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ISOLATION OF A DOMAIN OF THE PLASMA MEMBRANE IN CHINESE HAMSTER OVARY CELLS WHICH CONTAINS IODINATABLE, ACIDIC GLYCOPROTEINS OF HIGH MOLECULAR WEIGHT

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Summary

A light vesicle fraction, apparently derived from the plasma membrane, was obtained following breakage of Chinese hamster ovary (CHO) cells by means of a fluid pump disrupting device. The final preparation was enriched approx. 40-fold over the homogenate in K^+,Na^+-stimulated ATPase and phosphodiesterase I, but only approx. 10-fold in ^{125}I specific radioactivity after lactoperoxidase-catalyzed iodination. This preparation was compared with another plasma membrane fraction purified as large sheets via a two-phase centrifugation procedure. Two-dimensional polyacrylamide gel electrophoresis followed by Coomassie blue staining indicated that both fractions were fairly similar in polypeptide composition, although a few consistent differences were evident. However, staining of glycoproteins by the periodic acid-Schiff technique or by overlaying with ^{125}I-labeled concanavalin A showed that the vesicle fraction was highly enriched in groups of high molecular weight, acidic glycoproteins which stain only weakly with Coomassie blue. These glycoproteins also bound ^{125}I-labeled ricin I agglutinin and wheat germ agglutinin. They appear to be the major receptors for wheat germ agglutinin on the CHO cell surface. After surface labeling of cells by the ^{125}I-lactoperoxidase technique, the membrane sheet fraction contained a large number of iodinated polypeptides, whereas labeling in the vesicle fraction was restricted almost entirely to the high molecular weight, acidic glycoproteins. It is proposed that the vesicle fraction constitutes a specific domain of the cell surface which is coated on its exterior by this group of glycoproteins. These components probably mask underlying proteins of the plasma membrane from external labeling.

Introduction

It has been emphasized repeatedly that during plasma membrane isolation procedures, the method chosen to break cells can have a profound influence on the nature of the final preparation [1]. Because the plasma membranes of cultured mammalian cells are unlikely to be uniform throughout their surface, it seems probable that different breakage techniques might be employed to isolate particular functional domains [2—5]. For example, nitrogen cavitation breakage of thymocytes provides two right-side-out populations of plasma membrane vesicles which differ in their enzymatic composition and in the nature of their ability to bind the lectin concanavalin A [5]. One method that has been used extensively to disrupt lymphocytes is to extrude cells under pressure through a small orifice occupied by a spring-loaded ball [6,7]. This procedure leads to a minimal amount of nuclear disruption and a very high recovery and enrichment of certain putative marker enzymes for the plasma membrane in a light vesicle fraction. In this paper, we describe the application of this technique to CHO cells and indicate that it provides a fraction highly enriched in a group of acidic plasma membrane glycoproteins of high molecular weight. These glycoproteins, which stain only faintly with Coomassie blue, are externally disposed and bind the lectins concanavalin A, wheat germ agglutinin and ricin I agglutinin. A preliminary communication of these results has appeared [8].

Materials and Methods

Chemicals. Sodium dodecyl sulfate (SDS; specially pure) was the product of British Drug House (Poole, Dorset U.K.). Acrylamide, *N,N'*-dimethylenebisacrylamide and dithiothreitol were from Eastman-Kodak. Diallyltartardiamide was purchased from BioRad Labs. Nonidet P-40 was purchased from Particle Data Laboratories (Elmhurst, IL, U.S.A.). Ampholines were the product of LKB. Concanavalin A was obtained from Miles Laboratories, Inc. (Elkhart, IN, U.S.A.). $Na^{125}I$ (carrier free) was a product of the Amersham Corp. Sepharose 6B was from Pharmacia Fine Chemicals (Uppsala, Sweden). Lactoperoxidase, hemoglobin, and α-methyl mannoside were obtained from Sigma Chemical Co. *N*-Acetyl-D-glucosamine-Sepharose ('Selectin 1'), urea and chloroglycoluril (Iodogen) were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). Fetuin (Spiro method) was purchased from GIBCO. All reagents for electron microscopy were obtained from Polysciences, Inc. (Warrington, PA, U.S.A.). Ricin I agglutinin was from Vector Labs. Wheat germ agglutinin was purified on *N*-acetyl-D-glucosamine-Sepharose according to published procedures [9].

Cells. Chinese hamster ovary cells, derived from a proline negative clone, K-1, of Kao and Puck [10], were grown in glass or plastic roller bottles using McCoy's 5A medium supplemented with 10% (v/v) fetal calf serum [11,12]. Cells were removed from the culture vessel with calcium- and magnesium-free phosphate-buffered saline containing 0.02% (w/v) Na_2EDTA.

Membrane isolation. Two methods for isolation of plasma membranes were utilized. In the first [12] cells were swollen in 20 mM borate buffer, pH 9.2, containing 0.2 mM EDTA and subjected to gentle Dounce homogenization.

The membrane sheets generated by this procedure were collected by centrifugation and resuspended in a two-phase polymer system consisting of poly(ethyleneglycol) and dextran. Following centrifugation (1100 × *g*, 15 min) the membrane sheet fraction was collected from the resultant interface between the two phases [13].

In the second method [7,14], cells were suspended at a concentration of $5 \cdot 10^7$/ml in Tris-buffered saline (0.05 M Tris-HCl, pH 7.4, containing 0.15 M NaCl) and disrupted at 200 lb/inch^2 back pressure in a Stansted fluid pump equipped with a Model 716 valve (Stansted Fluid Power, Essex, U.K.). The preparation was centrifuged at 300 × *g* for 10 min to remove unbroken cells, nuclei and large membrane sheets. The supernatant fraction was centrifuged first at 4000 × *g* for 15 min to remove mitochondria, and again at 30 000 × *g* for 45 min. The resultant membrane pellet was resuspended in 10 mM Tris-HCl, pH 7.4, and homogenized 3—5 strokes with a loose fitting Dounce homogenizer. After centrifugation at 30 000 × *g* for 45 min, the membranes were resuspended in 25% (w/v) sucrose containing 10 mM Tris-HCl, pH 7.4. The membranes were overlayed on a 40% sucrose cushion adjusted to pH 7.4 (10 mM Tris-HCl), and centrifuged in a Beckman SW 27 rotor at 71 000 × *g* (av.) for 3 h. The membrane vesicles at the interface between the 25 and 40% sucrose layer were removed, diluted with 10 vols. of 10 mM Tris-HCl, pH 7.4, and centrifuged at 30 000 × *g* for 45 min. The denser, yellowish fraction which passes through the 40% sucrose layer on the gradient is believed to be enriched in endoplasmic reticulum [7]. This was also collected as above.

Membranes were solubilized and subjected to two-dimensional electrophoresis as described elsewhere [11,15].

Lectin staining. Lectins were labeled with ^{125}I either by the lactoperoxidase procedure [16] or using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (chloroglycoluril) [17] and desalted on Biogel P-2 columns. Wheat germ agglutinin was purified on *N*-acetyl-D-glucosamine-Sepharose [9]; concanavalin A by adsorption on Sephadex G-100, followed by elution with 0.5 M D-glucose and ricin I agglutinin by adsorption on Sepharose 6B followed by elution with 0.5 M D-galactose. Labeled, purified lectins (approx. 10^8 cpm/mg) were stored at −20°C. Slab gels were stained with ^{125}I-lectins as described elsewhere [15], dried and examined by autoradiography using Kodak XRP-1 film and X-Omat processing.

Control gels for concanavalin A were incubated as described earlier [14,15] in the presence of 0.5 M α-methyl mannoside and washed in Tris-buffered saline containing 0.5 M D-glucose and 0.01% (w/v) sodium azide. For ricin I agglutinin 0.5 M D-galactose was used as the competing hapten. Control gels for wheat germ agglutinin were incubated in the presence of 20 mg fetuin glycopeptides prepared according to the method of Spiro [18], and washed in three changes of Tris-buffered saline (300 ml) containing 0.01% sodium azide and 2 mg fetuin glycopeptides, followed by buffer alone.

Iodination of cell surface components. CHO cells were labeled with ^{125}I by the lactoperoxidase procedure. Cells in roller bottles were washed with phosphate-buffered saline and iodinated in situ using 25 ml phosphate-buffered saline containing 5 mg lactoperoxidase/0.5% (w/v) glucose/10^{-8} M potassium iodide/1—2 mCi Na^{125}I. The reaction was initiated by addition of 20 μl 0.06%

(v/v) hydrogen peroxide; subsequent additions of this reagent were made at 1 min intervals for 15 min. Thereafter, labeling reagent was decanted, the cells were washed with phosphate-buffered saline containing 10^{-3} M potassium iodide and removed as described above.

Total membrane proteins were labeled with ^{125}I by modification of the glycoluril method [17]. Membranes containing about 50 μg protein were suspended in 9.5 M urea/2% Nonidet P-40/5 mM K_2CO_3. After thorough mixing, the clear preparation was added to a freshly prepared glass tube containing 10 μg chloroglycoluril which had been dissolved in 100 μl $CHCl_3$ and dried under a stream of nitrogen. The labeling reaction was initiated by the addition of 500 μCi $Na^{125}I$ and allowed to proceed at room temperature for 25 min with intermittent shaking. The iodination was terminated by decanting the mixture; KI was then added to a final concentration of 0.25 M.

The specific activity of ^{125}I labeled preparations was determined following precipitation of cytosol or solubilized membrane fractions in the presence of 2% (v/v) Nonidet P-40 with an equal volume of cold, 50% (w/v) trichloroacetic acid. After centrifugation (400 $\times g$ for 15 min), the resulting pellets were washed with 5% (w/v) trichloroacetic acid and centrifuged again. The final pellet was counted in a Packard 5130 autogamma spectrometer and then dissolved in 0.1 ml 0.1 M NaOH prior to protein determination (see below).

Enzyme assays. Cell homogenates and isolated membrane fractions were assayed for various marker enzymes by the following spectrophotometric methods. The $(Na^+ + K^+)$-ATPase was assayed by the method of Brunette and Till [13]. Alkaline phosphodiesterase I (EC 3.1.4.1) was monitored according to Roberts and Yuan [12] using *p*-nitrophenyl 5′-thymidylate as the substrate. Alkaline phosphatase (EC 3.1.3.9) was assayed using *p*-nitrophenylphosphate as the substrate [12]. Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Veegar et al. [19], while NADH cytochrome *c* reductase (EC 1.6.2.1) was monitored by the procedure of Mackler and Green [20]. Leucine aminopeptidase (EC 3.4.11.1) activity was measured at 37°C as described [21]. Lactate dehydrogenase (EC 1.1.1.27) was assayed as described by Kornberg [22]. Nucleoside diphosphatase (EC 3.6.1.6) was quantitated by the procedure of Plaut [23]. Inorganic phosphate was quantitated by the procedure of Bartlett [24]. The specific activity of all enzymes releasing inorganic phosphate is expressed as μmol phosphate liberated/min per mg protein. All other specific activities are expressed as μmol substrate (or co-factor) consumed/min per mg protein.

Protein determinations. Protein was determined by a modification [12] of the procedure of Lowry et al. [25], using bovine serum albumin as standard.

Scanning electron microscopy. Suspended CHO cells, before and after disruption in the fluid pump, were fixed in 4% (w/v) glutaraldehyde buffered to pH 7.2 with phosphate-buffered saline for 1 h at room temperature. Following fixation, the cell preparations were washed three times in the same buffer, centrifuged (100 $\times g$ for 3 min), collected in membrane filters (0.45 μM; Amicon Corp.) and processed for scanning electron microscopy as described by Noonan and Ukena [26]. The fixed cells were osmicated, dehydrated through graded ethanol rinses, dried in a Sorvall critical point drying apparatus, then coated with gold and palladium in a Technics, Inc. Hummer

II sputtering system. Cells were viewed in a ETEC Omniscan scanning electron microscope.

Transmission electron microscopy. Membrane vesicles were fixed in 2.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2.5 mM $CaCl_2$ for 15 min at room temperature. Fixed vesicles were then washed in cacodylate buffer three times by gentle centrifugation and post fixed in 1.3% osmium tetroxide and 0.6% glutaraldehyde in cacodylate buffer for 15 min at room temperature. Following three washings, the vesicles were stained for 15 min at room temperature in 0.25% (w/v) uranyl acetate in 0.1 M sodium acetate, pH 5.0. After three more washings in distilled water, the vesicles were dehydrated in a graded series of ethanol solutions and then embedded in Spurs' low viscosity medium and sectioned. Silver sections were examined in a JEOL-100CX transmission electron microscope at 80 kV.

Results

Isolation of membranes

Whole cells (Fig. 1A) were subjected to disruption at 200 lb/inch^2 back pressure in the Stansted pump, a procedure which led to breakage of approx. 90% of the cells as monitored by trypan blue exclusion. As with lymphocytes, the nuclei of the broken CHO cells retained a thick layer of cytoplasm around their perimeter (Fig. 1B). Few nuclei were disrupted by the procedure.

Scanning electron microscopy of the preparation after breakage revealed some whole cells, isolated nuclei and rounded structures larger than nuclei which we interpret to be cells stripped of their plasma membranes (Fig. 1C). In addition, large sheets of membrane were present. Because of the method whereby the broken cells were collected (on filters with pore size 0.45 μM), small vesicles (see below) would not be retained. Subsequent purification of the membrane vesicles resulting from pressure pump breakage by means of differential and sucrose density gradient centrifugation yielded a whitish, vesicle fraction which banded at the 25—40% sucrose interface and a heavier, creamy yellow fraction which passed through the 40% sucrose layer and was collected as a pellet at the bottom of the tube. After density gradient centrifugation in a continuous (20—50% w/w) sucrose gradient, the lighter fraction gave a single band at a density corresponding to 1.11 g/cm^3. When examined in thin section by electron microscopy, this fraction was shown to consist of a fairly uniform population of vesicles bearing an internal 'fuzz' of fibrillar material (Fig. 1D).

Degree of purification

The recovery of protein and the relative specific activities of a number of marker enzymes in the fractions obtained from the broken cells is illustrated in Table I for one particular experiment in which 200 lb/inch^2 back pressure was employed for disruption. In addition, the ^{125}I-specific radioactivity after lactoperoxidase-catalyzed iodination of the unbroken cells has been followed through the isolation procedure. The final vesicular preparation contained 0.4% of the original amount of protein and exhibited a greatly enhanced specific activity (approx. 40-fold) of $(Na^+ + K^+)$-ATPase and alkaline phosphodiesterase I which are believed to be largely localized in the plasma membrane [27—

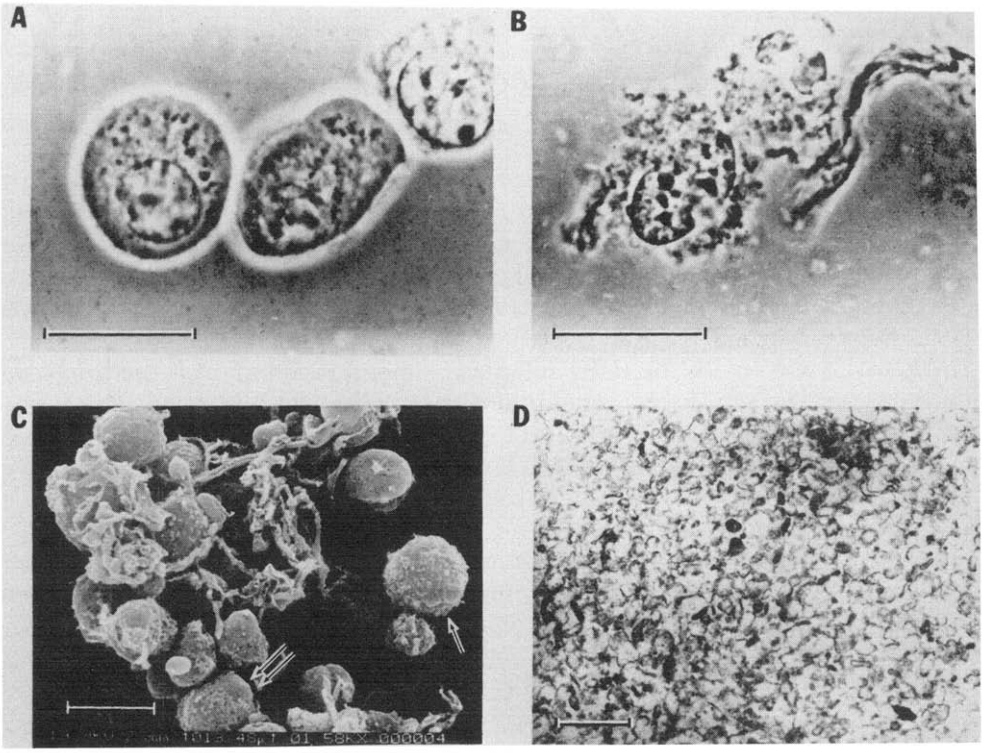

Fig. 1. A. Phase contrast micrograph of suspended CHO cells before disruption in the fluid pump. Bar indicates 13.5 μM. B. Phase contract micrograph of CHO cells after disruption in the fluid pump. Cells appear as a nucleus surrounded by cytoplasm. Furled sheets of membrane, presumably of surface origin, are also evident. Bar indicates 13.5 μM. C. Scanning electron micrograph of CHO cells after disruption in the fluid pump. Unbroken cells (single arrows) can be recognized by the presence of surface blebs and microvilli, while nuclei with attached cytoplasm (double arrow) are more frequent. Some tubular furls of membrane-like material are also evident and may be equivalent to the material seen in Fig. 1B. Bar indicates 13.5 μM. D. Transmission electron micrograph of a section from CHO cell vesicle preparation after pressure disruption and purification by differential and sucrose density gradient centrifugation. Following purification, vesicles were prepared for sectioning and electron microscopy as described in Materials and Methods. Bar indicates 1 μM.

TABLE I

ENRICHMENT OF MARKER ENZYMES IN CELL FRACTIONS AFTER FLUID PUMP DISRUPTION OF CHO CELLS

1 unit of enzyme activity is defined as that amount of enzyme which will catalyze 1 μmol of substrate to product per min under the assay conditions (see Materials and Methods). Although 5′-nucleotidase could be detected in the homogenate (approx. 0.01 units/mg protein), no activity could be detected in the purified vesicle fraction using standard colorimetric procedures.

	Fraction					
	Homogenate		25—40% Sucrose interface (plasma membrane)		40% Sucrose pellet (endoplasmic reticulum)	
Total protein mg (%)	313	(100)	1.3	(0.4)	0.6	(0.2)
^{125}I Labeled proteins cpm/μg (-fold)	560	(1.0)	8476	(15.1)	4125	(7.4)
Leucine aminopeptidase 10^6 X units/mg (-fold)	33.1	(1.0)	350	(10.6)	76.9	(2.3)
Alkaline phosphodiesterase I 10^3 X units/mg (-fold)	0.90	(1.00)	35.9	(40.0)	5.39	(6.0)
($Na^+ + K^+$)-ATPase units/mg (-fold)	0.22	(1.0)	8.3	(37.4)	2.8	(12.6)
NADH-Cytochrome *c* reductase units/mg (-fold)	240	(1.0)	567	(2.3)	455	(1.9)
Lactate dehydrogenase units/mg (-fold)	488	(1.0)	35.3	(0.07)	36.9	(0.08)

30]. By contrast, leucine aminopeptidase, which is also believed to an ectoenzyme on the cell surface [31,32], was enriched only about 11-fold over the homogenate. Contamination by soluble enzymes of the cytoplasm (lactic dehydrogenase) appeared minimal. Succinate dehydrogenase activity was not detectable in the light vesicle fraction (data not shown). The specific activities of various other enzymes were not greatly altered compared to the homogenate, e.g. inosine diphosphatase (1.2-fold) and alkaline phosphatase (1.0-fold). The increase in specific ^{125}I radioactivity of the vesicle fraction in this experiment was approx. 10-fold over the broken cell preparation.

In previous work with the CHO cell plasma membrane [11,12], the breakage technique that we employed generated a membrane sheet fraction which was purified by centrifugation in a poly(ethylene glycol)-dextran gradient. It consisted of approx. 3% of the total cell protein. In the rest of this study we compared the properties of this sheet fraction with the light vesicle fraction.

Two-dimensional electrophoresis of soluble polypeptides

Polypeptides from both membrane preparations were solubilized in alkaline urea solution and analyzed by two-dimensional electrophoresis [11,14] (Fig. 2A and B). In both preparations, the majority of the polypeptides have molec-

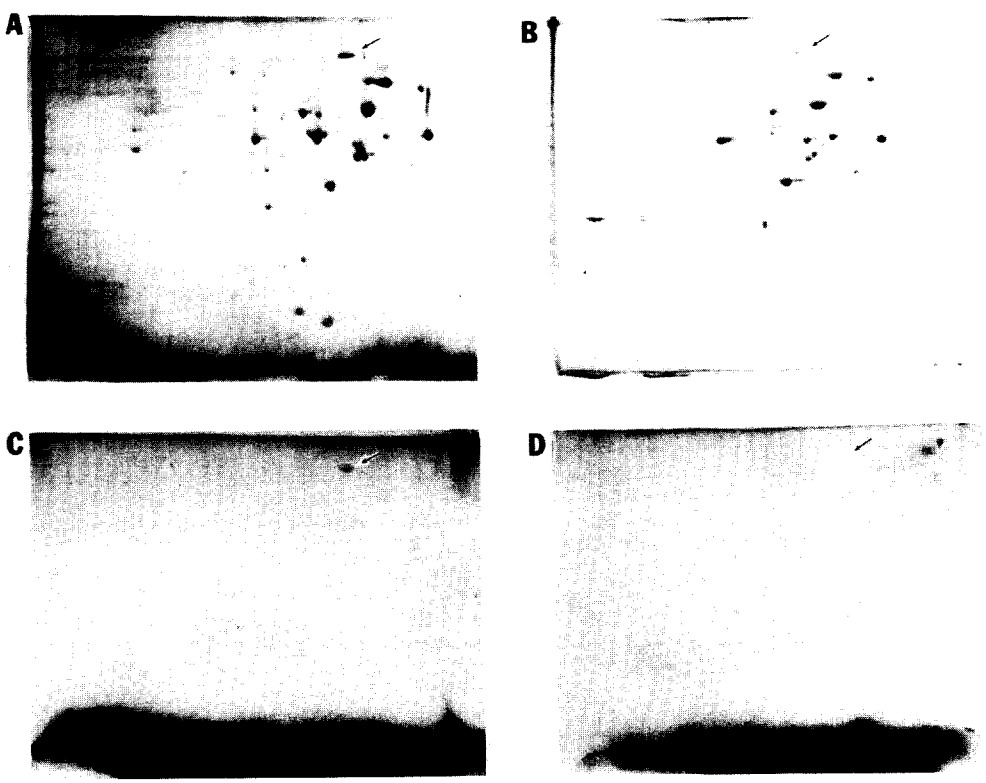

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis of CHO cell plasma membrane polypeptides. A and C are membrane vesicle fractions, while B and D are membrane sheet preparations. Proteins were detected by Coomassie blue staining (A and B) while the presence of carbohydrate was demonstrated by the periodic acid-Schiff procedure (C and D). Spot D_2 is arrowed.

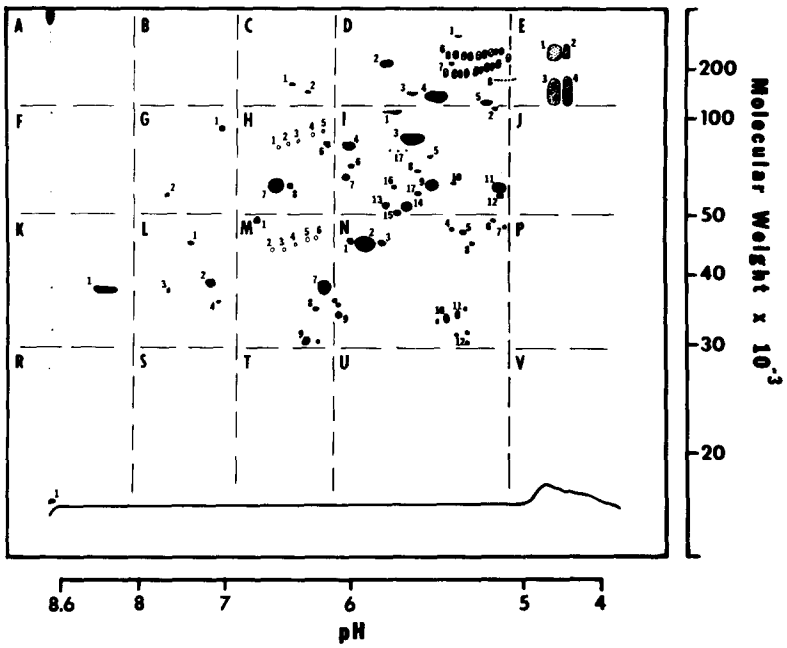

Fig. 3. Composite map of CHO cell plasma membrane polypeptides (vesicle fraction). The map is divided arbitrarily into alphabetically labeled grids with spots in each square designated by a number. The approx. pH gradient is shown on the horizontal axis at the bottom of the diagram and the molecular weight scale as a vertical line on the left.

ular weights between 17 000 and 200 000 and apparent isoelectric points between pH 4.5 and 7.0.

In order to aid identification of spots we have also constructed a diagramatic map to include the major Coomassie blue staining polypeptides from the vesicle membrane preparation (Fig. 3). The map has been divided arbitrarily into a number of alphabetically labeled rectangles based on p*I* (vertical lines) and molecular weight (horizontal lines). Polypeptides within each rectangle have been assigned numbers.

By comparison of many gels, it has become evident that the sheet and light vesicle fractions contained many major polypeptides in common, although their proportions appeared to vary. The similarities have been confirmed in mixing experiments. In these, a vesicle fraction membrane preparation was iodinated by the chloroglycoluril technique after solubilization. A small amount of this (1—10 μg containing approx. 100 000 dpm) was then mixed with a large amount of solubilized sheet fraction protein (250 μg). After electrophoresis, it was then possible to compare the autoradiograph of the dried gel with the Coomassie blue stain pattern. A few major polypeptides which are unequivocally present in the one preparation but not usually in the other have been detected. We have not attempted to catalog differences in components which stain weakly with Coomassie blue. However, certain spots are invariably absent from the vesicle fraction (e.g. spots J_3 and J_7 in Ref. 11), while the sheet fraction lacks K_1 and M_7 shown in Fig. 3. These experiments suggest that

although distinct differences in composition do exist between the fractions, a common group of polypeptides is present in both.

In order to determine which of the many components might be glycoproteins, gels were stained by the periodic acid-Schiff technique [33]. Because of the relative insensitivity of this method on slab gels, it is only effective if more than 0.5 mg total protein can be analyzed on a single gel. In the sheet fraction (Fig. 2C), the most intensely stained spot was D_2. In addition, faint staining was detected in D_4, D_5, I_5, I_7, I_{13} and in either N_2 or a spot which migrates close to that polypeptide. The gel for the vesicle fraction (Fig. 2D) was loaded less heavily. Nevertheless, it is clear that the stain was localized most intensely in the upper right hand corner of the gel in a group of acidic, high molecular weigh components. Several other spots including D_2 and those others noted in the sheet fraction were stained very faintly, and have photographed poorly.

^{125}I-Lactoperoxidase-catalyzed iodination

When ^{125}I labeled vesicle fraction was analyzed by two-dimensional electrophoresis and subjected to autoradiography, the major labeled species detectable after short-term exposure (6 h to 12 h) of the autoradiographs were the high molecular weight, acidic components in quadrants D and E (Fig. 4A). The pattern was very similar to that obtained after ^{125}I-labeled wheat germ agglutinin staining as described in a later experiment. After much longer exposure (24—47 h) of the vesicle fraction autoradiograph, additional labeled components were detected (Fig. 4B).

The iodination pattern of the sheet fraction after short term exposure (6—12 h) was much more complex (cf. Fig. 4C and A). Although traces of polypeptides D_6, D_7, and E_1—E_4 were detectable, at least 30 other spots had a similar or greater intensity. These included D_2, D_4, D_5, I_1, I_8 and I_{14}, all of which are detectable by Coomassie blue staining on the gels of the sheet fraction.

Staining of gels by ^{125}I-labeled concanavalin A

Gels were overlaid with ^{125}I-labeled concanavalin A and, after extensive washing, autoradiographs prepared of the dried gels (Fig. 5). Radioactive spots are assumed to represent glycoproteins containing either α-mannosyl or, more

Fig. 4. Autoradiographs of two-dimensional electrophoretic gels of CHO cell membrane polypeptides after lactoperoxidase-catalyzed surface iodination. The membrane vesicle fraction is shown after 6 h (A) and 20 h (B) exposure. A membrane sheet preparation is shown (C) after a 6 h exposure. Approx. 250 μg of protein was loaded on each gel.

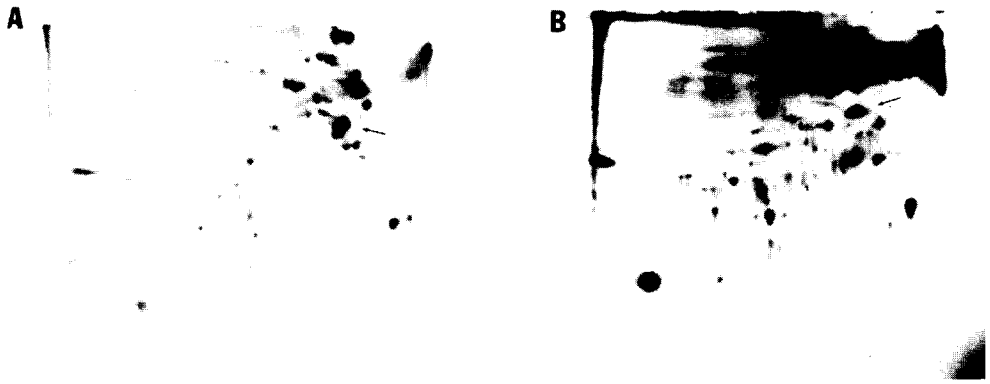

Fig. 5. Autoradiographs of two-dimensional electrophoretic gels of CHO cell membrane preparations that had been stained with ^{125}I-concanavalin A. A membrane sheet preparation is shown in A, while the vesicle preparation is depicted in B. Both results were obtained after approx. 10 h exposure to the X-ray film. Spot I_5 is arrowed in both figures. Approx. 200 μg of protein was loaded on each gel.

unlikely, glucosyl residues available to the lectin for binding. Control gels, stained in presence of the hapten α-D-methyl mannoside and washed extensively in buffer containing D-glucose, were completely blank (data not shown).

Both membrane fractions contained very large numbers of concanavalin A-binding species, many of which appeared to be common to both preparations. For example, polypeptide I_5, is a major stained spot in Fig. 5A but can also be detected easily in Fig. 5B. It is located to the lower right of a major Coomassie blue positive spot, I_3. The latter actually appears to exclude ^{125}I-labeled concanavalin A, leaving a clear area. Significantly, spot I_5 has stained with about equal intensity in both the sheet and vesicle preparations (Fig. 5A and B). On the other hand, several additional high molecular weight, acidic components could be identified in the vesicle fraction (Fig. 5B) by their binding of ^{125}I-labeled concanavalin A in the upper right hand corner of the gel, i.e. quadrants D and E. As discussed elsewhere [14], these glycoproteins stained faintly, if at all, with Coomassie blue. Thus, although both fractions seemed to share many components, the vesicle fraction was enriched with respect to this group of acidic glycoproteins in the molecular range 130 000—350 000 which were present in lower amounts in the sheet fraction.

Ricin I agglutinin and wheat germ agglutinin staining of vesicle fraction glycoproteins

Other lectins have been employed to detect the high molecular weight, acidic glycoproteins after two-dimensional electrophoresic separation of the polypeptides in the vesicle fraction. Here we show staining by ^{125}I-labeled ricin I agglutinin and ^{125}I-labeled wheat germ agglutinin (Fig. 6). The former shows specificity for β-D-galactosyl residues and can be clearly seen to bind to the acidic glycoproteins in quandrants D and E, as well as a number of other components on the gel, the most obvious of which is a spot running close to M_7 (Fig. 5A). The basic polypeptide K_1 also bound ricin I agglutinin. Labeling in the presence of 0.5 M D-galactose abolished the radioactive staining of all these components (data not shown).

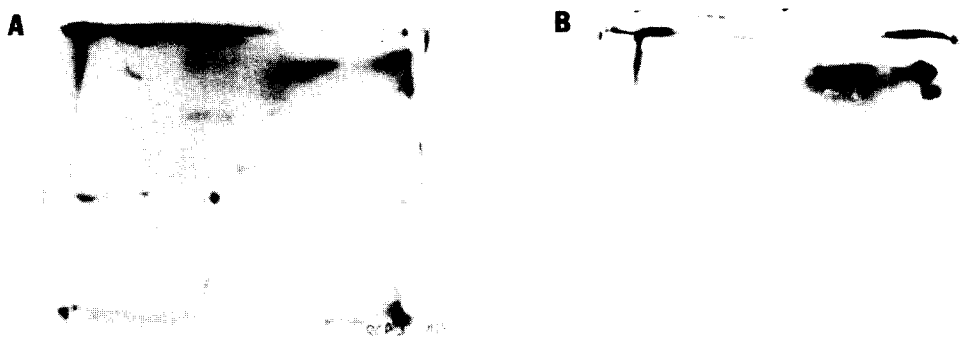

Fig. 6. A. Autoradiograph of two-dimensional electrophoretic map of CHO cell vesicle fraction after staining with ^{125}I-labeled ricin I agglutinin. Exposure time was 13 h. B. Autoradiograph of two-dimensional map of CHO cell vesicle fraction stained using ^{125}I-labeled wheat germ agglutinin. The exposure time was 6 h.

Wheat germ agglutinin bound to even fewer components than ricin I agglutinin and labeling was confined exclusively to the high molecular weight acidic glycoproteins (Fig. 6B). Only a smaller number of additional labeled components were detected after long-term exposure (48 h) of the ^{125}I-labeled wheat germ agglutinin gel (data not shown). Labeling in the presence of fetuin glycopeptides abolished radioactive staining of all these components.

The labeling pattern depicted in Fig. 6B is similar to that observed after ^{125}I-lactoperoxidase catalyzed iodination, except that an additional spot (E_1) was labeled. It should be emphasized that the separation of these high molecular weight acidic molecules is somewhat variable, since their isoelectric points are close to the limit of resolution of the isoelectric focusing technique employed here.

Discussion

Results presented in this paper support the concept that the vesicle fraction isolated after fluid pump disruption of CHO cells is derived from the plasma membrane. It was highly enriched in certain surface marker enzymes and, to a lesser extent, in ^{125}I after lactoperoxidase-catalyzed iodination of the cells prior to breakage. By similar criteria, it has been established earlier [12] that the sheet fraction preparation also represents a fraction enriched in plasma membrane, although the apparent degree of purification was somewhat less. Therefore, depending upon the procedure chosen for breakage and for isolation, two distinct, surface-derived fractions can be generated from CHO cells. We presently favor the idea that it is the method employed for purification which is critical, since we have recently been able to isolate a membrane fraction from the initial $300 \times g$ pellet after fluid pump disruption of the cells which appears to be identical to the sheet fraction described here (Baumbach, G.A., Horst, M.N. and Roberts, R.M., unpublished results). Similarly, a light vesicle fraction as well as a sheet fraction can be purified from swollen cells broken by Dounce homogenization according to the method of Brunette and

Till [13]. Further, Kramer and Canellakis [34] have recently reported isolating a plasma membrane 'ghost' fraction and a small vesicle fraction, also of plasma membrane origin, from HeLa cells. Finally, Willinger and Frankel [35] partially purified a dense membrane fraction from the initial low-speed centrifugation step and light vesicle fraction from the post-nuclear supernatant fraction after breakage of leucocytes using a Teflon-coated Potter-Elvehjem homogenizer. Thus, various methods of breakage can provide two physically distinct fractions, both of which appear to be derived from the cell surface. One possibility, therefore, is that the light vesicle fraction represents a specialized domain of the plasma membrane which vesiculated during cell breakage.

In the present work, a fluid pump disruption procedure has been employed to obtain a vesicle fraction derived from the plasma membrane. It should be stressed that the use of different disruption pressure (20—400 lb/inch^2) does cause variation in the relative amounts of marker enzymes (Baumbach, G.A., Horst, M.N. and Roberts, R.M., unpublished results), and this aspect of cell breakage should be carefully controlled. Using a fixed back pressure of 200 lb/inch^2, we have presented evidence here that the vesicle fraction is not only differentiated structurally from the rest of the surface, but also highly enriched in certain marker enzymes, ($Na^+ + K^+$)-ATPase and phosphodiesterase I, while other plasma membrane-associated enzymatic activities were less pure, e.g. leucine aminopeptidase. Thus, the vesicle fraction exhibits a subset of the total cell surface-associated enzyme activities, supporting the suggestion that the preparation be designated a domain of the plasma membrane. Additional evidence in support of the proposed domain concept has been obtained by mixing experiments where a few polypeptides present in one fraction but not usually in the other were detected. In part, of course, this might be due to differences in the relative purities of the two preparations, with the sheets being contaminated with higher amounts of material not derived from the plasma membrane.

The vesicle fraction is also enriched in a group of high molecular weight, acidic glycoproteins. These glycoproteins are presumably externally disposed since they are readily iodinatable by the lactoperoxidase technique. Curiously, however, few other vesicle fraction polypeptides were labeled extensively following lactoperoxidase-catalyzed iodination, whereas in the sheet fraction, which seemed to have a fairly similar polypeptide composition, many radiolabeled components were detected after iodination. Possibly the high molecular weight, acidic glycoproteins form a glycocalyx or protective coating over the domain of the surface that constitutes the vesicle fraction and shield underlying components in this region from labeling agents. Therefore, a small proportion of the total vesicle fraction protein contains a majority of the iodine label. This observation may explain the relative low ^{125}I specific activity of the vesicle preparation (Table I), and rules out direct comparison of marker enzyme and ^{125}I-labeled polypeptide fold-purifications.

The high molecular weight, acidic glycoproteins may be analogous to the epiglycanin of mouse TA3 mammary carcinoma ascites cells which appears to mask underlying antigenic proteins on the plasma membrane from immune recognition [36]. However, unlike epiglycanin, whose carbohydrate chains appear to be of the mucin type, the high molecular weight, acidic glycoproteins

of CHO cells bind concanavalin A, ricin I agglutinin and wheat germ agglutinin, which strongly suggests that they contain α-D-mannosyl, β-D-galactosyl and *N*-acetyl-D-glucosaminyl residues. These are all typical components of glycoproteins of the plasma type which have their saccharide groups linked through asparagine residues on the protein chain. Significantly, the high molecular weight, acidic glycoproteins appear to be the major wheat germ agglutinin receptors on the CHO cell surface (Fig. 5B).

Although we do not know the specific location of the vesicle fraction on the CHO cell, it is possible that it originated from surfaces blebs or microvilli. During cell breakage such structures might be expected to detach from the rest of the cell surface and round up. In this case, the high molecular weight, acidic glycoproteins are likely to form an outer coating on these structures. However, it is conceivable that the vesicle fraction might also derive from attachment sites, surface pits or some other structurally differentiated region of the plasma membrane. One additional possibility under investigation is that the vesicles are released from within the cell at the time of breakage and are thus of pinocytotic origin.

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