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ANALYSIS OF LECTIN-SPECIFIC CELL SURFACE GLYCOPROTEIN ON NEUROBLASTOMA CELLS

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The binding sites for the lectins wheat germ agglutinin, *Ricinus communis* agglutinin and concanavalin A on mouse neuroblastoma cell membranes were identified using SDS-gel electrophoresis in combination with fluorescent lectins. *Ricinus communis* agglutinin and wheat germ agglutinin were found to bind almost exclusively to a single polypeptide with an apparent molecular weight of 30 000. Concanavalin A labeled over 20 different polypeptides, most with molecular weights greater than 50 000. However, when the neuroblastoma cells were treated with concanavalin A so as to internalize all the concanavalin A binding sites visible at the level of the fluorescent microscope and the purified plasma membranes analyzed for their concanavalin A binding polypeptides, only four of the 20 glycopolypeptides were missing or significantly reduced in amount. Thus, these four high molecular weight concanavalin A-binding polypeptides appear to be the major cell surface receptors for concanavalin A. Binding studies with iodinated concanavalin A indicated that these polypeptides represented the high affinity concanavalin A binding sites ($K_d = 2 \cdot 10^{-7}$ M). Low affinity concanavalin A binding sites were present on the cell surface after internalization of high affinity concanavalin A binding sites.

Introduction

It has become increasingly evident that cell surface glycoproteins play important roles both in the recognition of extracellular components and other cells and in the regulation of vital cellular processes. One of the most widely used tools in the study of cell surface glycoproteins has been the plant lectins [1–3]. Lectins conjugated to various fluorescent dyes [4], enzymes [5] and macromolecular complexes [6,7] have been used to study the distribution of lectin-specific glycoproteins on cell surfaces by fluorescent microscopy, transmission and scanning electron microscopy. Lectins have also been tagged with radioactive isotopes and used to quantify the number and affinity of binding sites on different cells [8,9]. However, in most labeling studies the membrane glycoproteins with which the lectins interact have not been identified. In other studies the lectin receptors have been characterized to varying extents, but their molecular properties have not been related to their properties as seen in cell surface labeling studies.

In an earlier paper [10], we have shown that labeled receptors for concanavalin A, wheat germ agglutinin and *Ricinus communis* agglutinin on neuroblastoma cells redistribute and become internalized. However, the mode of redistribution of concanavalin A receptors was found to differ markedly from that of wheat germ agglutinin or *R. communis* agglutinin receptors. When a small percentage of the wheat germ agglutinin or *R. communis* agglutinin binding sites were labeled, only the labeled sites appeared to clear from the cell surface. In contrast, when less than 10% of the concanavalin A binding sites were labeled, both labeled and unlabeled concanavalin A sites were removed from the cell surface. This suggested that concanavalin A receptors might reside on different

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membrane components from wheat germ agglutinin and *R. communis* agglutinin receptors and be under different transmembrane control.

In an effort to understand the interactions of lectins with the neuroblastoma cell surface, we have used SDS-gel electrophoresis in conjunction with fluorescent lectin labeling to identify the membrane polypeptides which bind *R. communis* agglutinin, wheat germ agglutinin and concanavalin A. The results indicate that *R. communis* agglutinin and wheat germ agglutinin both bind to a single membrane polypeptide, whereas concanavalin A binds to a large number of membrane glycopolypeptides fractionated on SDS gels. Lectin-induced internalization studies, however, indicate that only a few of these glycopolypeptides serve as high affinity concanavalin A receptors on the surface of neuroblastoma cells.

Materials and Methods

Reagents. Phosphate-buffered saline and Dulbecco's phosphate-buffered saline were prepared as described previously [10]. Tris buffer used in concanavalin A labeling studies consisted of 8.77 g NaCl, 0.11 g $CaCl_2$. 2.42 g Tris adjusted to pH 7.4 with HCl and 0.02 g $MnCl_2$ per liter of distilled water. Buffer A at pH 8.5 contained HCl, 0.58 g NaCl, 1.22 g Tris, 1.02 g $MgCl_2 \cdot 6H_2O$ and 0.11 g $CaCl_2$ per liter of distilled water. Buffer B contained one-third these concentrations. Sucrose solution buffer at pH 7.5 was composed of HCl, 0.06 g Tris, 1.02 g $MgCl_2 \cdot 6H_2O$ and 0.11 g $CaCl_2$ per liter of distilled water.

Cell culture. Mouse neuroblastoma line neuro-2a cells (American Type Culture Collection) were routinely grown in Eagle's minimal essential medium (Gibco) supplemented with fetal calf serum (Gibco) as described [10]. Differentiated cells used for the membrane isolation were prepared by seeding $(2-5) \cdot 10^5$ cells in 100 mm Falcon tissue culture dishes. The cells were allowed to grow for 6–7 days in medium containing 10% fetal calf serum. The medium was then removed and serum-free medium was added [11]. The cells were used between 24 and 48 h later.

Lectin preparations. Ricinus communis agglutinin (molecular weight = 120 000) and wheat germ agglutinin were purified as described [12]. Concanavalin A was purchased from Sigma. Lectins were labeled with either fluorescein isothiocyanate or ^{125}I as previously reported [10]. Fluorescent lectins typically had 495 nm/280 nm absorbance ratios of 0.5–1.0. The specific radioactivity of ^{125}I-labeled concanavalin A was $9.4 \cdot 10^4$ cpm/μg.

Plasma membrane preparation. Neuroblastoma cell plasma membranes were purified by a modification of the method of Garvican and Brown [13]. Differentiated monolayer cells were removed from twenty 100 mm tissue culture dishes by treatment with 0.02% EDTA in phosphate-buffered saline for 30 min at 37°C. Cells were collected at $1\,000 \times g$ for 4 min and resuspended in phosphate-buffered saline. All subsequent operations were carried out at 0–4°C. The cells were washed once in buffer A and once in buffer B. They were then resuspended in buffer B (20 ml per 10^8 cells), allowed to swell for 12–15 min on ice and disrupted by several gentle strokes with the 'B' pestle in a glass Dounce homogenizer. The nuclei were stabilized by the addition of an equal volume of 30% (w/w) sucrose and removed by centrifugation at $480 \times g$ for 30 s. The supernatant was layered onto discontinuous sucrose gradients (four per 10^8 cells) consisting of 5.0 ml 42% (w/w) sucrose and 15 ml 30% sucrose. The gradients were centrifuged for 30 min at $5\,800 \times g_{max}$ in a SW27 Beckman rotor. The material at the 42%/30% interface was harvested, diluted with buffer A and collected at $2\,000 \times g$ for 30 min. The pellet was washed once and resuspended in 1 ml buffer A and 1 ml 30% sucrose. The membrane was then layered onto a 42%–30% linear sucrose gradient formed over 1.5 ml 43% (w/w) sucrose and 1.0 ml 42% sucrose in Beckman SW 27.1 rotor tubes. The gradients were centrifuged overnight (12–14 h) at $130\,000 \times g_{max}$.

The 42%–35% sucrose fraction was removed, diluted with buffer A and sedimented at $2\,000 \times g$ for 30 min. The pellet was resuspended in 1 ml buffer A and 1 ml 30% sucrose and stored at −20°C until needed.

Internalization of concanavalin A receptors. Monolayer cells in 100 mm tissue culture dishes were rinsed two times in concanavalin A buffer and incubated for 1 h at 37°C in 2.0 ml Tris-HCl buffer containing 100–200 μg/ml concanavalin A or ^{125}I-labeled concanavalin A (specific activity = $9.4 \cdot 10^4$). The concanavalin A solution was then removed, the cells were rinsed two times with Tris-HCl buffer and incubated for 1 h at 37°C in 5 ml Tris-HCl buffer. This treat-

ment brought about complete internalization of all visible concanavalin A receptors (i.e., cells subsequently treated with fluorescent concanavalin A did not appear labeled as seen under the fluorescent microscope). The cells were then scraped off the plates and a nuclei-free homogenate prepared as described above. The homogenate was layered onto continuous 42%–30% sucrose gradients formed on top of 2.0 ml 43% sucrose and 1.0 ml 42% sucrose in SW 27 or 27.1 rotor tubes and centrifuged at 5 800 × *g* for 30 min. The SW 27.1 gradients were then fractionated into 30 drop fractions. The 42%/30% interface was removed from the SW 27 gradients, collected at 2 000 × *g* for 30 min, resuspended in 1 ml 30% sucrose and 1 ml buffer A and stored at −20°C until needed.

Enzymatic assays. The following enzymatic activities were assayed by the methods indicated: (Na^+ + K^+)-activated ATPase [14], NADPH-cytochrome *c* reductase [15], UDPgalactose:*N*-acetylglucosamine galactosyltransferase [16], and acid phosphatase using *p*-nitrophenylphosphate as substrate [17].

Analytical methods. Protein was measured by the method of Bradford [18] using bovine γ-globulin as a standard, inorganic phosphate by the method of Bonting et al. [19] and DNA by the method of Burton [20]. Acid-precipitable ^{125}I was determined by precipitating 100-μl samples with 10% trichloroacetic acid. After 1 h on ice, the samples were centrifuged and the pellets were counted in a gamma counter. Monolayer cells were iodinated by the method of Truding et al. [21].

Gel electrophoresis. Plasma membrane samples were dissolved in denaturing buffer (0.015 M Tris-HCl, pH 8.0, 20% sucrose, 5% SDS, 10% β-mercaptoethanol and 0.025% bromophenol blue) to give a concentration of 5–6 μg protein per μl of solution. The samples were run for 8.5 cm on 6 or 10% [22] slab gels. The gels were then sliced longitudinally and either stained for protein with Coomassie blue [23] or treated with fluorescent lectins as described below.

Staining of gels with fluorescent lectins. The gels were stained following a modification of the method previously described [12,24]. Briefly, gel slices were fixed overnight in 25% isopropanol/10% acetic acid, rinsed in distilled water and washed for 4–5 h in several changes of phosphate-buffered saline. The gel slices were then incubated with 0.2 mg/ml fluorescent lectins, in the presence or absence of lectin inhibitors, (0.2 M methyl α-D-mannose for concanavalin A, 0.2 M D-galactose for *R. communis* agglutinin and 0.1 M *N*-acetyl-D-glucosamine, 10 mg/ml ovomucoid for wheat germ agglutinin) washed and photographed under ultraviolet light using Kodak Tri X film and a Wratten 61 barrier filter. The best results were obtained if the gels were labeled with fluorescent wheat germ agglutinin or fluorescent concanavalin A for 3 days and with fluorescent *R. communis* agglutinin for 4–5 days to allow diffusion of lectins into the gels. The intensity of labeling was reduced when less than 0.1 mg/ml fluorescent lectins was used; concentrations greater than 0.3 mg/ml resulted in considerable background fluorescence.

Labeling of cells with ^{125}I-labeled concanavalin A. Cells in 35 mm tissue culture dishes were rinsed three times with Tris-HCl buffer and either treated with 100–200 μg/ml concanavalin A in order to internalize all their visible concanavalin A receptors as described above and then labeled with ^{125}I-labeled concanavalin A (specific activity $9.4 \cdot 10^4$ cpm/mg) or were labeled directly with ^{125}U-labeled concanavalin A. In both cases, the cells on the dishes were treated for 20 min at 23°C with increasing ^{125}I-labeled concanavalin A concentrations (10–300 μg/ml) in a total volume of 250 μl. The plates were washed three times with Tris-HCl and the cells solubilized in 500 μl 1 M NaOH. The solutions were then counted in a gamma counter and the protein concentration of each determined by the method of Lowry et al. [25] using bovine serum albumin as the standard. The high concentration of NaOH prevented the use of the protein determination method of Burton [20]. The data was analyzed by the Scatchard method [26]. All assays were run in duplicate in parallel with controls containing 0.05 M methyl α-D-mannoside.

Lectin competition studies. Neuroblastoma cells suspended in Dulbecco's phosphate buffered saline were incubated at 24°C with increasing concentrations of lectin (0.1–5.0 mg/ml), in a total volume of 90 μl. After 15 min, 10 μl of ^{125}I-labeled lectin (0.25–0.5 mg/ml, specific activity $(1–2) \cdot 10^5$ cpm/μg) was added and the incubation was continued for 30 min. The cells were then washed and counted in a gamma counter.

Results

Purification of neuroblastoma plasma membranes

In this study, plasma membranes were isolated from a homogenate of mouse neuroblastoma cells by a series of differential and sucrose gradient centrifugation steps. Table I shows the total and specific activities of various plasma membrane and intracellular membrane markers determined in the cell homogenate, nuclear pellet, the 30% and 30%/42% interface fractions of the sucrose gradient and the final enriched-plasma membrane.

Both plasma membrane markers, $(Na^+ + K^+)$-ATPase and acid-precipitable ^{125}I [29] showed increases in specific activity in the membrane fraction. An increase in specific activity of these two markers was not seen in either of the two fractions which were discarded. The $(Na^+ + K^+)$-ATPase was enriched by a factor of 10 relative to the homogenate, a value which agrees with that reported by Charalampous [29] for his membrane preparation from the same neuroblastoma cell line. The increase in acid-precipitable ^{125}I specific radioactivity in the membrane fraction and the percent recovery of radioactivity in this fraction are very similar to the values for the $(Na^+ + K^+)$-ATPase. The low recovery and small fractional enrichment of NADPH-cytochrome *c* reductase, the endoplasmic reticulum marker, in the plasma membrane fraction suggests there is only slight contamination of the plasma membrane preparation by this organelle. The lack of detectable succinate-cytochrome *c* reductase activity in the plasma membrane fraction indicates that there is no significant mitochondrial contamination.

Some contamination of the preparation by lysosomes is suggested by the recovery of acid phosphatase activity in the plasma membrane fraction. In addition, 1.1% of the galactosyltransferase activity, a marker for the Golgi apparatus, was also found in the plasma membrane preparation. This value represented a fractional enrichment of 0.7.

The average yield of membrane protein (0.84 mg from 10^8 cells) is similar to that reported by Garvican and Brown [13] for a different neuroblastoma cell line. This value represents 1.5% of the protein present in the homogenate.

SDS-gel electrophoresis

When isolated differentiated neuroblastoma cell plasma membranes were dissociated in SDS and 45 μg of protein run on 10% Laemmli gels up to 30 bands could be seen when the gels were stained for protein with Coomassie blue (Fig. 1). If the gels were loaded with over 200 μg up to 60 bands could be distin-

TABLE I

DISTRIBUTION OF MARKERS IN THE DIFFERENT FRACTIONS RECOVERED DURING MEMBRANE ISOLATION

The different fractions were assayed for the markers as described in Materials and Methods. The top fraction is the top of the discontinuous sucrose gradient and the interface fraction is the 30%/42% interface of the same gradient. For the enzymes: TA, total activity (nmol per min) and SA, specific activity (nmol per min per mg protein). For the acid-precipitable ^{125}I: TA = total radioactivity (cpm $\times 10^{-6}$) and SA = specific radioactivity (cpm per mg protein).

Fraction	$(Na^+ + K^+)$-ATPase		Acid-precipitable ^{125}I		NADPH-cytochrome *c* reductase		Succinate-cytochrome *c* reductase		Acid phosphatase	
	TA	SA	TA	SA	TA	SA	TA	SA	TA	SA
homogenate	258.4	4.6	6.2	113.1	520.0	9.4	115.0	20.9	767.3	13.9
480 × g pellet	78.8	4.8	1.7	101.2	67.2	4.1	64.0	3.9	55.8	3.4
top	46.2	1.8	8.4	33.5	374.0	14.9	401.6	16.0	115.5	4.6
interface	82.0	43.6	1.7	877.7	34.6	18.4	57.3	30.5	15.4	8.2
Membrane	39.4	47.0	7.9	939.3	3.2	3.8	0.0	0.0	7.6	9.0

guished (not shown). Over 80% of these polypeptides had molecular weights between 30 000 and 120 000. If the same membrane preparation was run on a 6% Laemmli gel only six to eight polypeptides with molecular weights greater than 120 000 could be observed (Fig. 2).

Identification of membrane glycoproteins

When the gels of the differentiated neuroblastoma cell membranes were stained with fluorescent lectins, a variety of labeling patterns was seen (Fig. 1). Staining of the gels with fluorescent concanavalin A revealed over 20 polypeptides which appeared to specifically bind the lectin. The majority of these polypeptides had molecular weights greater than 50 000 (Fig. 2). All the concanavalin A-labeled bands also stained with Coomassie blue. Lentil lectin, which has a saccharide specificity very similar to that of concanavalin A, gave the same staining pattern as concanavalin A when gels were treated with fluorescent lentil lectin. Both fluorescent wheat germ agglutinin and fluorescent *R. communis* agglutinin bound almost exclusively to a single polypeptide band with a molecular weight of 30 000. This band also stained strongly with Coomassie blue. At higher plasma membrane protein concentrations several other polypeptides appeared to stain faintly with fluorescent wheat germ agglutinin and/or fluorescent *R. communis* agglutinin. Ricin (molecular weight = 60 000) gave the same staining pattern as *R. communis* agglutinin at all membrane protein concentrations. Fluorescent *Ulex europeus* lectin with a specificity for fucose did not stain gels of plasma membrane proteins. This is in agreement with the finding that fluorescent *U. europeus* lectin did not label neuroblastoma cells when analyzed by fluorescent microscopy (data not shown). Control gels, stained in the presence of a 0.2 M concentration of the appropriate inhibitor,

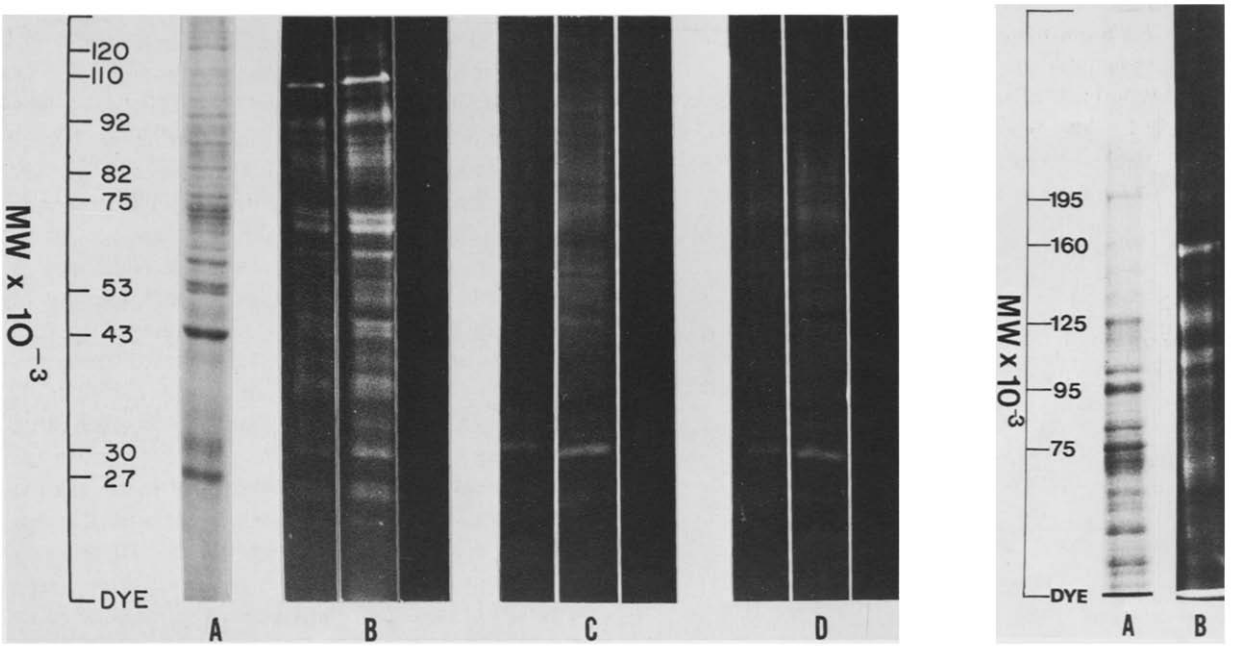

Fig. 1. SDS-gel electrophoresis of purified neuroblastoma plasma membranes on 10% polyacrylamide gels. A. 45 μg membrane protein stained with Coomassie Blue. In B–D fluorescent lectin (0.2 mg/ml) staining of 45 μg of membrane protein (left), 135 μg of membrane protein (center) and 135 μg membrane protein in the presence of the lectin inhibitor (right). B. Fluorescent concanavalin A ± 0.2 M methyl α-D-mannoside, C. Fluorescent *R. communis* agglutinin ± 0.2 M D-galactose and D. Fluorescent wheat germ agglutinin ± wheat germ agglutinin inhibitor (100 mM *N*-acetyl-D-glucosamine, 10 mg/ml ovomucoid).

Fig. 2. SDS-gel electrophoresis of purified neuroblastoma plasma membranes on 6% polyacrylamide gels. 135 μg of membrane protein was applied to each gel and stained with A, Coomassie Blue; B, fluorescent concanavalin A (0.2 mg/ml).

showed no fluorescence in any experiment. In addition, there was never any fluorescence below the dye marker where glycolipids migrate.

When gels of the plasma membrane polypeptides were oxidized with periodic acid and labeled with dansylhydrazine [30] in order to visualize membrane glycoproteins, eight fluorescent bands were observed (not shown). All of these bands corresponded to bands which labeled with fluorescent lectins. A broad fluorescent band below the dye marker was also seen. This appears to represent membrane glycolipids.

Internalization of concanavalin A receptors

When monolayer cells were treated with concanavalin A or ^{125}I-labeled concanavalin A and incubated at 37°C for 1 h, internalization of concanavalin A receptors occurred [10] and the cells could not be relabeled with fluorescent concanavalin A as visualized by fluorescent microscopy. Plasma membrane derived from these cells could be separated from internalized ^{125}I-labeled concanavalin A by centrifugation on a continuous sucrose gradient as shown in Fig. 3.

A single peak of radioactivity and several peaks of

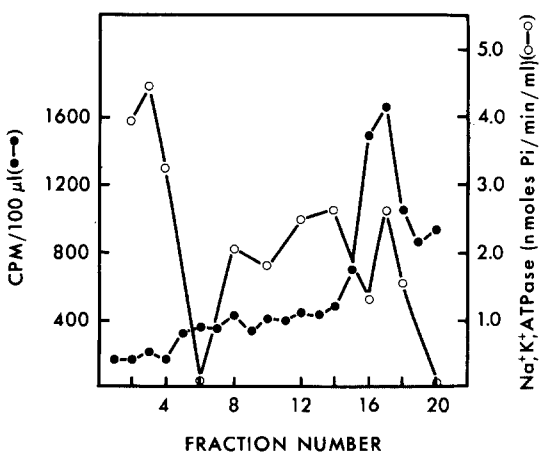

Fig. 3. Analysis of a continuous sucrose gradient containing membranes from cells which have internalized their visible concanavalin A receptors. Cells were treated for 1 h at 37°C with ^{125}I-labeled concanavalin A (100 µg/ml; specific activity = $9.4 \cdot 10^4$ cpm/µg) and subsequently incubated for a second hour at 37°C with buffer. A cell homogenate was prepared and run on the continuous sucrose gradient. The gradient was separated into 20 fractions (0.9 ml each) and aliquots were assayed for $(Na^+ + K^+)$-ATPase activity (○———○) and counted for ^{125}I (●———●).

$(Na^+ + K^+)$-ATPase activity were apparent. The pattern of $(Na^+ + K^+)$-ATPase activity is very similar to that seen when untreated cells were homogenized and run on a similar gradient. The major peak of $(Na^+ + K^+)$-ATPase activity near the bottom of the gradient, which corresponds to the plasma membrane fractions, contains little or no radioactivity whereas a peak of $(Na^+ + K^+)$-ATPase activity near the top of the gradient contains almost all the ^{125}I-labeled concanavalin A radioactivity. However, gradients run with homogenates of cells which had been treated only briefly with ^{125}I-labeled concanavalin A (i.e. under conditions in which no endocytosis of concanavalin A occurred) had peaks of radioactivity which corresponded to each of the peaks of $(Na^+ + K^+)$-ATPase activity.

Analysis of concanavalin A-treated plasma membranes on SDS-polyacrylamide gels

When the plasma membrane fractions from concanavalin A-treated cells were compared to untreated cells by SDS gel electrophoresis a number of differences in the Coomassie blue and fluorescent concanavalin A staining patterns were revealed. Several high molecular weight Coomassie blue-staining bands were decreased or absent in gels of the plasma membrane fractions from concanavalin A-treated cells (Fig. 4A). Several high molecular weight polypeptides which stained with fluorescent concanavalin A in gels of membranes from untreated cells were missing or reduced in amount in gels of membranes from concanavalin A-treated cells (Fig. 4B). Most of these corresponded to the absent Coomassie blue-staining bands. The remainder of the fluorescent concanavalin A staining patterns, however, was very similar for both untreated and concanavalin A-treated plasma membranes. When gels of the upper gradient fractions (fraction numbers 16–20) were run on SDS-gels and treated with fluorescent concanavalin A, the staining pattern was similar to that seen with untreated cells. However, since this fraction also contains small membrane vesicles and fragments not due to the concanavalin A treatment, the origin of the missing concanavalin A binding polypeptides in these fractions is inconclusive.

Lectin competition studies

Competition of the different lectins for the high

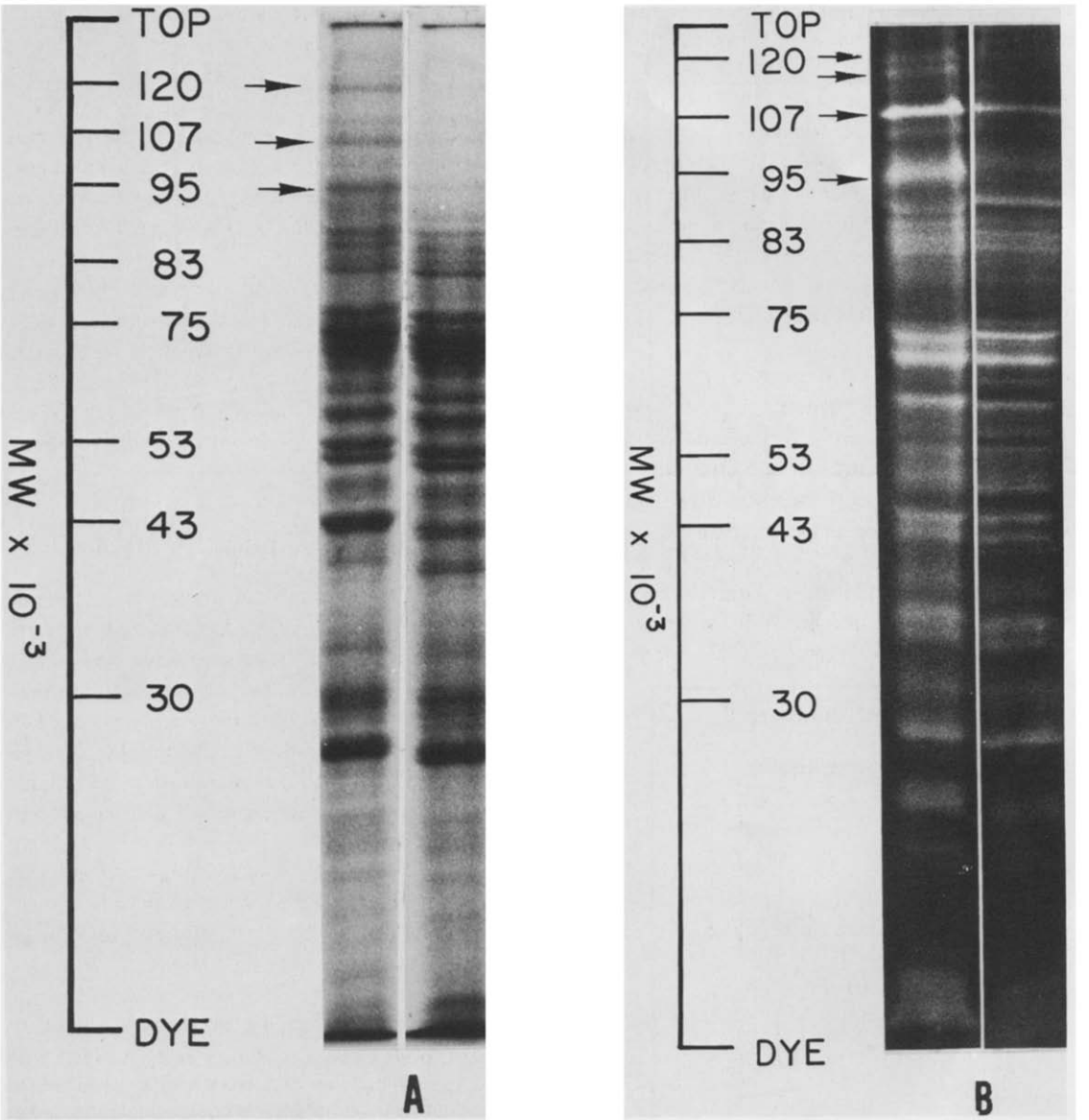

Fig. 4. SDS-gel electrophoresis of purified plasma membranes from untreated and concanavalin A-treated cells. In A and B, untreated membranes are on the left and membranes from cells which had internalized all their visible concanavalin A receptors are on the right. A, 45 μg membrane protein stained with Coomassie Blue; B, 135 μg membrane protein stained with fluorescent concanavalin A. The arrowheads indicate the bands absent or diminished in the gels of the concanavalin A-treated plasma membranes.

affinity ($K_d \sim 10^{-7}$) binding sites [10] on neuroblastoma cells was studied. All three unlabeled lectins were able to reduce the binding of the corresponding ^{125}I-labeled lectin by 80–90% as shown in Fig. 5. This is in agreement with the reduction in lectin binding observed when the appropriate lectin inhibitor is used. Concanavalin A, on the other hand, had no effect on the binding of ^{125}I-labeled wheat germ agglutinin and, similarly, wheat germ agglutinin did not affect ^{125}I-labeled concanavalin A binding. *R.*

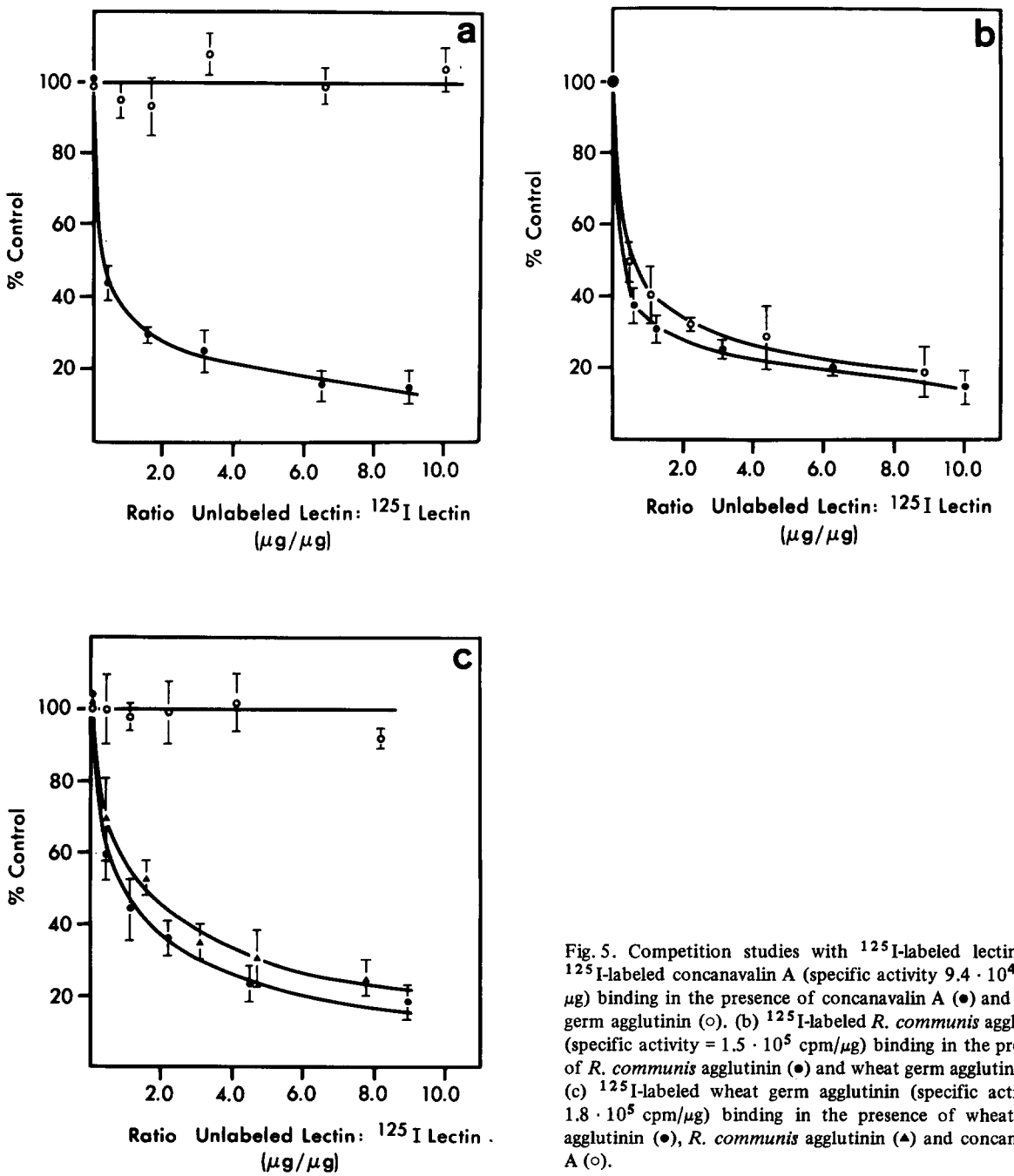

Fig. 5. Competition studies with ^{125}I-labeled lectins. (a) ^{125}I-labeled concanavalin A (specific activity $9.4 \cdot 10^4$ cpm/μg) binding in the presence of concanavalin A (•) and wheat germ agglutinin (○). (b) ^{125}I-labeled *R. communis* agglutinin (specific activity = $1.5 \cdot 10^5$ cpm/μg) binding in the presence of *R. communis* agglutinin (•) and wheat germ agglutinin (○). (c) ^{125}I-labeled wheat germ agglutinin (specific activity = $1.8 \cdot 10^5$ cpm/μg) binding in the presence of wheat germ agglutinin (•), *R. communis* agglutinin (▲) and concanavalin A (○).

communis agglutinin, however, strongly inhibited ^{125}I-labeled wheat germ agglutinin binding and wheat germ agglutinin inhibited ^{125}I-labeled *R. communis* agglutinin. Competition studies between *R. communis* agglutinin and concanavalin A could not be carried out since concanavalin A binds to carbohydrate residues on *R. communis* agglutinin [12].

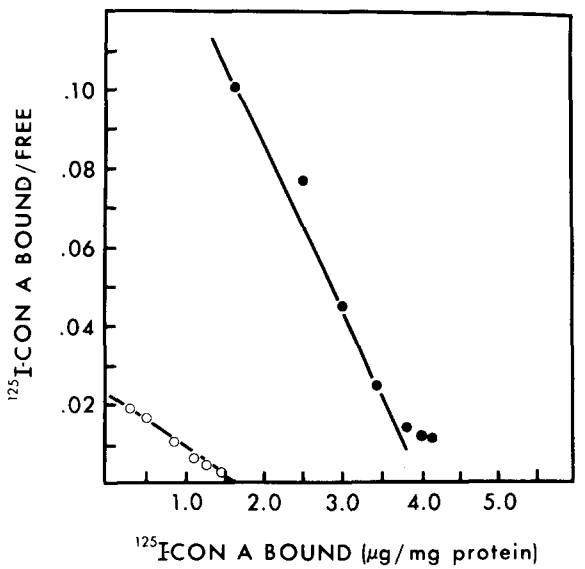

Fig. 6. Scatchard analysis of the binding of ^{125}I-labeled concanavalin A to neuroblastoma cells. Cells were labeled for 30 min at 23°C with the lectin, washed three times in buffer and solubilized in 500 μl 1 N NaOH. Aliquots were taken for radioactivity counting and protein assays. ^{125}I-labeled concanavalin A binding to untreated (•———•) and to cells which were pre-treated with concanavalin A so as to internalize all their concanavalin A receptors (o———o).

^{125}I-labeled concanavalin A binding to untreated and concanavalin A-treated cells

The binding of ^{125}I-labeled concanavalin A to neuroblastoma cells grown on tissue culture dishes is shown in the form of the Scatchard plot in Fig. 6. For untreated cells a major class of sites (approx. $2 \cdot 10^7$ sites) having an apparent dissociation constant (K_d) $2 \cdot 10^{-7}$ M was found. When ^{125}I-concanavalin A binding studies were carried out on cells which had been pretreated with concanavalin A under conditions in which concanavalin A receptors become internalized, a smaller number of sites having a lower apparent K_d of $8 \cdot 10^{-7}$ M was observed.

Discussion

Based on the data from the assays for the different marker enzymes, the membrane preparation appears to have little contamination by other cellular components. The major source of impurities in the preparations are the Golgi apparatus and the lysosomes. However, both acid phosphatase, the lysosomal marker, and galactosyltransferase, the marker for the Golgi apparatus, have been reported to be present in the plasma membranes of several different cell types as well as in their respective organelles [13,31,32]. Thus, the recovery of these enzyme activities in the isolated plasma membrane may be a reflection of their presence in the plasma membrane as well as an indication of a small amount of contamination of the plasma membrane preparation by these two organelles.

The recoveries of the two plasma membrane markers in the plasma membrane fraction are similar to that reported for two other plasma membrane markers (5′-nucleotidase and [^{3}H]fucose) used with the same membrane isolation technique, but on another neuroblastoma cell line [13]. Similar recoveries of plasma membrane marker activities also have been found with several other cell lines when the same type of membrane purification was used [28,33]. The major losses of plasma membrane appear to be due to the trapping of some membrane material in the nuclear pellet and to the formation of small membrane vesicles during the homogenization. These vesicles collected in the upper portions of both the discontinuous and continuous sucrose gradient along with other cellular components. The vesicles appeared to represent the entire cell membrane since SDS-polyacrylamide gels of this fraction gave a Coomassie blue staining pattern very similar to that of the enriched plasma membranes. In addition, autoradiograms of gels run on the plasma membrane and vesicle fractions of iodinated cells were identical.

The general molecular weight distribution of the Coomassie blue-staining membrane polypeptides is similar to that seen by the other groups who have analyzed neuroblastoma cell membrane polypeptides by SDS-gel electrophoresis [13,21,29,34]. In all cases the majority of proteins had apparent molecular weights greater than 50000 and less than 150000. Several Coomassie blue bands can be tentatively identified. The major band at 43 kdaltons comigrates with chicken gizzard actin and is probably actin. HeLa cell plasma membranes have also been found to contain fairly large quantities of actin [33]. The band in the range of 180 kdaltons on the 6% gels comigrates with myosin. The double bands at 54 kdaltons have been identified in another study [34] as tubulin.

Fluorescent lectin labeling of neuroblastoma plasma membrane proteins separated on SDS-gels indicated that *R. communis* agglutinin and wheat germ agglutinin both bind to a 30-kdalton polypeptide. The view that ricinus communis agglutinin and wheat germ agglutinin bind to the same 30-kdalton polypeptide is supported in lectin competition studies which showed that wheat germ agglutinin inhibits the binding of *R. communis* agglutinin to cells and vise versa in microscopic studies [10] which revealed that *R. communis* agglutinin and wheat germ agglutinin receptors on neuroblastoma cells redistributed in a similar manner.

The staining pattern for fluorescent concanavalin A was considerably more complex than that for wheat germ agglutinin and *R. communis* agglutinin. Fluorescent concanavalin A labeled over 20 polypeptides many of which had molecular weights greater than 50 000. This wide array of concanavalin A-specific polypeptides would not appear to be consistent with the coordinate redistribution and internalization of cell surface concanavalin A as seen by fluorescent microscopy since this would suggest that all the different concanavalin A-specific polypeptides are directly or indirectly linked to one another.

We further investigated this by comparing plasma membrane proteins of concanavalin A-labeled cells which had been induced to internalize all high affinity cell surface concanavalin A receptors with plasma membrane proteins of untreated cells. SDS-gel staining pattern indicated that only a few high molecular weight polypeptides were reduced or missing as a result of concanavalin A-induced internalization. Thus, concanavalin A labeling of cells causes a selective internalization of a relatively small number of polypeptides, at least four of which contain binding sites for concanavalin A.

These experiments further indicate that most of the polypeptides which stain with fluorescent concanavalin A on SDS-gels are for the most part not labeled with concanavalin A at the cell surface. This may be due to steric inaccessibility of the lectin binding sites on the membrane bound glycoproteins. The four concanavalin A specific glycopolypeptides which are lost as a result of internalization appear to contain the major high affinity cell surface concanavalin A binding sites.

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