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SUBFRACTIONATION OF LIVER MEMBRANE PREPARATIONS BY SPECIFIC LIGAND-INDUCED DENSITY PERTURBATION

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Summary

Immunofluorescence and immunoferritin staining with monospecific antibodies to dipeptidyl peptidase IV purified from rat liver plasma membrane showed that the antigenic sites of this glycoprotein were exposed only on the outer surface of the liver cell. In a vesiculated plasma membrane preparation the peptidase was located exclusively on right-side-out elements, which differed in their degrees of ferritin staining, and could be separated into subfractions of different buoyant densities corresponding to their concentration of dipeptidyl peptidase IV. The concomitant density perturbation of nucleotide pyrophosphatase was similar, but not identical, to that of the peptidase itself, indicating that these two marker enzymes are somewhat differently distributed in the plane of the liver plasma membrane. Since essentially all the galactosyl transferase in plasma membrane and none of that in Golgi membrane could be density-perturbed with the antipeptidase, the activity in the plasma membrane preparation could not be ascribed to contamination with discrete Golgi elements. On the other hand, the small amount of dipeptidyl peptidase IV found in the Golgi preparations was itself perturbed by the antipeptidase, indicating that it represented contaminating right-side-out plasma membrane vesicles. In preliminary experiments similar separations were also obtained with wheat germ agglutinin as the plasma membrane ligand. Density perturbation, mediated by the recognition of specific surface markers,

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Abbreviations: SDS, sodium dodecyl sulfate: Ig, immunoglobulin.

should be a useful adjunct in the separation and characterization of subcellular components in other systems.

Introduction

We have isolated two plasma membrane glycoprotein enzymes, alkaline nucleotide pyrophosphatase and dipeptidyl peptidase IV, from rat liver, and have used monospecific antibodies against these proteins to outline the kinetics of their translocation to, and disappearance from, the hepatocyte plasma membrane in vivo [1,2]. Indirect immunoferritin staining of plasma membrane fragments with antibiodies against dipeptidyl peptidase IV was asymmetrical, and fragments differed in their overall staining intensity. Since this indicated that different regions of the hepatocyte plasma membrane differed in their concentration and/or disposition of the antigen, we attempted to separate such fragments on the basis of the change in their buoyant density which should result from their binding different amounts of ferritin. We describe here some results of such 'density perturbation' [3] experiments.

Methods and Materials

'Crude' and 'light' plasma membrane from rat liver was prepared as described previously [4]. Golgi membranes were isolated by the method of Leelavathi et al. [5]. To obtain Golgi preparations labeled primarily in their membrane proteins each of three adult female rats was injected intraperitoneally with 1 mCi L-[4,5-^{3}H]leucine in phosphate-buffered saline 24 h before killing the rats.

The purification and characterization of dipeptidyl peptidase IV (EC 3.4.14.1) and nucleotide pyrophosphatase (EC 3.6.1.9) from rat liver plasma membrane, as well as the production and characterization of rabbit antibodies against these proteins are described elsewhere [1,2].

Wheat germ agglutinin purified as described by Rosen et al. [6] gave a single band of 17 000 daltons on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis [7]. To obtain rabbit antiserum against the agglutinin 100 μg of antigen were emulsified in 1 ml saline/Freund's complete adjuvant (1 : 1) and distributed subcutaneously in each of two rabbits. 4 weeks later the animals were boosted subcutaneously and intramuscularly with the same amount of antigen in incomplete adjuvant and immune serum was collected weekly, with additional boosting as necessary. Immune serum gave a single precipitin line against the agglutinin in the presence of 0.1 M *N*-acetylglucosamine; under these conditions no precipitate was formed with pre-immunization serum.

Gamma globulins were precipitated from rabbit sera with 42% saturated ammonium sulfate, dialyzed against 0.01 M potassium phosphate, pH 7.4 and passed over a DEAE-cellulose column equilibrated with the same buffer. The unadsorbed peak was pooled as immunoglobulin G (IgG), precipitated with ammonium sulfate, dialyzed against phosphate-buffered saline, and stored at 4 °C in the presence of 0.02% sodium azide.

Goat antiserum to rabbit IgG, obtained by immunization and periodic boosting with 1-mg portions of purified non-immune-rabbit IgG, was mixed with an equal volume of packed Sepharose 6B, which contained 1.2 mg/ml rabbit IgG, attached by the cyanogen bromide procedure [8]. After 10 min at room temperature this mixture was poured into a glass column (5 cm internal diameter) and washed free of unadsorbed protein with phosphate-buffered saline, pH 7.4. A volume of 0.1 M acetic acid corresponding to 75% of the bed volume was run into the packed absorbent; after 30 min the acetic acid elution was resumed and 20-ml fractions were monitored for protein and adjusted to pH 5.0 with solid sodium acetate. Peak fractions were combined, dialyzed against three changes of phosphate-buffered saline, concentrated to 100 ml on a PM-10 membrane (Amicon), and precipitated with 42% saturation ammonium sulfate to yield affinity-purified goat antirabbit-IgG immunoglobins. Before use the precipitate was collected by centrifugation and exhaustively dialyzed against three changes of phosphate-buffered saline to remove all traces of ammonium ions.

The ferritin conjugate of goat antirabbit-Ig was prepared essentially as described by Kyte [9]. Ferritin, EM grade (Polyscience), 100 mg pelleted at 100 000 $\times g$ for 2 h, was resuspended in 1.5 ml phosphate-buffered saline containing 50 mg of goat antirabbit-Ig immunoglobulin. To this solution 0.15 ml of 0.5% glutaraldehyde (Polyscience) was added dropwise, with stirring, at room temperature and the reaction mixture left unstirred for an hour. After addition of excess ammonium bicarbonate and centrifugation at 10 000 rev./min for 10 min in the Sorval SS-34 rotor to remove protein aggregates the supernatant was chromatographed at 4°C on a 90 × 1.5 cm column of Sepharose 6B (Pharmacia, Inc.) in phosphate-buffered saline, at a flow rate of 6 ml/h. The fractions comprising the leading shoulder of the ferritin monomer peak were pooled, taking care not to include polymerized material emerging at the void volume. The ferritin conjugate was pelleted at 100 000 $\times g$ for 2 h, resuspended in 1—2 ml phosphate-buffered saline and recentrifuged in the SS-34 rotor at 10 000 rev./min for 10 min immediately prior to use.

For density perturbation, membrane preparations, 5 mg protein in 1.0 ml phosphate-buffered saline, were incubated with the rabbit antipeptidase IgG (1.5—2.0 mg) for 30 min at 37°C; for light plasma membrane and Golgi membrane this represented about a 2- and 30-fold excess of antibody, respectively [1]. After removing excess rabbit IgG by three washes with phosphate-buffered saline the membranes were resuspended to 1.0 ml and incubated with 1.5—2.0 mg ferritin conjugate for 30 min at 37°C. For analysis by sedimentation the membranes were then pelleted, resuspended to 1.0 ml and layered onto discontinuous gradients of 1.5 ml each (25, 32, 34, 40, 50 and 60% (w/w) sucrose) and centrifuged in the Beckman SW 41 rotor for 2 h at 41 000 rev./min. Membrane bands were collected by aspiration and analyzed either directly, or after dilution and pelleting in the SW 41 rotor. Controls consisted of membranes treated with non-immune rabbit IgG. When samples were to be analyzed by flotation to equilibrium the concentration of conjugate was increased 4—5-fold. Membranes were then pelleted and resuspended in 2 ml 50% sucrose, layered on 1 ml 60% sucrose, overlayed with 2.5 ml each at 40, 33 and 25% sucrose and centrifuged for 28 h at 38 000 rev./min in the Beckman SW 41 rotor.

For treatment with wheat-germ agglutinin membranes in 2 ml phosphate-buffered saline were incubated for 30 min at 37°C with 100 μg/ml of the lectin. They were then washed three times, suspended in 1 ml of the same medium and incubated with 2 mg of rabbit antiagglutinin IgG for 30 min at 37°C, washed free of excess rabbit IgG, treated with 1 mg/ml conjugate, and sedimented through discontinuous sucrose gradients as described above. Controls were treated with non-immune IgG, or washed with 0.2 M *N*-acetyl-glucosamine before treatment with the ferritin conjugate.

For immune-electron microscopy crude and light plasma membrane samples were taken through the first part of the perturbation procedure. After treatment with the conjugate the stained membranes were washed three times by centrifugation and resuspension in phosphate-buffered saline to remove any unbound conjugates. These final pellets, as well as material recovered from sucrose gradients, were fixed in glutaraldehyde and processed for electron microscopy as described [4].

Adult hepatocytes, isolated essentially as described by Leffert et al. [10], were seeded on glass cover slips and maintained for 2—4 days at 37°C in ornithine-supplemented arginine-free Dulbecco's modified Eagle's medium with 20% calf serum, under an atmosphere of 5% CO_2/95% air. For fluorescence microscopy cells were washed, fixed with formaldehyde and stained for dipeptidyl peptidase IV by a conventional double-immune procedure, using rabbit antipeptidase IgG and rhodamine-conjugated antirabbit-Ig Ig [11]. This preparation maintained an intact surface membrane barrier to macromolecular reagents, since visualization of intracellular actin with a modified heavy meromyosin procedure [11] required prior treatment with detergent.

Protein was determined by a modified Lowry procedure [10]. The phosphodiesterase I activity of nucleotide pyrophosphatase was assayed as described previously [4]. Assays for UDPgalactose galactosyltransferase were done as before [4], with addition of 5 mM adenosine monophosphate to inhibit nucleotide pyrophosphatase-catalyzed hydrolysis of UDPgalactose.

As detailed elsewhere [1], the enzymatic identity of the dipeptidyl peptidase IV protein was discovered only after completion of the work described here, during which this protein was determined by quantitative immunoprecipitation/SDS polyacrylamide gel electrophoresis, as follows. Untreated membranes were solubilized in 5% Tris-sarcosyl (K. and K. Labs.)/1 M sodium chloride, pH 8.0 and centrifuged at 10 000 rev./min in the SS-34 rotor for 10 min. The supernatants were treated with excess rabbit antipeptidase IgG, incubated at 37°C for 1 h and then left at 4°C overnight. Following one wash with 5% sarcosyl/1 M NaCl and two washes with phosphate-buffered saline the immunoprecipitates so formed were subjected to quantitative SDS polyacrylamide gel electrophoresis, and the amounts of the 120 000 dalton peptidase peptides determined by gel scanning as described elsewhere [1]. For membranes which had been subjected to the density perturbation procedure the pellets obtained on centrifugation of the original sarcosyl lysates contained peptidase protein which had already complexed to the antipeptidase/ferritin conjugate; these pellets were processed separately for quantitative SDS polyacrylamide gel electrophoresis. The lysate supernatants were then treated with a second portion of antipeptidase IgG to precipitate any remaining peptidase which had

been inaccessible to the antibodies in the intact vesicles.

For liquid scintillation counting 25-μl aliquots of membrane suspensions were treated at 90°C for 30 min with 1 ml Protosol. After cooling and addition of 10 ml Econofluor the samples were counted in a Packard Tri Carb 3300 to less than ±5% error.

Results

Dipeptidyl peptidase IV constitutes about 1% of the total protein in rat liver crude plasma membrane [1], and many sheets and vesicles in this preparation bound the ferritin conjugate after treatment with rabbit antipeptidase Ig, but not with non-immune Ig (Fig. 1). Most importantly, it is seen in Fig. 1 that such staining occurred only on one side of any membrane element, even when both sides clearly were accessible to the reagents. Furthermore, immunofluorescent staining of intact glutaraldehyde-fixed substrate-attached hepatocytes

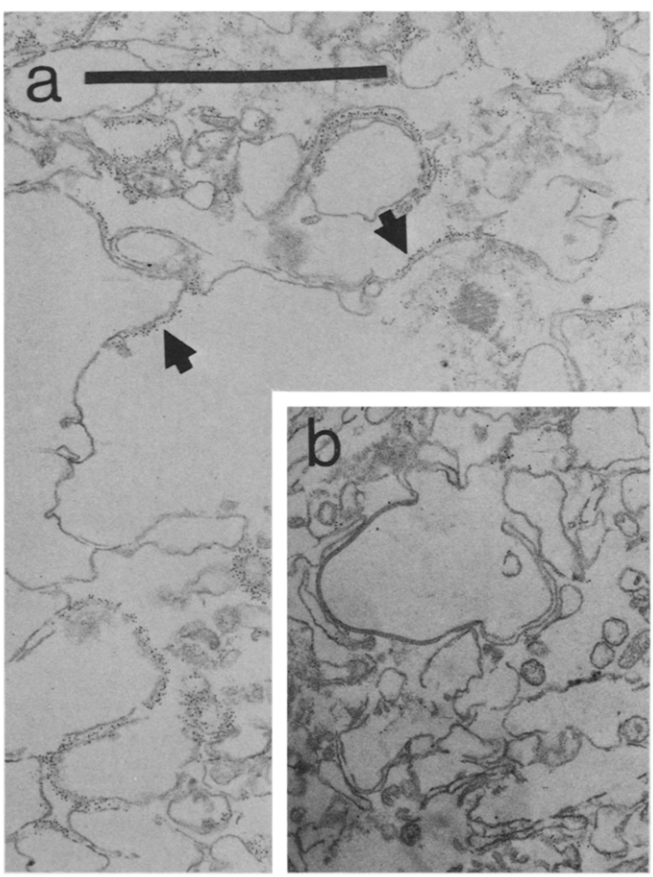

Fig. 1. Indirect immunoferritin staining of crude plasma membrane fraction from rat liver. Crude plasma membrane was treated with (a) rabbit antibodies to dipeptidyl peptidase IV or (b) rabbit non-immune IgG; they were then washed free of excess IgG, reacted with ferritin-conjugated goat antirabbit-IgG Ig and processed for electron microscopy as described under Methods. Arrows show immunoferritin labeling on one side only of open plasma membrane vesicles and sheets. Bar, 1 μm.

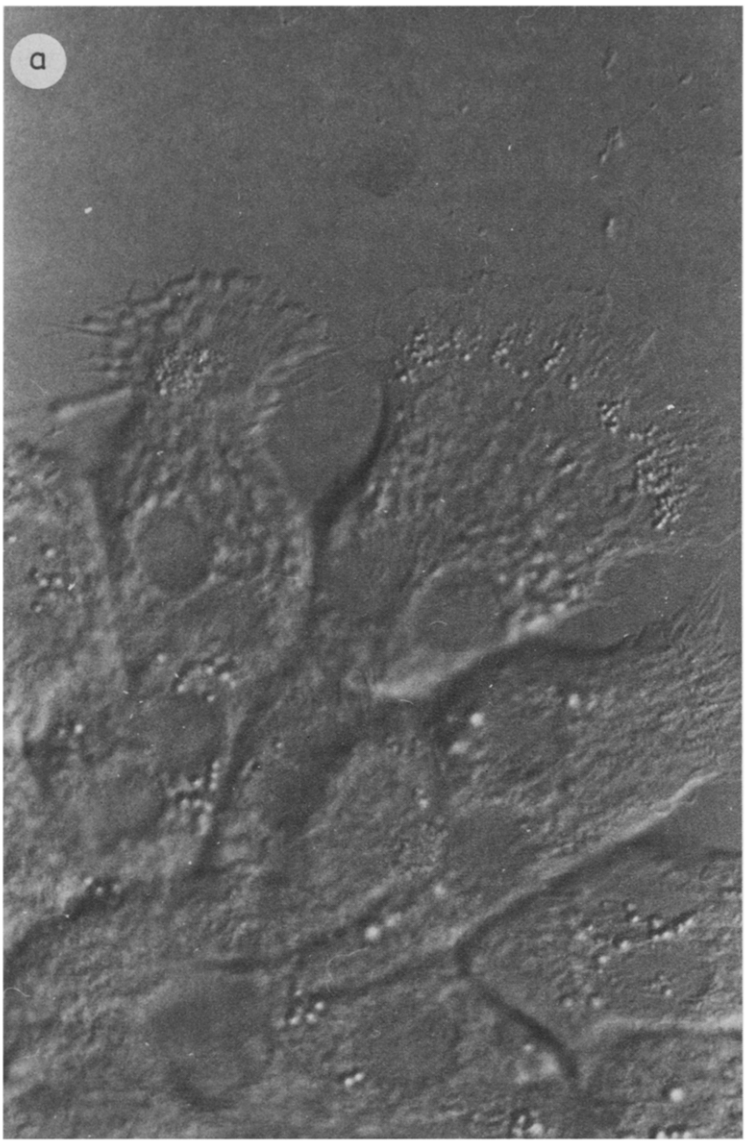

Fig. 2.

showed typical surface staining of dipeptidyl peptidase IV (Fig. 2), while that of actin, a major intracellular component, required prior disruption of the cell membrane with detergent (Lourard, D. and Elovson, J., unpublished). Thus, it may be concluded that dipeptidyl peptidase exposes its antigenic determinants, as well as its carbohydrate moieties [13] exclusively on the outer surface of the plasma membrane. Parenthetically, the carbohydrates themselves are unlikely to constitute these determinants, since heat denaturation irreversibly abolished the antigenicity of the peptidase.

Vigorous Polytron vesiculation of crude plasma membrane yields a light fraction which is further enriched about 3-fold for both nucleotide pyrophos-

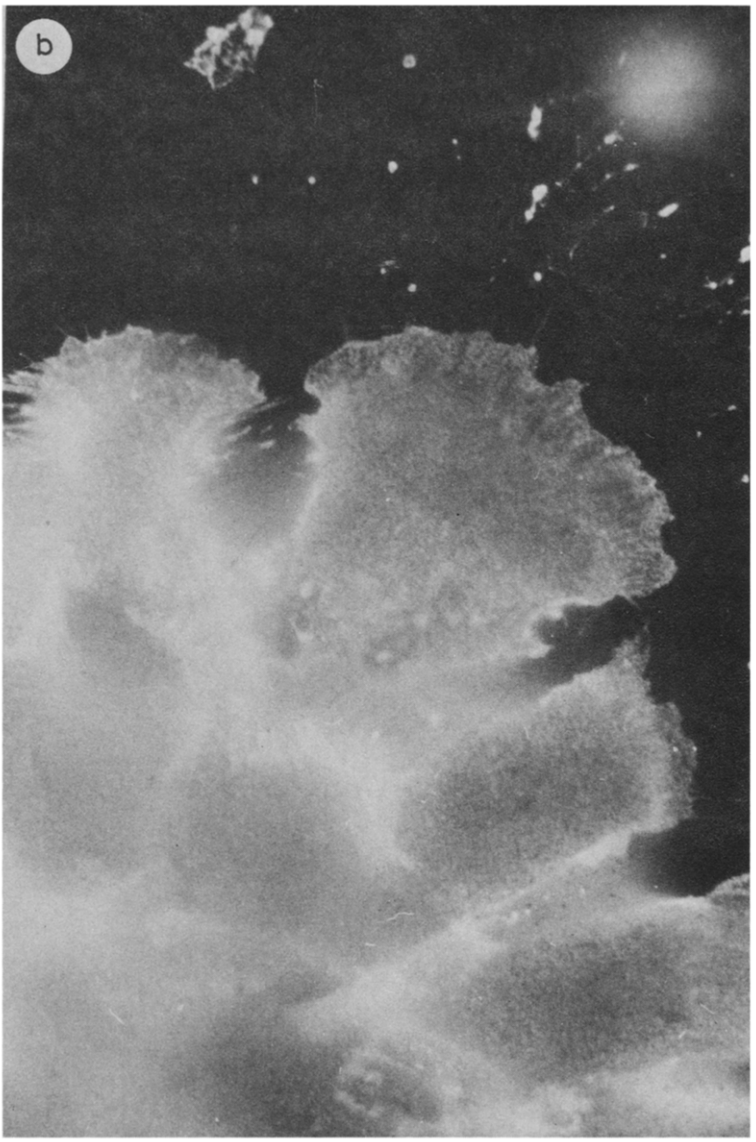

Fig. 2. Indirect immunofluorescence staining of hepatocyte surface dipeptidyl peptidase IV. Hepatocytes were seeded on glass cover slips, cultured, fixed and stained as described under Methods. (a) Nomarski optics showing typical appearance of mostly binucleated hepatocytes on periphery of microcolony. (b) Same field showing immunofluorescence focused on brightly staining microvilli on upper cell surface.

phatase and dipeptidyl peptidase IV [4]. As seen in Fig. 3 the smaller vesicles in this preparation also showed extensive specific staining after treatment with antipeptidase, again exclusively on their surface; some vesicles also appeared more heavily stained, while others were essentially free of ferritin. A priori such unstained vesicles could: (a) be derived from regions of the plasma membrane which were devoid of dipeptidyl peptidase IV; (b) be inside-out, with

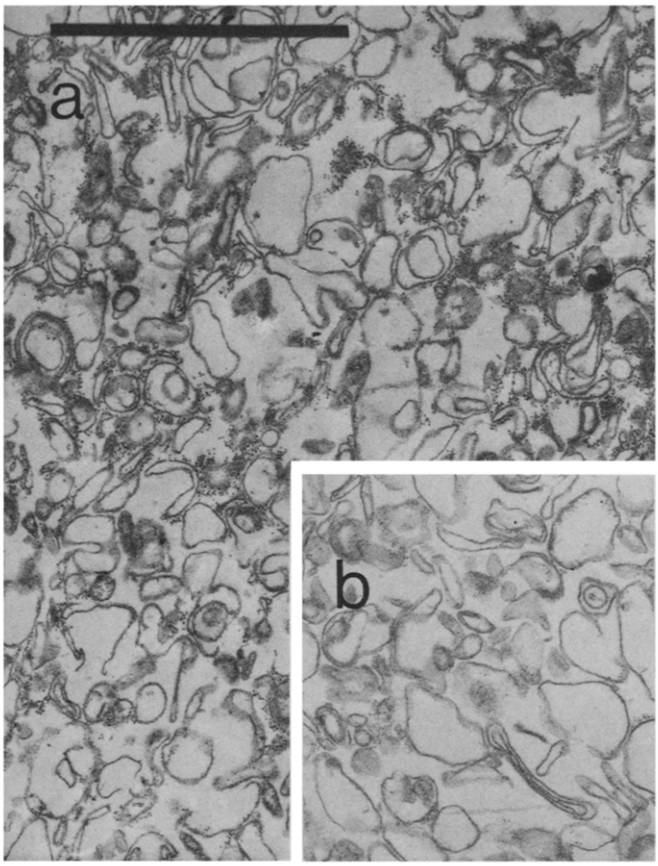

Fig. 3. Indirect immunoferritin staining of light plasma membrane fraction from rat liver. Light plasma membrane fractions were treated as in Fig. 1. Note ferritin staining only on outside of vesicles. Bar, 1 μm.

the determinants inaccessible on their inner surface; or (c) constitute contaminating vesicles derived from intracellular membranes (Golgi or smooth endoplasmic reticulum) which contain little peptidase [2]. To decide between these alternatives, separation of such vesicles was undertaken on the basis of the change in buoyant density accompanying binding of different amounts of ferritin conjugates.

Fig. 4 shows that such separation was obtained. Light plasma membrane preparations treated with the ferritin conjugate alone, or after prior exposure to non-immune rabbit IgG, had the same banding pattern on a sucrose density gradient as did the untreated material; however, when first treated with rabbit antibodies to dipeptidyl peptidase IV, part of the preparation banded at higher sucrose densities, correlating with increased immunoferritin staining, again exclusively on the outer vesicle surface (Fig. 5). As shown in Table I, this represented perturbation of one-half of recovered protein, and about one-third and two-thirds of the galactosyltransferase and pyrophosphatase, respectively, to higher densities, with a marked decrease in the ratio between the

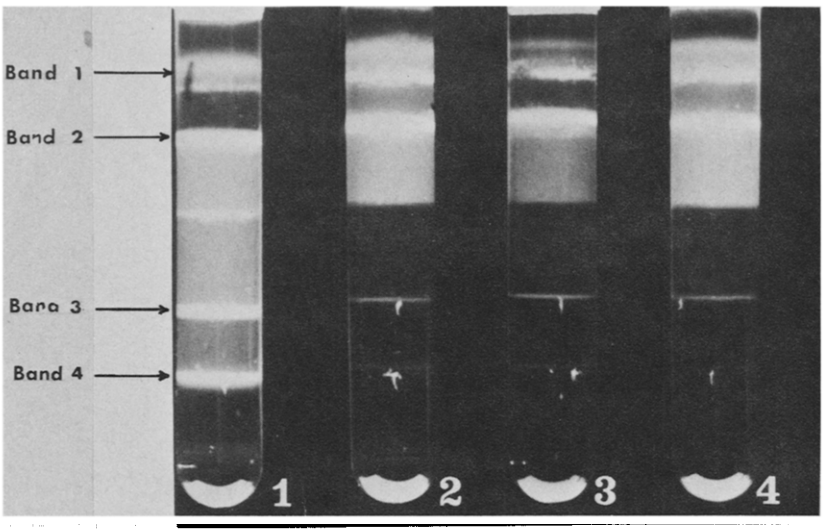

Fig. 4. Sedimentation pattern of light plasma membrane subfractions after density perturbation. Samples of light plasma membrane were treated as described under Methods. Tube 1, rabbit antipeptidase followed by ferritin-conjugated goat antirabbit-IgG Ig; Tube 2, rabbit non-immune IgG followed by the conjugate; Tube 3, conjugate only; Tube 4, no treatment. Samples were layered on discontinuous sucrose density gradients and centrifuged for 2 h at 39 000 rev./min in a SW 41 rotor. Band 1, buffer/25% sucrose interface; Band 2, 25%/32% sucrose interface; Band 3, 34%/40% sucrose interface; Band 4, 40%/50% sucrose interface.

latter two enzymes with increased buoyant density of the perturbed vesicles. The low overall recoveries may be attributed to the many washing steps in the perturbation procedure. Very similar results were obtained in a second experiment in which the distribution of the dipeptidyl peptidase IV itself also was determined. Since, as seen in Fig. 6, there was a direct relationship between the concentration of peptidase in these subfractions and their degree of perturbation it may be concluded that the vesicles indeed sorted out according to their content of this specific surface determinant. Furthermore, since all the peptidase in these fractions was recovered as the immune complex bound to the ferritin conjugate (see Methods), which had formed exclusively on the outer surface of membrane vesicles (Fig. 5), it follows that all vesicles which contained dipeptidyl peptidase IV also were right side out and, conversely, that vesicles which remained unstained and unperturbed contained little or no peptidase rather than having it exposed on their inner surface, inaccessible to the antibodies. The sidedness and origin of these vesicles is not known, but their content of nucleotide pyrophosphatase suggested they are derived from the plasma membrane itself, rather than from Golgi or endoplasmic reticulum membrane, as discussed below.

Density perturbation was also performed with wheat-germ agglutinin as a more generally available cell surface probe, using rabbit antiagglutinin antibody to couple the ferritin conjugate to the bound lectin, as described in Methods. As shown in Fig. 7 such treatment also resulted in perturbation of protein and enzyme markers similar to that obtained with antibodies to dipeptidyl peptidase IV. Here substitution of rabbit non-immune IgG for antiagglutinin Ig

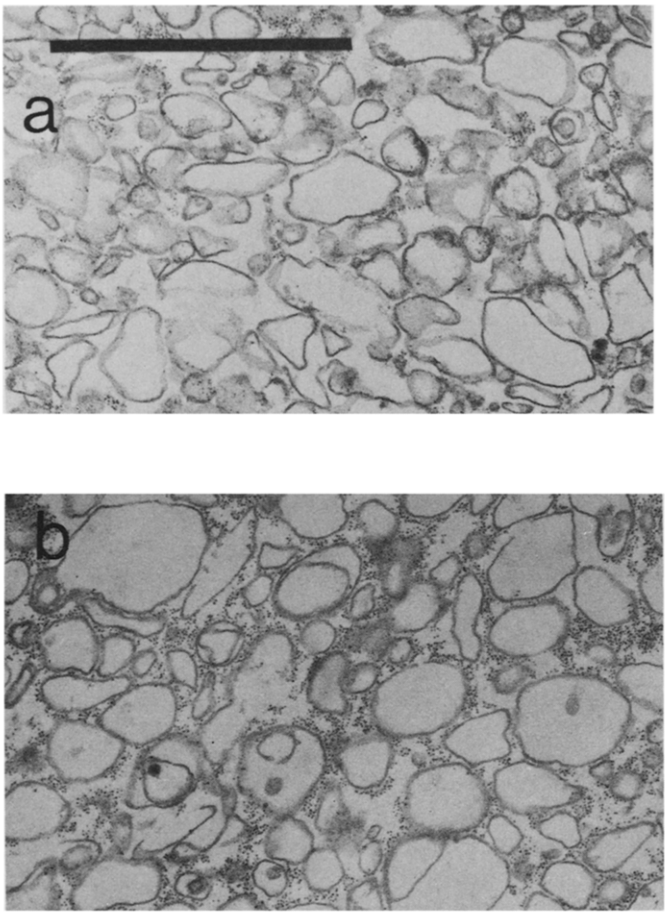

Fig. 5. Electron microscopy of light plasma membrane subfractions after density perturbation. Bar, 1 μm. (a) and (b) show bands 1 and 4, respectively, from tube 1 in Fig. 4.

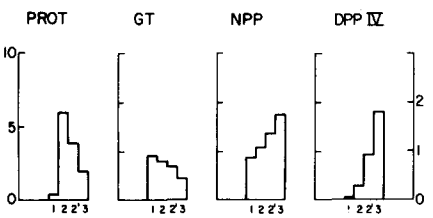

Fig. 6. Distribution of total protein (PROT), galactosyltransferase (GT), nucleotide pyrophosphatase (NPP), and dipeptidyl peptidase IV (DPPIV) following antipeptidase-induced density perturbation of light plasma membrane. Fractions indicated on abscissa were collected after 2 h sedimentation through a sucrose density gradient as in Fig. 4. Fraction 1, buffer/25% sucrose interface; Fraction 2, 25%/32% sucrose interface; Fraction 2, 32%/34% sucrose interface; Fraction 3, 34%/40% sucrose interface. Protein values are given on left-hand ordinate as mg/fraction; other ordinates show the enrichment of galactosyl transferase, nucleotide pyrophosphatase, and dipeptidyl peptidase IV in different fractions relative to that in the original light plasma membrane fraction.

TABLE I

ANALYSIS OF LIGHT PLASMA MEMBRANE FRACTIONS OBTAINED BY DENSITY PERTURBATION WITH ANTIBODIES AGAINST DIPEPTIDYL PEPTIDASE IV

Band [a]	Treatment of samples											
	Antipeptidase IgG [b]				Non-immune IgG [c]				No additions [d]			
	Protein (%) [g]	GT [e] (%) [g]	NPP [f] (%) [g]	GT / NPP	Protein (%)	GT (%)	NPP (%)	GT / NPP	Protein (%)	GT (%)	NPP (%)	GT / NPP
1	9	0	3	0	24	8	41	0.2	30	14	35	0.4
2	40	70	29	2.4	76	92	59	1.56	70	86	65	1.32
3	27	23	37	0.62	— [h]	—	—	—	—	—	—	—
4	23	7	32	0.22	—	—	—	—	—	—	—	—
Recovery (%) [i]	46	37	55		36	38	59		51	82	82	

[a] Bands 1—4, Fig. 4.
[b] Tube 1, Fig. 4.
[c] Tube 2, Fig. 4.
[d] Tube 4, Fig. 4.
[e] GT, galactosyltransferase.
[f] NPP, nucleotide pyrophosphatase.
[g] Values for protein or enzyme activity in each fraction, expressed as per cent of total recovered from gradient.
[h] No measurable material recovered.
[i] Protein and enzymatic activity recovered from gradient, expressed as per cent of that in the original sample of light plasma membrane.

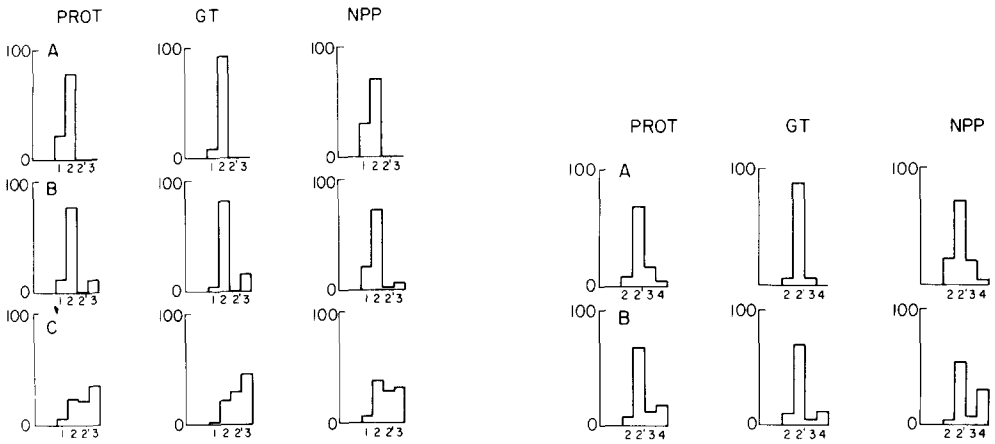

Fig. 7. Distribution of protein (PROT), galactosyltransferase (GT), and nucleotide pyrophosphatase (NPP) following wheat-germ agglutinin-induced density perturbation of light plasma membrane. Part A: plasma membrane treated sequentially with agglutinin, non-immune IgG and ferritin-conjugated goat antirabbit IgG Ig. Part B: plasma membrane treated with agglutinin, washed with 0.2 M *N*-acetylglucosamine, then treated with rabbit antiagglutinin IgG and the ferritin-conjugate. Part C: plasma membrane treated with agglutinin, rabbit antiagglutinin IgG and conjugate. Fractions 1, 2, 2′ and 3 as in Fig. 6. The heights of the bars represent values for protein or enzymic activities in each fraction, expressed as percent of the total recovered from the gradient.

Fig. 8. Distribution of protein (PROT), galactosyltransferase (GT), and nucleotide pyrophosphatase (NPP) following wheat-germ agglutinin-induced density perturbation of a Golgi preparation. Part A: Golgi fraction treated as in Fig. 7, part A. Part B: Golgi fraction treated as in Fig. 7, part C. Separations and ordinates as in Fig. 7, Fractions 2, 2′, 3 and 4 as in Figs. 6 and 7.

abolished the perturbation, showing that the vesicle-bound wheat-germ agglutinin did not simply interact with IgG via the latter's carbohydrate moiety; perturbation was also greatly reduced when treatment with the agglutinin was followed by a wash with 0.2 M *N*-acetylglucosamine.

The wheat-germ agglutinin procedure was also applied to a rat liver Golgi preparation (Fig. 8), whose carbohydrate components should be on the inner cisternal surface, inaccessible to the lectin. Perturbation of protein and galactosyl transferase was indeed minimal for this preparation, but a significant portion of its nucleotide pyrophosphatase was perturbed to the 40—50% interface, and probably represented contaminating plasma membrane vesicles. The distribution of dipeptidyl peptidase IV was not analyzed in these experiments.

To approach the reverse question, that is, the possible contamination of light plasma membrane with Golgi elements, the following samples were prepared and thoroughly washed to remove unbound rabbit IgG. (A) control samples of light plasma membrane and Golgi preparation, separately treated with non-immune IgG; (B) the same samples separately treated with antipeptidase IgG; (C) a sample of plasma membrane treated with antipeptidase IgG, washed, and mixed 1 : 1 with a Golgi preparation, which had been similarly treated with non-immune IgG; and (D) plasma membrane and Golgi preparations mixed 1 : 1 before treatment with antipeptidase IgG. To maximize perturbation the membrane samples were then treated with high concentrations of ferritin con-

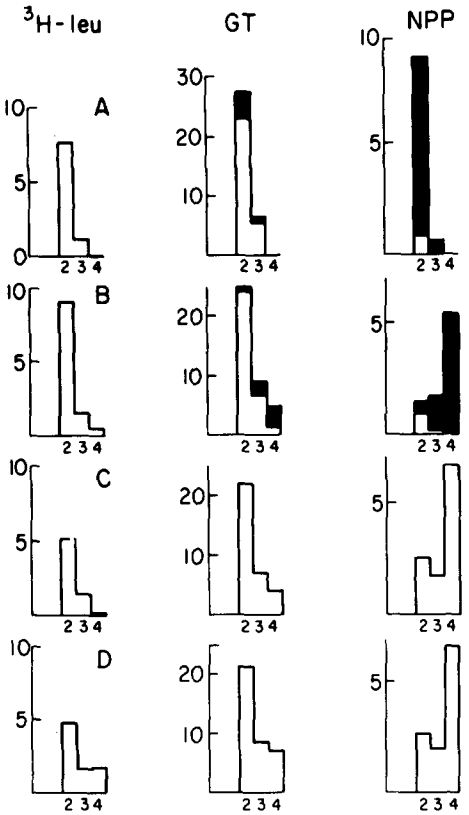

Fig. 9. Distribution of ^{3}H-labeled Golgi protein ([^{3}H]Leu), galactosyltransferase (GT), and nucleotide pyrophosphatase (NPP) following density perturbation of mixed light plasma membrane and Golgi preparations with rabbit antibodies to dipeptidyl peptidase IV. Parts A and B: Golgi material (open bars) and light plasma membrane (solid bars), treated separately with (A) non-immune and (B) antipeptidase IgG. Part C: antipeptidase-treated plasma membrane mixed 1 : 1 with non-immune IgG-treated Golgi material. Part D: 1 : 1 mixture of plasma membrane and Golgi material treated with antipeptidase IgG. Samples were treated with high concentrations of ferritin-conjugated goat antirabbit-IgG Ig and fractionated by flotation through a sucrose density gradient as described under Methods. Fractions 1, 3 and 4 as in previous Figs. Ordinates: [^{3}H]Leu, cpm X 10^{-3}/fraction; galactosyltransferase, mU/fraction; nucleotide pyrophosphatase, U/fraction.

jugate, recovered by centrifugation at high speed, and separated by flotation through an isopycnic sucrose density gradient, where the excess unreacted conjugate pelleted out. To provide a marker for the Golgi preparation it was processed from rats injected with [^{3}H]leucine 24 h before killing [14]. The upper two panels of Fig. 9 show the results when preparations of plasma membrane (solid bars) and Golgi (open bars) were analyzed separately. Compared to its non-immune control (Panel A, open bars) antipeptidase-treated Golgi elements (Panel B, open bars) showed minimal perturbation, while antipeptidase-treated plasma membrane (Panel B, solid bars), showed nearly complete shift of nucleotide pyrophosphatase, protein (not shown) and galactosyl transferase to the most perturbed position. Fig. 9 further shows that a mixture of non-immune IgG-treated Golgi and antipeptidase IgG-treated plasma membrane behaved as the sum of unperturbed Golgi plus perturbed plasma mem-

brane (compare Panel C to Panels A and B); although the plasma membrane did carry some Golgi material down to the 40%/50% interface when the two were mixed and treated together with antipeptidase (Panel D) this shift was small, and it is clear that the overwhelmingly perturbed plasma membrane galactosyltransferase activity (Panel B) could not be accounted for as non-specifically entrapped Golgi contaminants. Conversely, the small amount of perturbed material at the 40%/50% interface in the antipeptidase-treated Golgi preparation accounted for all the dipeptidyl peptidase IV recovered from that gradient, again in the form of immune complex bound to the ferritin conjugate (data not shown). Thus, since it was fully perturbed and separated away from the bulk of Golgi material, the dipeptidyl peptidase IV present in the original Golgi preparation most likely is derived from contaminating plasma membrane vesicles.

Discussion

Density perturbation was first used by Roizman and Spear [15] who found that viral antigen-containing membrane vesicles bound enough unconjugated antiviral antibodies to separate from unreactive vesicles on a density gradient. Wallach [3] perturbed lymphocytes plasma membrane vesicles with phage-conjugated concanavalin A, and more recently, Lim et al. [16] used rabbit antiserum to perturb human erythrocytes ghosts with goat antirabbit-IgG Ig coupled to synthetic methacrylate microspheres; in neither of the latter two studied, however, was the technique used to actually separate different vesicle populations.

As done here, as an extension of the immune-staining method for electron microscopy, density perturbation allowed separation of membranes depending on their content and disposition of a specific surface antigen. With high concentrations of the ferritin conjugate an essentially all-or-none separation was obtained between vesicles with and without available dipeptidyl peptidase IV antigen. With lower concentrations of ferritin conjugate, plasma membrane subfractions could be separated which showed quantitative differences in dipeptidyl peptidase IV content, presumably corresponding to different areas of the hepatocyte plasma membrane. The concomitant perturbation of the other plasma membrane marker, nucleotide pyrophosphatase, was generally similar but not identical to that of the peptidase itself, indicating that the distribution of these two glycoproteins in the plane of the membrane was somewhat different. Thus, density perturbation should be a useful complement to affinity separations with immobilized ligands which, when applied to membrane vesicles, usually give all-or-none separations and often present problems of efficiency and recovery. Thus, Bennet and Cuatrecasas were unable to adsorb right-side-out vesicles of adipocyte plasma membranes to wheat-germ agglutinin columns [17] while, conversely, Crumpton et al. [18] were unable to elute lymphocyte membrane vesicles from immobilized *Lens culinaris* lectin.

It was of interest that dipeptidyl peptidase IV was located exclusively on the outer surface of vesicles in the light plasma membrane fraction. Since 80% of the membranes in this fraction were susceptible to density perturbation with antipeptidase this method demonstrates that the vigorous means used to

prepare this fraction gave rise to vesicles almost all of which retained the righ-side-out orientation relative to the original cell surface. Since no unperturbed vesicles were found in the two studies cited above [3,16] it would seem that disruption of erythrocyte ghosts or pig lymphocytes by nitrogen gas decompression also produced right-side-out plasma membrane vesicles. On the other hand, Crumpton et al. found about half of the plasma membrane vesicles obtained by mechanical disruption of pig lymphocytes to be inside-out [18], while Bennet and Cuatrecasas [17] found that isolated adipocytes gave plasma membrane vesicles with both orientations and Zachowiski et al. [19] used a concanavalin A polymer to separate lymphoma cell plasma membrane into equal amounts of right-side-out and inside-out vesicles. The reasons for these different results are not known.

We also used density perturbation as a new approach to the problem of cross-contamination of subcellular fractions, specifically to ask whether the galactosyltransferase activity in liver plasma membrane preparations should be regarded as a Golgi marker, or whether it constituted a genuine component at the liver surface membrane [20]. Here treatment with antipeptidase perturbed essentially all the plasma membrane activity with mininal effect on the Golgi activity and although mixing experiments showed some nonspecific aggregation it may be concluded that the plasma membrane galactosyltransferase indeed resided in the right-side-out plasma membrane vesicles themselves. Unfortunately this does not settle the actual subcellular origin of this activity, since the vigorous shearing used to prepare light from crude plasma membrane may have caused fusion with any Golgi contaminants, with formation of artificial Golgi-plasma membrane hybrids [21] accounting for the integral galactosyltransferase activity in the light plasma membrane vesicles. This, however, is an ambiguity in the preparation of the light plasma membrane, which does not detract from the usefulness of density perturbation as a method to separate membrane vesicles according to specific differences in their surface component.

The question of cross-contamination also concerned the origin of dipeptidyl peptidase IV and nucleotide pyrophosphatase found in Golgi preparations, since other investigators have concluded that plasma membrane markers such as 5′-nucleotidase [22], receptors for insulin [23] and asialoglycoprotein [24], and adenylate cyclase [25] also are genuine Golgi components. The peptidase in our Golgi preparation was selectively perturbed by antipeptidase and therefore most likely represented low-level contamination by plasma membrane material. On the other hand the antipeptidase gave minimal perturbation of nucleotide pyrophosphatase in the Golgi fraction, and although the lectin procedure was somewhat more effective, it remains to be established whether this activity is due to contamination with plasma membrane vesicles which do not contain the peptidase, or whether the pyrophosphatase in fact also is a genuine Golgi component. Preliminary experiments indicate that pyrophosphatase in a heavy Golgi subfraction [26] is located in right-side-out elements, presumably plasma membrane contaminants, while activity in the Golgi secretory vesicle fraction is cryptic, as expected for a genuine Golgi component. (Huang, Y.O. and Elovson, J., unpublished results).

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