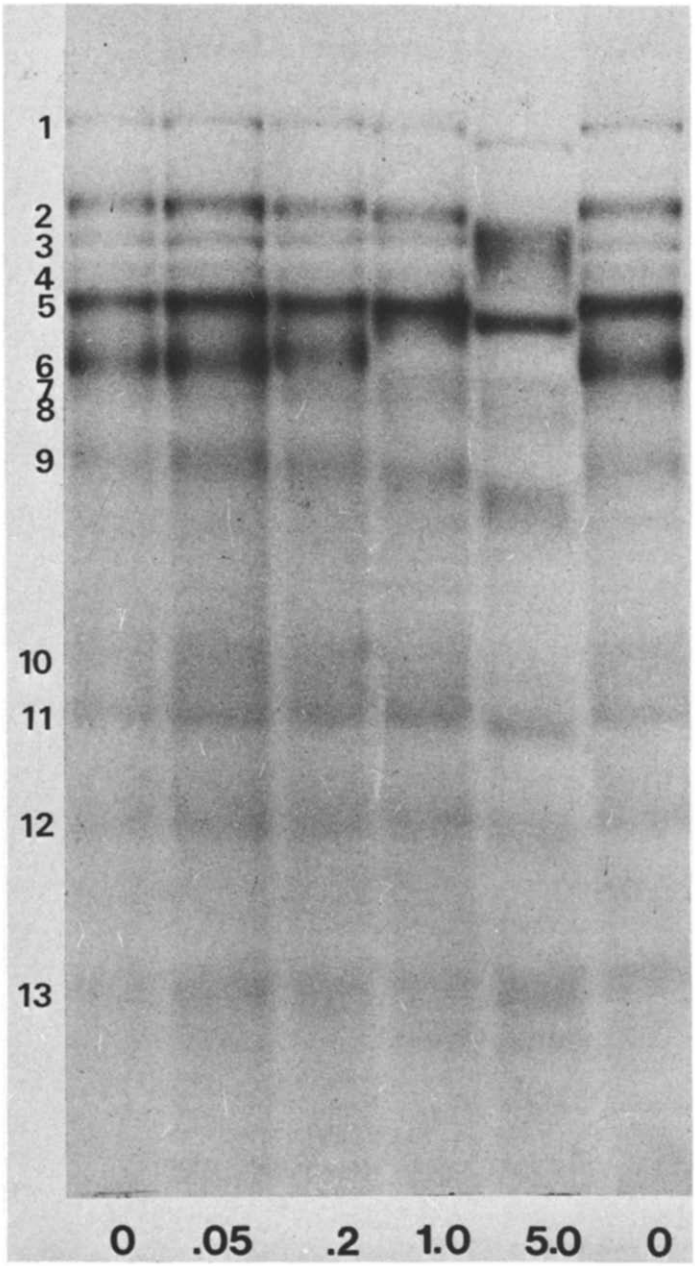

Fig. 4. The effect of neuraminidase treatment upon the sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of HeLa cell ^{125}I-labeled cell surface polypeptides. $5 \cdot 10^5$ ^{125}I-labeled HeLa cells in 1 ml of phosphate-buffered saline containing 1 mM $CaCl_2$, pH 7.0, were incubated for 10 min at 37°C with increasing concentrations of *V. cholerae* neuraminidase (units per ml given below each lane). The cells were washed, solubilized in sodium dodecyl sulfate, and electrophoresed in a 6% slab gel which was then dehydrated and exposed to X-ray film.

mM EDTA completely inhibited the reaction, and (c) the presence of 1% bovine serum albumin, which was added to provide a competing substrate for proteolytic enzymes did not alter the results.

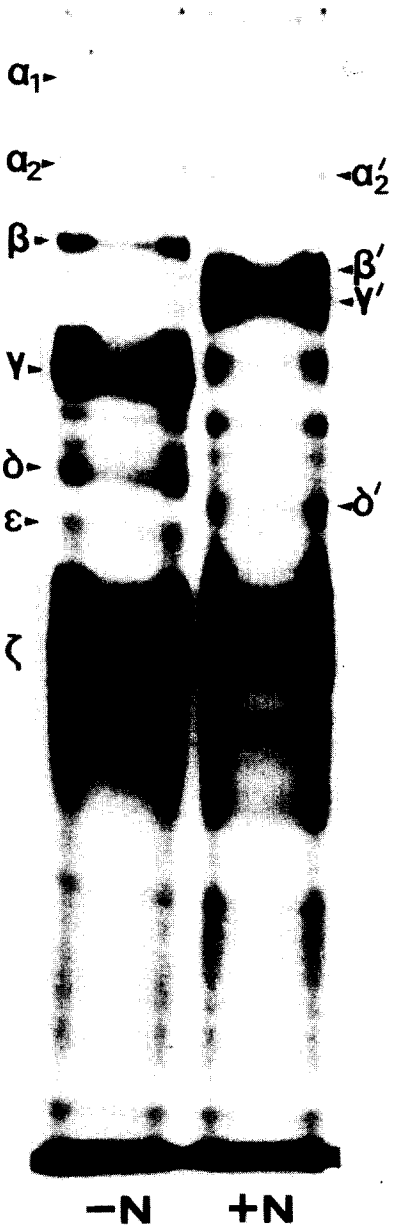

Fig. 5. The effect of neuraminidase treatment upon the sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of HeLa cell [^{3}H]glucosamine-labeled components. HeLa cells were cultured for 48 h in the presence of 5 μCi/ml of [^{3}H]glucosamine. Cells were incubated with 10 U/ml of *V. cholerae* neuraminidase in 1 ml of phosphate-buffered saline containing 1 mM $CaCl_2$ (pH 7.0), solubilized in sodium dodecyl sulfate, and electrophoresed in a 6% slab gel; detection by fluorography. +N, neuraminidase treatment; −N, no neuraminidase treatment.

Wheat germ agglutinin-induced endocytosis of cell surface proteins

The modification in the electrophoretic mobility of exposed cell surface sialoglycoproteins following neuraminidase digestion was used as a basis for

detecting wheat germ agglutinin-induced endocytosis of cell surface sialoglycoproteins; if internalization of surface membrane sialoglycoproteins occured during incubation with wheat germ agglutinin, the accessibility of these membrane proteins to neuraminidase in the medium would be lost.

Fig. 6A shows the labeling pattern of control HeLa cells ^{125}I-iodinated by the lactoperoxidase reaction. Neuraminidase treatment of these iodinated cells increased the electrophoretic mobility of ^{125}I-labeled bands 1, 2, 4, 5, 7, 8 and 9, but decreased the electrophoretic mobility of band 6.

When ^{125}I-iodinated HeLa cells were exposed to wheat germ agglutinin at 37°C for 1.5 h, thoroughly washed with 0.1 M *N*-acetylglucosamine, digested with neuraminidase and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the expected neuraminidase-induced alterations in electrophoretic mobility of the sensitive sialoglycoproteins was greatly suppressed (Fig. 6B). Concanavalin A, another lectin which specifically binds D-glucopyranose or D-mannopyranose residues, was found to produce an almost identical protective effect (Fig. 6B).

In an analogous series of experiments performed with [^{3}H]glucosamine-labeled HeLa cells, wheat germ agglutinin protected [^{3}H]glucosamine-labeled surface membrane sialoglycoproteins from neuraminidase digestion. We have already established that there are a number of high molecular weight [^{3}H]glucosamine-labeled sialoglycoproteins on the surface of HeLa cells, some of which appear to be ^{125}I labeled by the lactoperoxidase reaction [17]. Neuraminidase digestion of intact [^{3}H]glucosamine-labeled cells resulted in a modified electrophoretic mobility of bands α_1, α_2, β, γ and δ (Fig. 7). Several bands (α_2, β and δ) showed an increased electrophoretic mobility while α_1 was decreased in labeling and the γ band displayed a pronounced decrease in its electrophoretic migration.

The time dependence of the wheat germ agglutinin protective effect on the sialoglycoproteins of the [^{3}H]glucosamine-labeled cells is presented in Fig. 7. The sensitivity of [^{3}H]glucosamine bands to neuraminidase digestion was rapidly lost, being already apparent after the first 15 min of incubation with wheat germ agglutinin; this protection from neuraminidase became progressively more pronounced by 30 min and 60 min and by 2 h it was nearly complete. However, if in an otherwise identical experiment, the incubation with wheat germ agglutinin was performed for 2 h at 4°C rather than at 37°C, all of the neuraminidase sensitive bands remained accessible to digestion by neuraminidase (Fig. 7).

Experiments were performed to analyze what changes occurred in the composition of externally exposed plasma membrane polypeptides following wheat germ agglutinin-induced endocytosis. The rationale for these experiments was that, if discrete wheat germ agglutinin receptor membrane glycoproteins were selectively translocated from the cell surface into the cytoplasmic compartment during endocytosis, the residual plasma membrane remaining at the cell surface would be depleted of these receptors and would be enriched with non-receptor molecules. Alternatively, if the plasma membrane was being uniformly internalized, then no qualitative changes in the residual surface membrane polypeptide population would be detected.

In the experiment presented in Fig. 8 the distribution of the surface glyco-

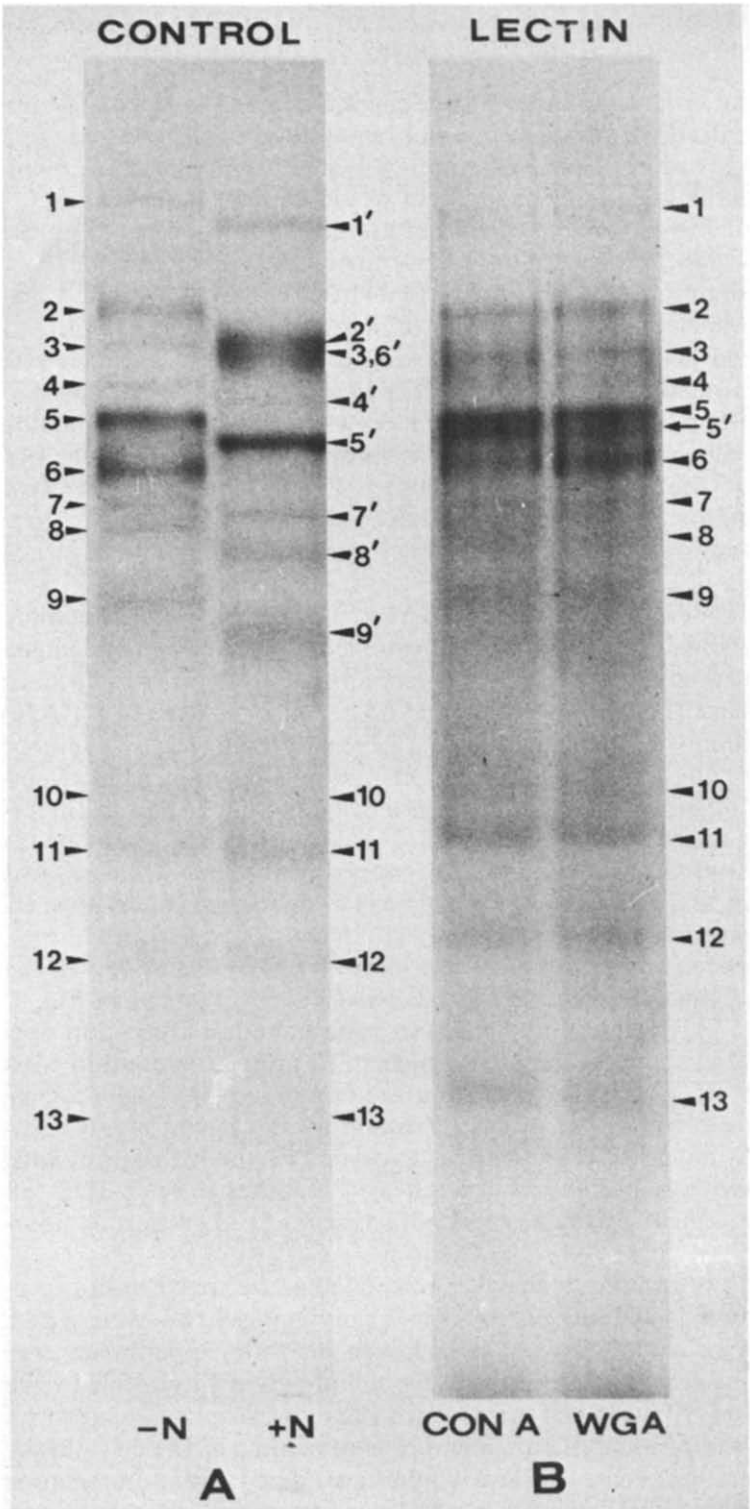

Fig. 6. Lectin-induced endocytosis of ^{125}I-iodinated surface polypeptides. In A, iodinated cells were incubated at 37°C for 1.5 h and incubated for 10 min in the absence (—N) or presence (+N) of neuraminidase (10 U/ml per $5 \cdot 10^5$ cells). In B, iodinated cells were incubated with either concanavalin A or wheat germ agglutinin (25 μg/ml per $5 \cdot 10^5$ cells) at 37°C for 1.5 h and were then exposed to 0.1 M methyl-β-D-mannopyranoside or 0.1 M *N*-acetylglucosamine, respectively, for 30 min at 4°C. After the cells were washed with phosphate-buffered saline, they were digested with neuraminidase as described in A. The cells were then solubilized with sodium dodecyl sulfate and electrophoresed in a 6% slab gel; detection by autoradiography.

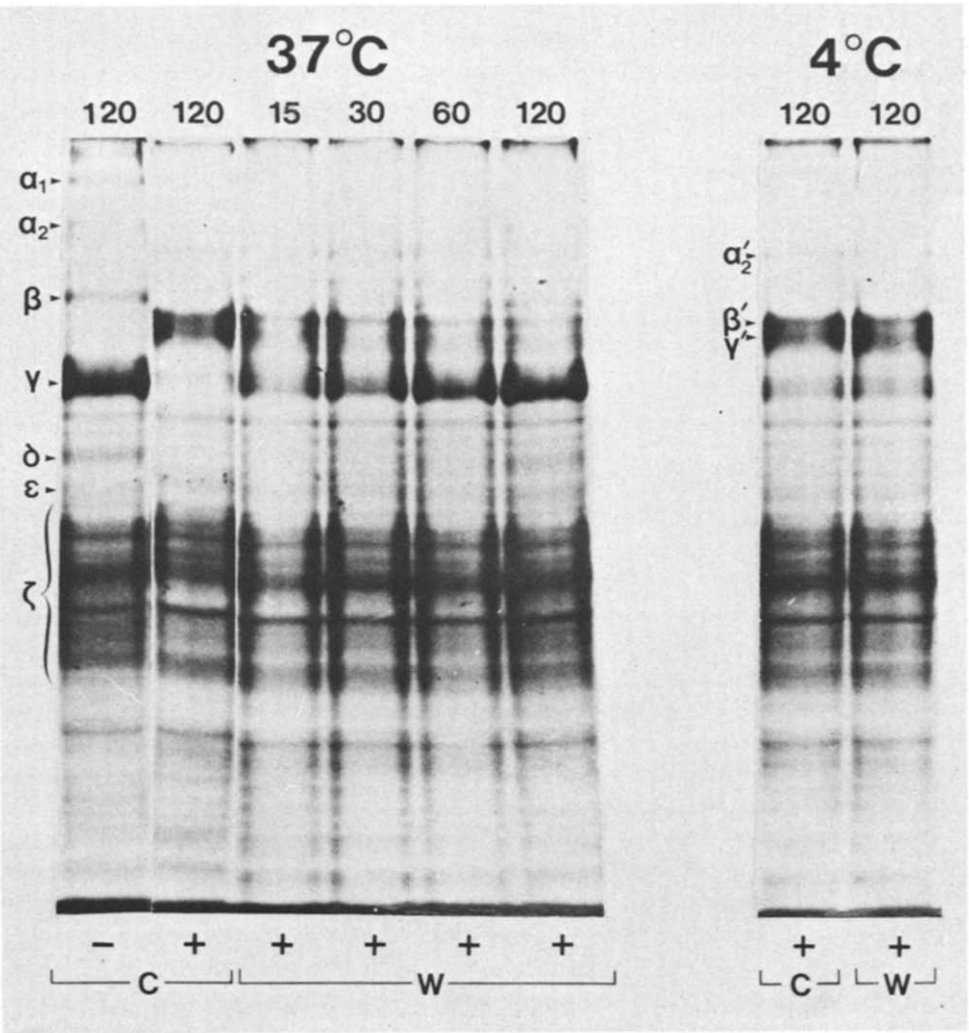

Fig. 7. Endocytosis of [^{3}H]glucosamine-labeled surface membrane glycoproteins. HeLa cells were cultured in the presence of [^{3}H]glucosamine for 48 h (5 μCi/ml). Cells ($5 \cdot 10^5$) were then incubated with (W) or without (C) wheat germ agglutinin (25 μg/ml) at either 37 or 4°C for the indicated time intervals (given incubated). The cells were then in min above each lane with (+) or without (—) neuraminidase (10 U/ml) at 37°C for 10 min (left panel) or at 4°C for 30 min (right panel). The cells were solubilzed in sodium dodecyl sulfate and electrophoresed in a 6% slab gel; detection by fluorography.

proteins among two subcellular fractions was determined. ^{125}I-labeled HeLa cells were exposed to wheat germ agglutinin for 2 h at 37°C, washed with *N*-acetylglucosamine, and disrupted in a Dounce homogenizer. The homogenate (H) was then fractionated to yield the purified plasma membrane ghosts (M) and the small vesicle fraction (V), containing microsomes, mitochondria, lysosomes, pinosomes and other vesicular material. The radioactivity profiles of ^{125}I-labeled proteins in these two fractions when compared to the whole cell homogenate in Fig. 8A are found to be identical. This result indicates that during wheat germ agglutinin-induced internalization of the surface membrane,

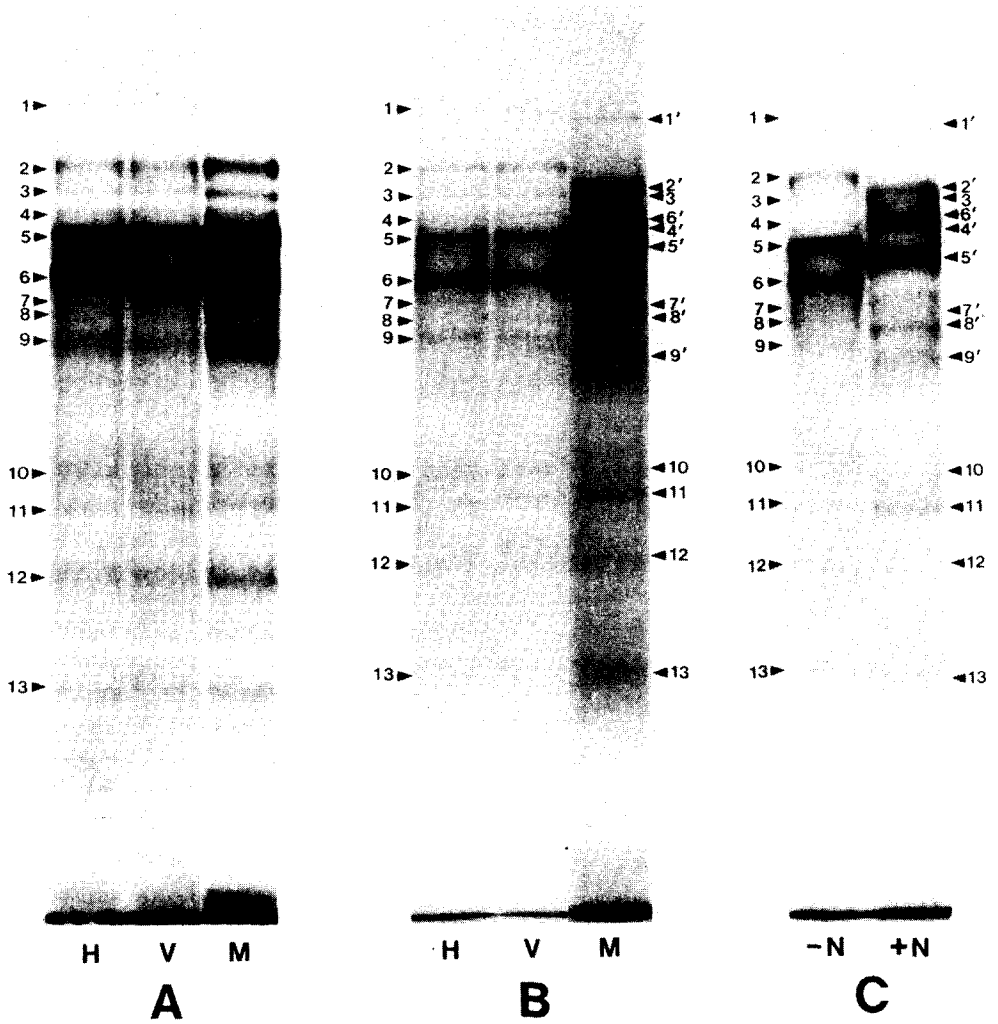

Fig. 8. Subcellular fractionation of wheat germ agglutinin-treated cells. In A, ^{125}I-iodinated cells were incubated at 37°C for 2 h with wheat germ agglutinin (25 μg/ml per $5 \cdot 10^5$), and then treated with 0.1 M *N*-acetylglucosamine. In B, the iodinated cells were treated identically except that after exposure to *N*-acetylglucosamine, the cells were digested with neuraminidase (10 U/ml per $5 \cdot 10^5$ cells for 10 min at 37°C). The cells in A and in B were then homogenized to give the homogenate (H), and the small vesicle fraction (V) and the plasma membrane fraction (M) were isolated as described in Materials and Methods. In C, control ^{125}I-labeled cells were incubated in the absence of wheat germ agglutinin for 2 h at 37°C. After washing with 0.1 M *N*-acetylglucosamine, the cells were treated with neuraminidase as described for group B cells. The samples were solubilized in sodium dodecyl sulfate and electrophoresed in a 6% slab gel; detection by autoradiography.

all of the labeled species are endocytosed in the same relative proportions to those existing in the native plasma membrane. Furthermore, whereas 20—40% of the total radioactivity could be recovered in the plasma membrane ghost fraction prepared from control cells, only about 5—10% of the total ^{125}I-incorporated radioactivity was recovered in the plasma membrane ghost fraction isolated from wheat germ agglutinin-treated cells.

TABLE I

RECOVERY OF ^{125}I-LABELED PLASMA MEMBRANE PROTEINS IN ELUATE FRACTIONS AFTER CHROMATOGRAPHY ON A WHEAT GERM AGGLUTININ-AGAROSE COLUMN

Results are from the experiment described in Fig. 9.

Fraction	cpm	%
Total cpm applied	146 000	100
A. Unbound	53 600	36.7
B. Wash	13 200	9.0
C. *N*-Acetylglucosamine eluted	71 100	48.6
Total recovered	137 900	94.3

A second experiment was performed, which was identical to the one just described, except that the wheat germ agglutinin-treated cells were digested with neuraminidase immediately prior to the homogenization and subcellular fractionation (Fig. 8B). As anticipated from the previous results, the neuraminidase treatment was largely ineffective in altering the electrophoretic mobilities of sensitive ^{125}I-labeled polypeptides following wheat germ agglutinin treatment (Fig. 8B, lane H). The labeling pattern found in the small vesicle fraction (V), which lacks the large plasma membrane ghosts, was identical to that of the homogenate (H).

In contrast, those residual sialoglycoproteins which copurified with the plasma membrane fraction (M), had been accessible to the neuraminidase treatment

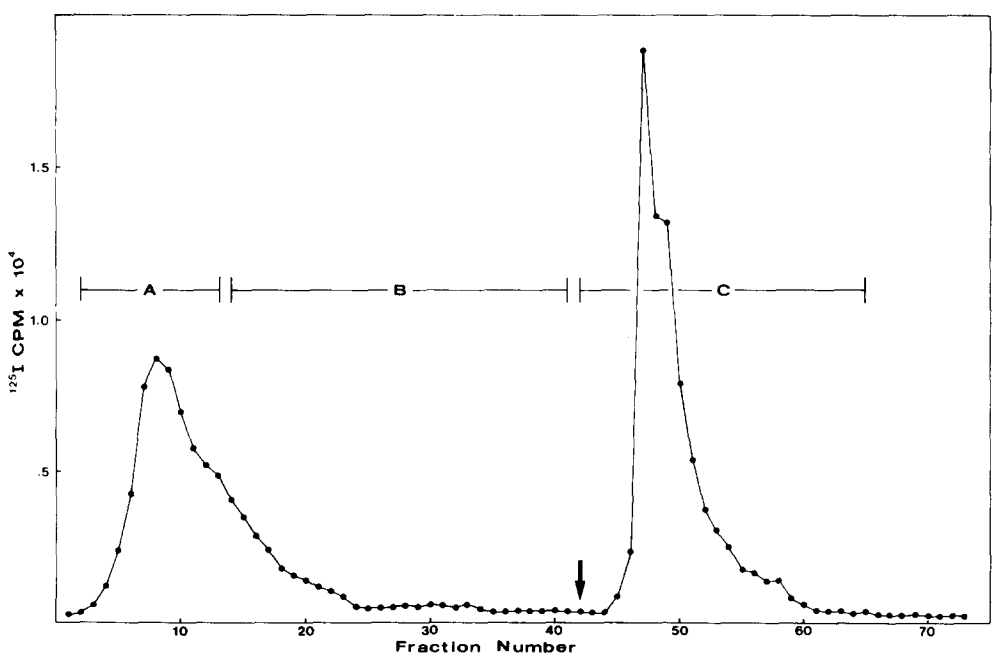

Fig. 9. Affinity chromatography of ^{125}I-labeled plasma membrane proteins from iodinated HeLa cells on a wheat germ agglutinin-agarose column. Approx. 100 μg protein of plasma membrane ghosts was solubilized in sodium dodecyl sulfate and chromatographed as described in Materials and Methods. Arrow indicates elution with 0.1 M *N*-acetylglucosamine. 0.35-ml fractions were collected, analyzed for radioactivity, then pooled to yield groups A, B and C as indicated.

and therefore were exposed on the cell surface. However, these ^{125}I-labeled polypeptides degraded by neuraminidase represented only a small proportion of the total ^{125}I-labeled polypeptide population present in the cells and do not obviously affect the overall pattern of the homogenate (Fig. 8B, compare lane M to lane H). These results indicate that the majority of the ^{125}I-labeled polypeptides have been internalized during wheat germ agglutinin and are associated with the vesicular fraction (V).

Isolation of HeLa cell surface wheat germ agglutinin receptors

Plasma membranes isolated from iodinated HeLa cells were solubilized and chromatographed on a wheat germ agglutinin-agarose column as described in Materials and Methods. The recovery was 94.3% of the total added radioactivity (Table I). The first peak of radioactivity to be eluted with the sodium dodecyl sulfate-salts buffer (see Materials and Methods), represents unbound ^{125}I-labeled material followed by some trailing of radioactivity (Fig. 9). Upon elution with 100 mM *N*-acetylglucosamine in the same sodium dodecyl sul-

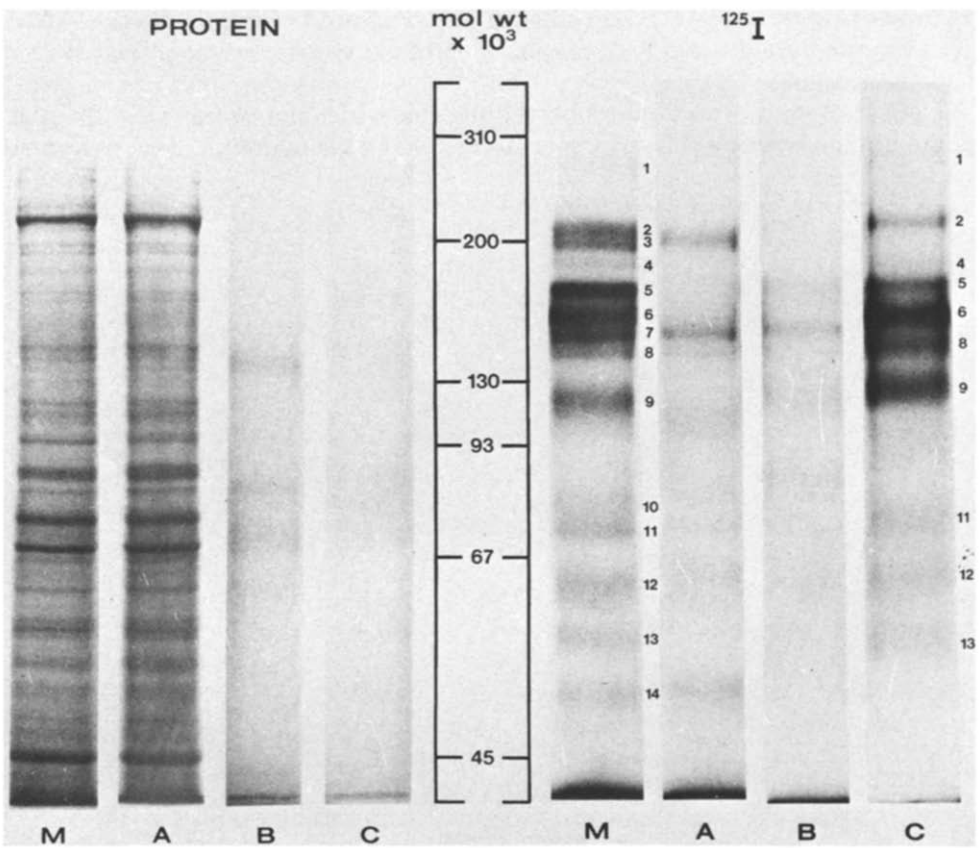

Fig. 10. Sodium dodecyl sulfate gel electrophoretic patterns of ^{125}I-labeled plasma membrane proteins fractionated by affinity chromatography in a wheat germ agglutinin-agarose column. Samples were obtained as described in Fig. 9 and electrophoresed on a 6% acrylamide slab gel. The gel was stained for protein with Coomassie Blue (left) and autoradiographed (right). M, plasma membrane ghosts; A, unbound fraction; B, column wash fraction; and C, *N*-acetylglucosamine fraction.

fate-salts buffer, a second peak of radioactivity emerged which represented wheat germ agglutinin-reactive proteins. The eluted fraction were divided into three groups: A, unbound fraction; B, column wash fraction; and C, wheat germ agglutinin-reactive fraction. Nearly half of the ^{125}I-labeled material (48%) was recovered in the wheat germ agglutinin-reactive fraction C (Table I).

The distribution of protein and ^{125}I-labeled bands in fractions A, B and C as assessed by sodium dodecyl sulfate gel electrophoresis is presented in Fig. 10. All of the major Coomassie Blue staining bands were recovered in fraction A; fractions B and C contained only a few very weak and diffusely stained Coomassie Blue bands. ^{125}I autoradiograms indicated that the iodinated membrane polypeptides fell into three general groups with respect to their wheat germ agglutinin receptor activity. There was a group of iodinated protein represented by bands 3, 7 and 14 which exhibited little or no affinity to the wheat germ agglutinin column and are found almost exclusively in fraction A. On the other extreme, bands 1, 2, 6, 8, 9, 12, 13 and possibly band 4 were firmly bound by the wheat germ agglutinin column and were isolated in the *N*-acetylglucosamine-eluted fraction C. Finally, an intermediate group consisting of bands 5, 10 and 11 demonstrated weak affinity for the wheat germ agglutinin column and were found in both fractions B and C.

Fraction C, which was eluted with *N*-acetylglucosamine, contained between 5 and 10% of the total membrane protein applied to the column as determined by the Lowry procedure [28] and 48% of the ^{125}I label. This represents a yield of about 800 μg of wheat germ agglutinin receptor glycoproteins from 10^9 cells. Little Coomassie Blue-stainable material was present in this fraction except for two weakly stained bands with apparent molecular weights of 165 000 and 210 000 which corresponded to iodinated bands 2 and 6, respectively.

Discussion

Through the use of a variety of techniques we have shown that wheat germ agglutinin can induce an extensive internalization of the lectin-surface membrane receptor glycoprotein complexes on HeLa cells. ^{125}I-labeled wheat germ agglutinin binding studies revealed that much of the cell-bound lectin was apparently irreversibly bound and that this irreversible binding proceeded in a time- and temperature-dependent fashion. Similar results have been obtained from ^{125}I-labeled ricin binding studies with Balb/c 3T3 fibroblasts [13] and Ehrlich ascites tumor cells [12]. Oliver et al. [30] have reported that ^{125}I-labeled ricin and ^{125}I-labeled concanavalin A are rapidly taken up by neutrophils and the endocytosis of these lectins was suggested.

Our studies with fluorescein- and horseradish peroxidase-labeled wheat germ agglutinin confirmed that the lectin had undergone endocytosis. Fluorescein isothiocyanate-conjugated wheat germ agglutinin was observed to concentrate at the perinuclear region of the cell after incubation at 37°C. Intracellular vesicles containing the horseradish peroxidase-wheat germ agglutinin reaction product were also localized in large numbers in this region. Steinman et al. [31] have studied pinocytosis in mouse L cells using horseradish peroxidase as a cytochemical marker. These investigators found that pinocytotic vesicles accumulated at the perinuclear region near the nucleus, where they eventually fused

with each other and with lysosomes. It has also been observed that when L cells internalize latex particles, the phagosomes that are formed will migrate to the perinuclear region and fuse with lysosomes [32]. The appearance of fluorescein isothiocyanate-conjugated wheat germ agglutinin and horseradish peroxidase-wheat germ agglutinin at the perinuclear region of HeLa cells is therefore consistent with the behavior of endocytotic vesicles in other enzymes.

We have used the sensitivity of HeLa cell surface sialoglycoproteins to neuraminidase digestion as a method for detecting the loss of these sialoglycoproteins from the cell surface during lectin-induced endocytosis.

A majority of the ^{125}I-labeled surface polypeptides were shown to be sialoglycoproteins since neuraminidase treatment modified their electrophoretic properties in sodium dodecyl sulfate gels. Most of the alterations observed after neuraminidase digestion were characterized by a moderate increase in electrophoretic mobility, probably reflecting a corresponding decrease in molecular weight. The major glycoprotein of the human erythrocyte plasma membrane, glycophorin, has been reported to undergo a similar increase in electrophoretic mobility after desialation with neuraminidase [21,33]. Segrest et al. [33] attributed this result to the decrease in the molecular size of glycophorin subsequent to the removal of sialic acid residues.

γ-protein was exceptional in that neuraminidase digestion resulted in a dramatic decrease in its electrophoretic mobility. This behavior can be related to studies carried out on the tryptic glycopeptide of glycophorin. Segrest et al. [33] have shown that this glycopeptide, after desialation with neuraminidase, exhibited a similar decrease in mobility and that this behavior was due to the abnormally low binding of sodium dodecyl sulfate by this molecule. ɔrmally

When HeLa cells were incubated with wheat germ agglutinin at 37°C, the surface sialoglycoproteins became resistant to subsequent neuraminidase digestion; *N*-acetylglucosamine treatment failed to reverse this protection. Such a result suggested that cell surface glycoproteins had undergone internalization concurrently with the wheat germ agglutinin. The kinetics of this protective effect paralleled the rates of formation of irreversibly bound ^{125}I-labeled wheat germ agglutinin. At 4°C, wheat germ agglutinin failed to protect the sialoglycoproteins from neuraminidase digestion when the cells were treated with *N*-acetylglucosamine. This temperature dependence of the protective effect mirrored the inability of ^{125}I-labeled wheat germ agglutinin to become irreversibly bound to cells at 4°C. Our morphological observations confirmed that the labeled lectin (fluorescein isothiocyanate-conjugated wheat germ agglutinin or horseradish peroxidase-wheat germ agglutinin) was internalized upon incubation at 37°C but not at 4°C. These results correlate with others indicating that endocytosis is a temperature-dependent process [34].

A variety of experimental evidence suggests that the surface membrane glycoproteins of the HeLa cell are induced to undergo endocytosis by wheat germ agglutinin in a non-selective fashion. This was demonstrated when the ^{125}I-labeled polypeptide profiles of the wheat germ agglutinin-treated cells were compared to those in the purified plasma membrane ghost fraction and in the small vesicle fraction isolated from the wheat germ agglutinin-treated cells. There were no detectable differences in the relative proportions of individual polypeptide species found in the homogenate or in the two membrane frac-

tions. Since we have demonstrated that a majority of the neuraminidase-sensitive sialoglycoproteins have been internalized, we conclude that every ^{125}I-labeled polypeptide species is endocytosed to the same relative extent.

It is of interest to note that nearly all of the ^{125}I-labeled membrane polypeptides possess at least some reactivity with wheat germ agglutinin, as determined by chromatography of solubilized plasma membrane on wheat germ agglutinin-agarose columns.

Iodinated band 3, however, was found to lack any such activity and therefore would not be expected to undergo lectin-mediated endocytosis. Yet this surface polypeptide is not detectably enriched in the plasma membrane ghost fraction isolated from wheat germ agglutinin-treated cells. It is possible that iodinated band 3 is intimately associated with a wheat germ agglutinin-reactive glycoprotein and this complex becomes endocytosed subsequent to wheat germ agglutinin binding. It is just as likely that a random, massive endocytosis of plasma membrane fragments occurs and that both wheat germ agglutinin-reactive and non-reactive surface membrane polypeptides are internalized in proportion to their concentration in the plasma membrane. The ability of concanavalin A to induce a similar protective effect at 37°C, supports the idea that lectin-mediated endocytosis of the cell surface proceeds in a non-specific manner.

There is evidence from another system which indicates that membrane internalization proceeds in a non-selective fashion. Hubbard and Cohn [32], studying the phagocytosis of latex particles by L cells, demonstrated that all of the cell surface ^{125}I-labeled cell surface polypeptides were uniformly endocytosed.

On the other hand, Berlin and colleagues have reported that during phagocytosis by neutrophils, certain amino acid transport sites were specifically excluded from the latex phagosomes [30] while cell surface ricin- and concanavalin A-binding sites were enriched in the endocytosed vesicles [35]. In this case, it would appear that certain cell surface structures can be preferentially selected for internalization.

A well-studied example of what appears to be the selective internalization of a defined surface membrane protein is that of anti-immunoglobulin-induced endocytosis of the B lymphocyte surface membrane immunoglobulin. The B lymphocyte surface immunoglobulin has been shown to undergo pinocytosis while other discrete surface antigens, such as H-2, does not [6]. It is possible, as Cohn and co-workers [36,37] have recently suggested, that the selection of surface receptors which are destined to be internalized may occur during the ligand-induced segregation of receptors into patches and polar caps. Subsequent endocytosis is thereby restricted to the membrane components in the patch or cap region. In the case of the HeLa cells, it is possible that the internalization of cell surface membrane proteins by wheat germ agglutinin proceeds in a non-selective fashion because patching or capping does not occur.

Acknowledgments

We wish to thank Dr. F.H. Ruddle for the HeLa cells and Ms. Rose Schultz for assistance with the electron microscopy. This work was supported in part by American Cancer Society grant BC-75 and by U.S. Public Health Service training grant CA05012. E.S.C. is a U.S. Public Health Service Research Career Professor K06-GM-3070.

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