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Isolation of the dorsal, ventral and intracellular domains of HeLa cell plasma membranes following adhesion to a gelatin substrate

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The plasma membrane is a complex organelle responsible for many cellular functions. In addition to mediating the exchange of components with the extracellular fluid, the plasma membrane is involved in cell adhesion to matrix proteins in vivo and in vitro. In vitro, adherent cells have three distinct plasma membrane domains to carry out these functions: one attached to the substrate (ventral); another exposed to the media (dorsal); and an intracellular domain involved in endocytosis and secretion. A technique has been developed for the rapid isolation of these specific domains from HeLa cells immediately following adhesion to a gelatin substrate. The isolation procedure utilizes the tight binding of cationic colloidal silica to the dorsal plasma membrane domain of attached cells. Following silica binding and cell lysis, the silica-coated dorsal plasma membrane domain is readily separated from intracellular plasma membrane components by virtue of the high density of the silica pellicle, and the intact ventral plasma membrane domain remains attached to the gelatin substrate. Fluorescence and electron microscopy and biochemical studies using ^{125}I-lactoperoxidase labeling, ^{125}I-labeled wheat germ agglutinin binding, and [^{3}H]-fucose incorporation into plasma membrane glycoproteins confirmed the separation of these three topologically distinct plasma membrane domains. The fractions isolated by the technique contained essentially all of the plasma membrane components present in intact cells. This unique membrane-isolation procedure is now being used to analyze membrane flow during plasma membrane domain formation accompanying cell adhesion to an extracellular matrix.

Introduction

Cells which occupy biological interfaces in vivo, such as vascular and capillary endothelial cells and renal epithelial cells, maintain a luminal and abluminal surface. This structural polarity reflects the functional needs of the cell to adhere to the underlying tissue and to act as a selective biological barrier. In vitro many cells assume a similar polarized structured when they adhere to a substratum [1]. Thus cell adhesion is accompanied by the differentiation of the plasma membrane into dorsal and ventral plasma membrane domains which are analogous to the luminal and abluminal surfaces observed in vivo. In addition to these two external plasma membrane domains, cultured cells maintain an intracellular pool of plasma membrane components which is in equilibrium with the external plasma membrane [2]. The ability to analyze these different plasma membrane domains has been important for the understanding of the function, synthesis and maintenance of the plasma membrane during cell adhesion.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; F-actin, filamentous actin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LRBSC, lissamine rhodamine B sulfonyl chloride; Mes-buffered saline, 20 mM 4-morpholineethanesulfonic acid/135 mM NaCl, pH 6.0; phosphate-buffered saline, 15 mM sodium phosphate/140 mM NaCl (pH 7.2); PMSF, phenylmethylsulfonyl fluoride.

Most procedures developed to isolate the plasma membrane of adherent cells have been for morphological investigation of the ventral domain. Observation of this domain from cells such as polymorphonuclear leukocytes [3] or the cellular slime mold *Dictyostelium discoideum* [4] has been accomplished by merely opening up the cells with a stream of buffer. In the case of cultured cells, such as fibroblast or epithelial cells, the stream of buffer either completely detached the cells from the substrate or they were left intact unless pre-incubated with the tanning reagent $ZnCl_2$ [5–7]. Unfortunately, the tanning technique was restricted to fully spread cells that had been attached to a substratum for at least 6 h, thus precluding analysis of plasma membrane domain formation during the adhesion process. Morphological studies of the dorsal membrane have been accomplished with phagocytosing macrophages by placing a polylysine-coated cover slip on top of the cell and pulling it away [8]. To date, biochemical studies of the ventral plasma membrane of cells in culture have been restricted to the isolation of a subsection of the membrane which most likely makes up the focal adhesion sites [9–11].

The internal plasma membrane domain has been morphologically and biochemically characterized by a variety of techniques. Ultrastructural studies [12] and enzyme availability techniques [13,14] have shown that the internal plasma membrane pool is in equilibrium with the external plasma membrane. Various labeling and isolation schemes have shown that the internal plasma membrane pool has a ^{125}I-lactoperoxidase-labeled polypeptide composition very similar to the external plasma membrane domain [15–17].

The goal of this study was to develop a technique to directly analyze the dorsal, ventral and internal plasma membrane components of cells immediately following adhesion to a solid substrate. Positively-charged colloidal silica and polyacrylic acid were used to create a rigid and dense pellicle on the exposed dorsal plasma membrane domain by a modification of a technique used for the isolation of plasma membrane from cells in suspension [18]. By swelling the coated cells in a low ionic strength buffer containing 50% glycerol and then causing hypotonic lysis, the intact ventral plasma membrane domain was retained on the gelatin substrate. The high-density silica-coated dorsal plasma membrane fragments were then isolated by low-speed centrifugation of the cell lysate and the internal plasma membrane components could be obtained by sedimentation at higher g values.

Materials and Methods

Cell culture and tritium labeling. Suspension cultures of HeLa-S_3 cells were grown to mid-log phase ($(3–5)\cdot 10^5$ cells/ml) in RPMI-1640 media (K.C. Biologicals, Lenexa, KS) supplemented with 5% calf serum (Gibco, Grand Island, NY), 0.3% $NaHCO_3$, 100μg/ml dihydrostreptocycin, 60 μg/ml penicillin and 0.0002% butyl *p*-hydroxybenzoate at 37°C in a humidified 5% CO_2 incubator. Tritium labeling was accomplished by growing cells in the same media with L-[5,6-^{3}H]fucose (56 Ci/mmol; New England Nuclear, Boston, MA), or with L-[4,6(n)-^{3}H]-leucine (47 Ci/mmol; New England Nuclear) at the concentrations given in the specific experiments.

Preparation of gelatin substrates. Polystyrene tissue-culture dishes (Nunc, Kamstruck, Denmark, or Falcon, B.D. Labware, Oxnard, CA), or polystyrene cover slips (Lux, Miles Lab., Naperville, IL), were modified by the method of Jacobson and Ryan [19]. Briefly, the dishes were sulfonated with concentrated sulfuric acid (1 h at 70°C), and gelatin (type I from swine skin, Sigma Chemical Co., St. Louis, MO) was coupled to the sulfonate groups with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (Sigma). The gelatin-coated dishes were dried and stored at room temperature for up to 4 months. No differences in attachment and spreading were noted between substrates prepared with commercial gelatin or non-denatured rat tail collagen.

Cell attachment. Cells used for attachment studies were removed from culture, washed by centrifugation in ice-cold 15 mM sodium phosphate, 140 mM NaCl, pH 7.2 (phosphate-buffered saline) and then in 37°C RPMI-1640 buffered with 20 mM Hepes, pH 7.2. The cells were resuspended in 37°C Hepes-buffered RPMI-1640 and allowed to attach to the gelatin substrates at 37°C.

Scanning electron microscopy. Whole cells were fixed in 2% glutaraldehyde (Polysciences Inc., Warrington, PA) in phosphate-buffered saline for

12–24 h at 4°C. Ventral plasma membranes were fixed under conditions which stabilized cytoskeletal structures [20]. Briefly, ventral membranes were fixed in 1% glutaraldehyde, 1% paraformaldehyde (Tousimis, Rockville, MD), 50 mM lysine, 100 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 20 mM Hepes, pH 6.8, for 15 min at room temperature, followed by 45 min of fixation in fresh fixative without lysine, and then postfixed in 1% OsO_4 (Polysciences) in 100 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 20 mM Hepes, pH 6.8. All samples were dehydrated, critical-point dried, coated with gold and examined as described by Fairman and Jacobson [21].

Plasma membrane labeling with lissamine rhodamine B sulfonyl chloride. Following harvesting and washing in cold phosphate-buffered saline as described above, cells were labeled with LRBSC by a modification of the method of Geiger et al. [7]. LRBSC (Molecular Probes, Junction City, OR; 1 mg/ml in acetone) was added to cells suspended at $1 \cdot 10^6$ cells/ ml in phosphate-buffered saline, pH 8.0, to a final concentration of 20 μg/ml. After a 2 min incubation at room temperature the cells were washed extensively in phosphate-buffered saline and allowed to attach as described above. Labeled cells and ventral plasma membranes were fixed in 3% paraformaldehyde in Hanks' balanced salt solution, plus glucose, without $CaCl_2$, pH 7.2. Dorsal plasma membranes were allowed to settle onto a gelatin film (prepared by coating polystyrene with 3% gelatin) in the presence of 3% paraformaldehyde in Hanks' balanced salt solution. All samples were washed in phosphate-buffered saline, mounted in 3% *n*-propylgallate (to retard photobleaching [22]) in 90% glycerol (v/v), 10 mM sodium phosphate, pH 7.8, examined and photographed on a Leitz Dialux 20 phase-fluorescence microscope.

Rhodamine-phalloidin staining. Samples were prepared and fixed as described for LRBSC staining, washed in phosphate-buffered saline and then stained with rhodamine-phalloidin (Molecular Probes; 50 ng/ml) in phosphate-buffered saline for 30 min, washed with phosphate-buffered saline, mounted in 50% glycerol in phosphate-buffered saline and observed as described for LRBSC-labeled samples.

Labeling plasma membrane proteins with ^{125}I. Cell surface proteins were labeled with ^{125}I using lactoperoxidase by a modification of the method of Morrison [23]. Cells used for labeling were washed twice with cold phosphate-buffered saline, once with cold 135 mM sodium phosphate, pH 7.4, and then resuspended in this phosphate buffer at a concentration of $(5–7) \cdot 10^6$ cells per ml with 200 μg/ml lactoperoxidase (Calbiochem-Behring, San Diego, CA), 2 μM K ^{127}I and 400 μCi/ml ^{125}I (Amersham, Arlington Heights, IL). The labeling reaction was initiated by the addition of hydrogen peroxide to the cell suspension to yield a final concentration of 48 μM. After a 5-min incubation at room temperature, the reaction was quenched by the addition of cold phosphate-buffered saline supplemented with 10 μM $Na_2S_2O_3$. The cells were then washed with cold phosphate-buffered saline and allowed to attach as described above. Labeling resulted in a 0.3–1.5% incorporation of radioactivity and was reduced 100-fold by the elimination of hydrogen peroxide or lactoperoxidase from the labeling mixture.

Labeling monolayers with ^{125}I-labeled wheat germ agglutinin. Following attachment, cell layers were placed on ice, rinsed with ice cold Hepes-buffered RPMI-1640, pH 7.2, containing 0.1 mg/ml bovine serum albumin, and then incubated in this same medium containing wheat germ agglutinin (Sigma) labeled with ^{125}I (Amersham) by the method of Cuatrecasas [24]. After 2 min on ice, the monolayers were rinsed twice with Hepes-buffered RPMI-1640, pH 7.2, containing 0.1 mg/ml bovine serum albumin.

Proteinase treatment of labeled cells and monolayers. After labeling and washing, or following labeling, adhesion and washing, cells were incubated at 37°C in phosphate-buffered saline containing 2 mg/ml trypsin (Sigma) or in phosphate-buffered saline containing 1 mM $MgCl_2$ and 2 mg/ml pronase (Sigma). Proteolysis was stopped by the addition of an equal volume of Hepes-buffered RPMI-1640, pH 7.2, containing 5% calf serum and 0.2 mM PMSF. The cell-associated trichloroacetic acid-precipitable radioactivity was determined as described below. The percent of labeled material remaining after proteolysis was based on the trichloroacetic acid-precipitable material present in untreated cell suspensions or monolayers.

Sample preparation and biochemical analysis. When preparing membrane fractions for biochemical analysis, all washing, coating and lysing steps were performed in buffer containing 0.2 mM PMSF. Isolated ventral plasma membranes and whole cells were scraped into 5% sodium dodecyl sulfate containing 0.5 mM PMSF and 2.5 mM *N*-ethylmaleimide, and the dorsal plasma membrane fraction and the particulate cytoplasmic fraction were resuspended in the same inhibitor cocktail. All of these fractions were then heated to 75°C for 5 min and sonicated for 10 s at setting 6 on a Branson sonifier (Model W-185, Branson Ultrasonics Co., Danbury, CT). The microbeads and other insoluble materials were then removed by centrifugation at $14500 \times g$ for 5 min. DNA content was determined using a modification of the method of Kissane and Robbins [25], using calf thymus DNA as a standard. Briefly, samples and standards were ethanol-precipitated, heated for 30 min at 60°C with 0.05 ml of 0.2 g/ml of charcoal-decolorized 3,5-diaminobenzoic acid dihydrochloride (Aldrich, Milwaukee, WI), then diluted with 1 M HCl, and the fluorescence intensity of the deoxyribose derivative was measured. Protein was determined by the method of Lowry et al. [16], using bovine serum albumin as a standard; and protein-associated radioactivity was determined by precipitation in cold 10% trichloroacetic acid, using bovine serum albumin as a carrier (^{125}I-containing samples were precipitated in the presence of 50 mM K ^{127}I). Trichloroacetic acid-precipitated tritium samples were collected on nitrocellulose filters, dissolved in 2-ethoxyethanol and counted in a scintillation counter. Trichloroacetic acid-precipitated ^{125}I samples were collected by centrifugation and counted in a gamma counter.

Results

Membrane domain isolation

The method used for the isolation of the plasma membrane domains of cells attached to a solid substratum is shown in Fig. 1. Following attachment to a gelatin substrate, the cell monolayer was washed twice with phosphate-buffered saline, once with Mes-buffered saline, and incubated for 10 s with a 1% colloidal suspension of positively charged silica (30–50 nm diameter [18])* in Mes-buffered saline. Following removal of the excess silica and washing with Mes-buffered saline, the cell layers were incubated for 10 s in 1 mg/ml polyacrylic acid (M_r 50000, Polysciences) in Mes-buffered saline. The coating steps were concluded by removal of the excess polyacrylic acid followed by two washes with Mes-buffered saline and one with phosphate-buffered saline. At this stage in the procedure, scanning electron microscopy showed that the smooth surface and filopodia of attached HeLa cells (Fig. 2a and b) were coated by a continuous layer of silica microbeads (Fig. 2c and

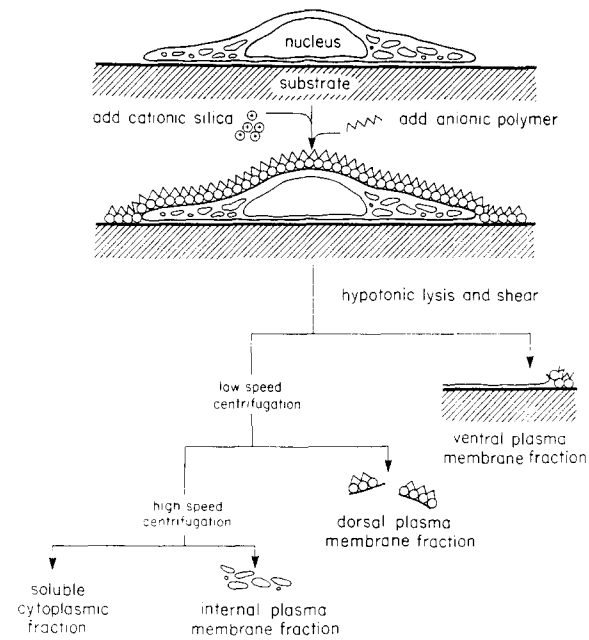
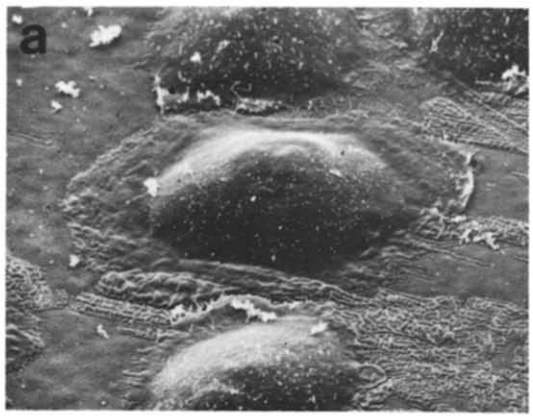


Fig. 1. Schematic diagram of the silica microbead method for the isolation of dorsal, ventral and internal plasma membrane fractions of adherent HeLa cells. Following adhesion, cell monolayers were coated with silica and overcoated with polyacrylic acid. This silica coating permitted the rigid dorsal plasma membrane to be sheared from the hypotonically lysed cell and sealed the edges of the ventral plasma membrane, preventing its removal from the substrate. Following lysis and shearing, the silica-coated dorsal plasma membrane fragments were isolated by low-speed centrifugation and the cytoplasmic plasma membrane fraction containing intracellular plasma membrane components was then sedimented using high-speed centrifugation.

* Positively charged colloidal silica will be provided to interested investigators upon request.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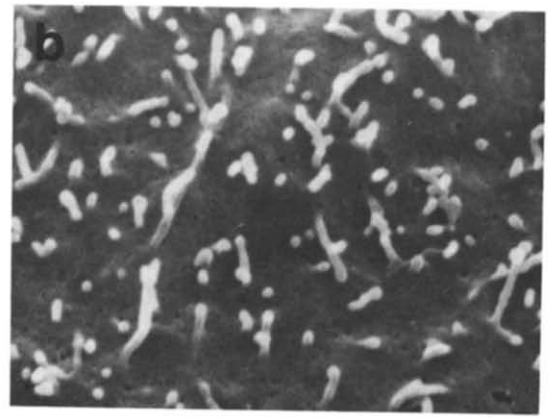
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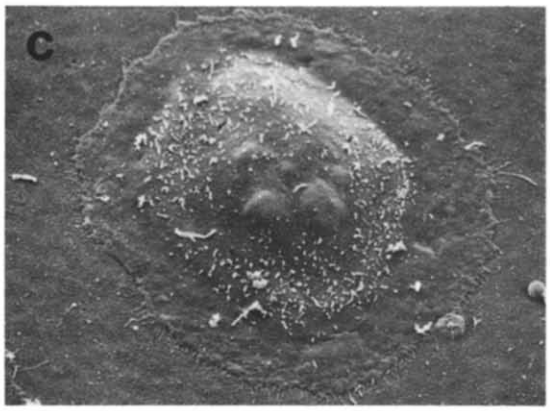
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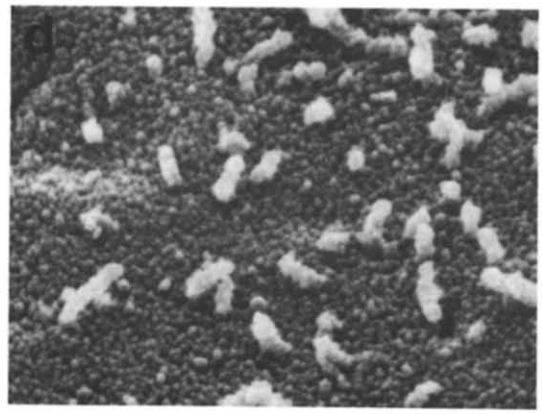
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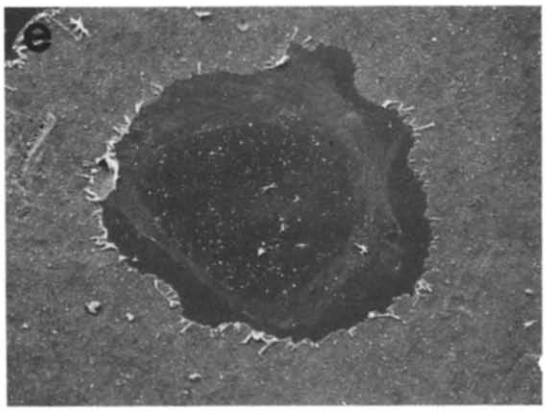
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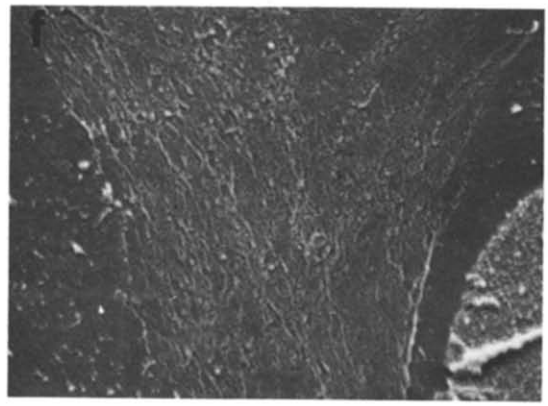
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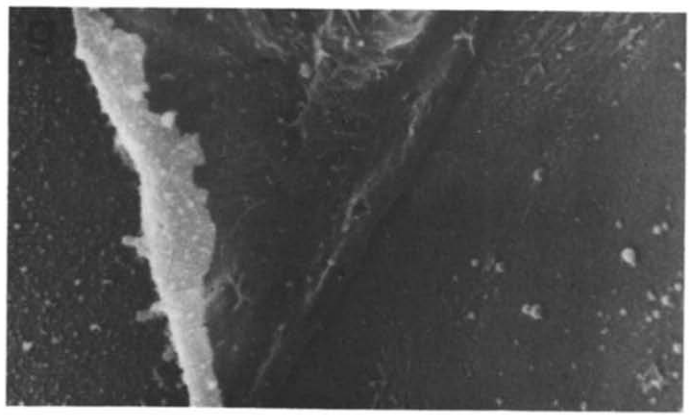
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d). These micrographs also revelaed that the coating procedure did not alter the gross morphology of the cell.

Lysis of the coated cells was accomplished by a combination of shear and hypotonic shock. The cells were incubated for 5 min at room temperature in a 50% (v/v) glycerol solution buffered with 2.5 mM imidazole at pH 7.25. The glycerol solution was removed and the cell layer was quickly rinsed with cold 2.5 mM imidazole, pH 7.25, and then covered with 1 ml of the same hypotonic buffer, causing cell lysis. The coated dorsal plasma membrane fragments and intracellular components were sheared from each 35-mm diameter culture dish by 12 successive squirts of the hypotonic incubation buffer forced from a 5-ml syringe equipped with a blunt 18-gauge needle. Morphological analysis revealed that this treatment resulted in a satisfactory removal of the dorsal plasma membrane domain with little damage to the ventral plasma membrane. (When large dorsal plasma membrane sheets were required, the syringe was replaced with a pasteur pipette.) The ventral plasma membranes which remained on the culture dish were then washed two times with cold phosphate-buffered saline. Fig. 2e is a low-magnification scanning electron micrograph of a ventral plasma membrane isolated by the silica microbead technique. The ring of light-colored materials which occupies the perinuclear region of the former cell has been tentatively identified as a cytoskeletal structure based on its co-distribution with rhodamine-phalloidin stained structures (Fig. 4b) and its fibrillar appearance under higher magnification (Fig. 2f). The vesicular structures seen in the central region of the isolated ventral plasma membranes (Fig. 2e, f and g) were similar in size to the clathrin-coated an uncoated vesicles associated with the ventral plasma membranes of macrophages [20] and HeLa Cells [27]. Fig. 2g is a micrograph of a dorsal plasma membrane fragment generated by incompletely shearing a hypotonically lysed monolayer. This micrograph shows that the silica microbeads remained attached to the external surface of the dorsal plasma membrane and stabilized it during cell rupture.

Immediately following cell lysis the lysate was centrifuged in a clinical centrifuge for 10 min at $970 \times g$ yielding a pellet containing the silica-coated dorsal plasma membrane fragments and a supernatant containing the cytoplasmic fraction. This cytoplasmic fraction was further divided by centrifugation in a microfuge at higher speeds (15 min at $14\,500 \times g$), to obtain the particulate cytoplasmic fraction, which contained intracellular plasma membrane components, and the soluble cytoplasmic fraction. All fractions were then immediately fixed for microscopy or prepared for biochemical analysis (see Methods).

Fluorescent labeling of cell surface proteins with LRBSC prior to membrane isolation demonstrated that continuous plasma membrane sheets were obtained using the silica microbead technique. The entire ventral plasma membrane domain was brightly stained with LRBSC (Fig. 3a) even though only the perimeter of the isolated ventral plasma membrane was visible under phase-contrast optics. The use of this label demonstrated that a homogeneous population of intact ventral plasma membranes, devoid of large attached dorsal fragments or other cellular debris, were isolated by the silica microbead technique. The bright ring of material at the edge of the ventral plasma membrane shown in Fig. 3a was identified as dorsal plasma membrane which had remained to the substrate. The bright spots in the center of the ventral plasma membrane presumably represent vesicles (internalized following labeling with LRBSC) similar to those observed using scanning electron microscopy (Fig. 2e, f and g). Fig. 3b shows a dorsal plasma

Fig. 2. Scanning electron micrographs of HeLa cells which had attached to a gelatin substrate for 1 h and the plasma membrane fragments isolated from cells which had attached under the same conditions. (a) An untreated cell; ×1230. (b) Higher magnification of the dorsal cell surface of an untreated cell; ×12300. (c) A cell coated with silica and polyacrylic acid; ×1230. (d) Higher magnification of the dorsal surface of a silica and polyacrylic acid coated cell showing the continuous microbead coat; ×12300. (e) Micrograph of a ventral plasma membrane showing the perinuclear cytoskeletal material and membrane associated vesicles; ×1230. (f) Higher magnification of a ventral plasma membrane showing the collapsed cytoskeletal fibers and vesicular structures; ×5980. (g) A large fragment of dorsal plasma membrane which remained attached to the substrate due to incomplete shearing. The right side of this micrograph shows the ventral plasma membrane, the center of the micrograph shows the cytoplasmic surface of the dorsal plasma membrane, and the left side of the micrograph shows the microbead coating of the external dorsal plasma membrane; ×5980.

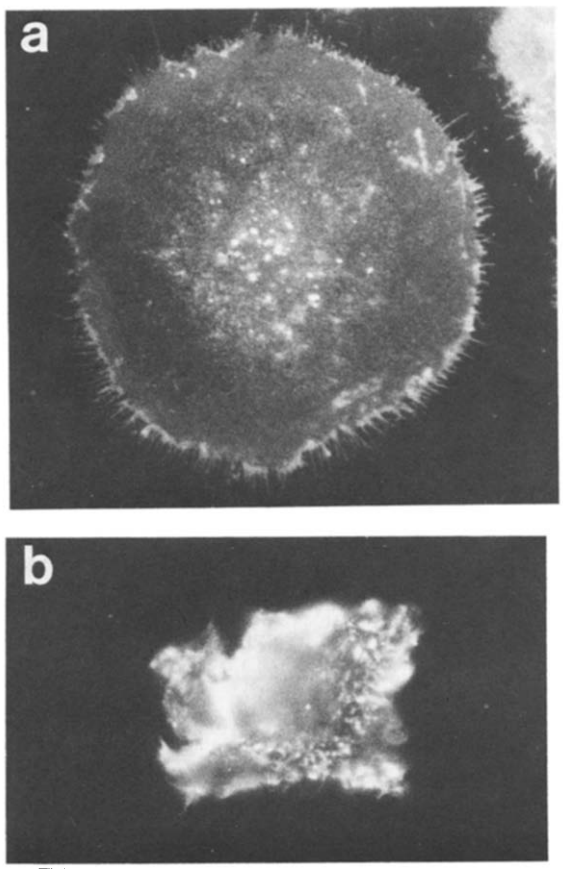

Fig. 3. Fluorescence micrographs of plasma membranes isolated from LRBSC-labeled HeLa cells which had been allowed to attach to a gelatin substrate for 1 h. (a) A ventral plasma membrane; ×765. (b) A dorsal plasma membrane fragment; ×765.

membrane fragment isolated from cells prelabeled with LRBSC. Although specific structures were not visible in these dorsal plasma membrane preparations, the labeled material was in the form of open sheets similar to those obtained using silica microbeads for the isolation of plasma membrane from cell suspensions of *D. discoideum* [18].

Actin-plasma membrane association

Filamentous actin (F-actin) present in whole cells and plasma membrane fragments was stained with rhodamine-phalloidin, one of several phalloidin derivatives which have been used to visualize F-actin [6,28]. Fig. 4a shows that three distinct F-actin structures were present in HeLa cells which had spread on a gelatin substrate for 1 h. These

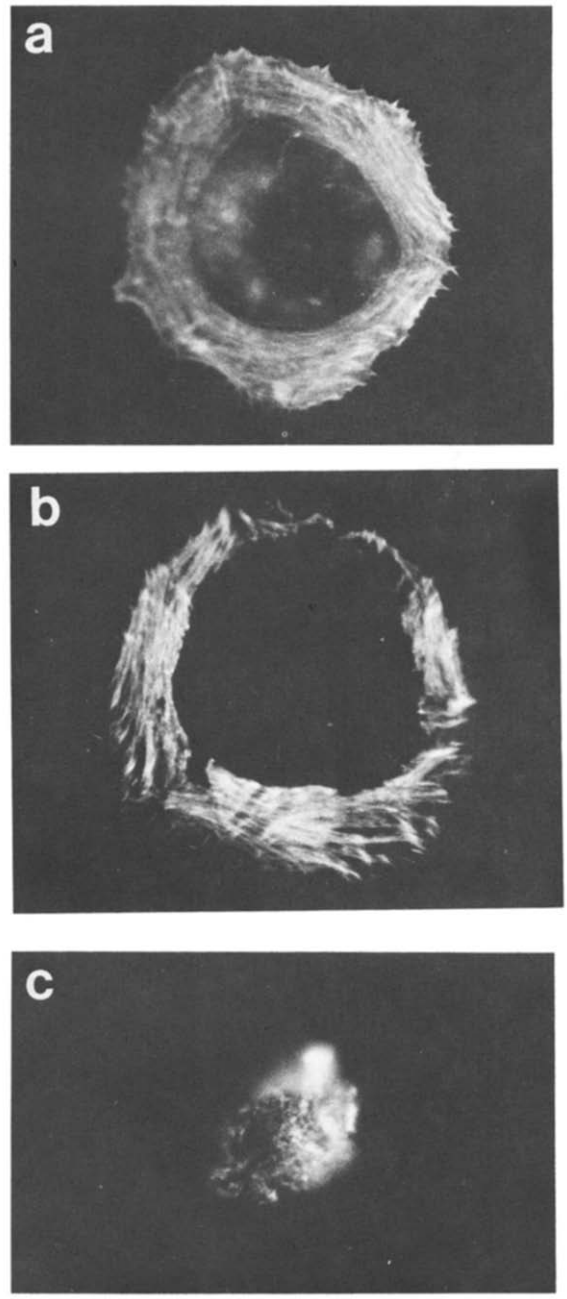

Fig. 4. Rhodamine-phalloidin staining of F-actin aggregates in HeLa cells and plasma membrane fragments isolated by the silica microbead procedure. (a) The F-actin staining-pattern present in an intact HeLa cell which had attached to a gelatin substrate for 1 h; ×900. (b) The F-actin staining-pattern seen in about 20% (see text) of the ventral membranes isolated from HeLa cells which had attached to a gelatin substrate for 1 h; ×900. (c) Typical F-actin staining-pattern seen in dorsal plasma membrane fragments isolated from HeLa cells which had attached to a gelatin substrate for 1 h; ×900.

structures included large transcellular microfilaments, filopodia which extended radially from the perimeter of the cell, and F-actin aggregates present beneath the cell nucleus which Carley et al. [29] have identified as a characteristic structure present in many other types of transformed cells. Fig. 4b shows a ventral plasma membrane which has retained the filopodial fibers and the large transcellular microfilaments found in intact cells. All of the ventral plasma membranes isolated exhibited some of the radial filopodia although only about 20% of the isolated plasma membranes contained the large microfilament arrays shown in Fig. 4b. This finding may reflect the fact that these transformed cells do not elaborate an extensive cytoskeletal network which is tightly bound to the plasma membrane after 1 h of adhesion. None of the isolated ventral plasma membranes contained the large subnuclear F-actin aggregates present in intact cells. Fig. 4c shows that dorsal plasma membrane fragments also contained F-actin, although specific structures could not be resolved in these preparations.

Biochemical analysis

Table I shows typical values for protein recoveries in the dorsal, ventral and cytoplasmic fractions. The data in Table I include results from the Lowry protein assay and from the determination of trichloroacetic acid-precipitable [^{3}H]leucine label. The protein recoveries based on the two assays were similar for all of the fractions examined except for the ventral plasma membrane. The disordance for the ventral fraction could have been due to the fact that Lowry-positive material was released from gelatin-coated dishes that did not have cells and were treated with silica and polyacrylic acid. It is unlikely that the Lowry-positive material was gelatin, since it was not present in SDS-polyacrylamide gels of the extract of the dishes. Additionally, the amount of protein present in the ventral plasma membrane samples as estimated by either Coomassie blue or silver staining of SDS-polyacrylamide gels was always much less than that determined using the Lowry assay (not shown). The protein content of the ventral plasma membrane fraction measured by the Lowry

TABLE I

PROTEIN CONTENT IN THE FRACTIONS AND LYSIS BUFFERS OBTAINED USING THE SILICA MICROBEAD PROCEDURE: COMPARISON OF PROTEIN DETERMINATION BASED ON THE LOWRY ASSAY AND TRICHLOROACETIC ACID-PRECIPITABLE [^{3}H]LEUCINE LABEL

Samples were derived from cells labeled for 48 h in the presence of [^{3}H]leucine (see Methods) and then allowed to attach to a gelatin substrate for 1 h at 37°C. Membrane fractions were prepared from five 35-mm dishes containing $4.5 \cdot 10^5$ cells each. Protein content was determined by the Lowry assay and [^{3}H]leucine-labeled protein was determined by trichloroacetic acid precipitation (see Methods). Recoveries were based on the amount of protein or ^{3}H-label present in the coated monolayer samples. The coated monolayer samples were prepared from two 35-mm dishes containing $4.5 \cdot 10^5$ cells and an average of 0.16 mg of cellular protein per dish. n.d., not determined due to low protein concentration and the presence of high concentrations of interfering substances.

Sample	Protein recovered using the Lowry assay (%)	Protein recovered using [^{3}H]leucine labeling (%)
Coated monolayer	(100)	(100)
Ventral plasma membrane fraction (V)	12.6	5.0
Dorsal plasma membrane[a] fraction (D)	7.1	6.6
Particulate cytoplasmic fraction (P)	9.9	10.2
Soluble cytoplasmic fraction (S)	43	47
Fractions V + D + P + S	72	69
50% glycerol incubation	n.d.	5.5
2.5 mM imidazole rinse	n.d.	14.6
Final phosphate-buffered saline washes	n.d.	1.6
Fraction V + D + P + S + 50% glycerol incubation + imidazole rinse + phosphate-buffered saline washes	–	91

[a] In this experiment, the dorsal plasma membrane fraction was obtained by a lower-speed centrifugation (670 × g) for 4 min.

assay exceeded the [^{3}H]leucine-labeled protein content for this fraction by more than 2-fold and corrections were made to take this into account when determining degrees of ventral membrane purification.

The protein content of the dorsal and particulate cytoplasmic samples varied from preparation to preparation, with each fraction comprising 7–14% of the protein recovered from whole cells (Table I). The total amount of protein recovered in the combined fractions (V + D + P + S) ranged from 75 to 90% of the amount of protein present in coated monolayer samples, with the greatest variability appearing in the protein recovered in the cytoplasmic fractions. The [^{3}H]leucine label recovered in the pre-lysis glycerol incubation step and the imidazole rinse accounts for the protein missing from the combined fractions and may explain the variability observed in the protein content of the cytoplasmic fractions. [^{3}H]Leucine labeling also facilitated the direct analysis of the protein content of the coating buffers, and showed that the silica and polyacrylic acid caused no release of protein from the cells (data not shown). Finally, [^{3}H]leucine label content was used to demonstrate that the solubilization techniques utilized for the preparation of samples for biochemical analysis (see Methods) recovered over 95% of the [^{3}H]leucine-labeled protein present in each fraction (data not shown).

The DNA content of the ventral plasma membrane fraction was less than 3% of that present in whole cells, while 15% of the DNA was isolated in the dorsal plasma membrane fraction, and 25% of the DNA was recovered in the particulate cytoplasmic fraction. The relatively high levels of DNA found in the latter two fractions were probably due to co-sedimentation of nuclei with the plasma membrane components.

Recovery of plasma membrane labels

Table II details the recovery of plasma membrane components in the fractions isolated by the silica microbead isolation procedure. The first column of Table II shows the recovery of bound ^{125}I-labeled wheat germ agglutinin. Since this ligand was only allowed to bind to the surface of the spread cells, it provided a label for the external *N*-acetylglucosamine-containing glycoproteins, and thus should be completely excluded from the cytoplasmic components of the plasma membrane. The results in Table II demonstrated that the isolation procedure was capable of recovering 95% of the external *N*-acetylglucosamine-containing glycoproteins in the external plasma membrane fractions (D + V). The higher recovery of the ^{125}I-labled wheat germ agglutinin label in the dorsal versus the ventral plasma membrane fraction was probably due to the limited ability of wheat germ agglutinin to diffuse underneath the attached cells. The second column in Table II shows the recovery of proteins enzymatically labeled with ^{125}I (using

TABLE II

RECOVERY OF DIFFERENT PLASMA MEMBRANE LABELS IN FRACTIONS OBTAINED USING THE SILICA MICROBEAD ISOLATION PROCEDURE

Samples were derived from cells incubated with ^{125}I-labeled wheat germ agglutinin for 2 min on ice following adhesion, cells labeled with ^{125}I using lactoperoxidase prior to adhesion, and cells labeled with 2 μCi/ml [^{3}H]fucose for 24 h prior to adhesion. In all cases cells were allowed to attach to a gelatin substrate for 1 h at 37°C. Radioactive label content of each fraction was determined by trichloroacetic acid precipitation (see Methods). Percent recoveries were based on the amount of label present in the combined fractions.

Fraction	Bound ^{125}I-wheat germ agglutinin recovered (%)	^{125}I-lactoperoxidase label recovered (%)	[^{3}H]fucose glycoproteins recovered (%)
Ventral plasma membrane fraction (V)	28	34	23
Dorsal plasma membrane fraction (D)	67	38	23
Particulate cytoplasmic fraction (P)	4	13	26
Soluble cytoplasmic fraction (S)	1	13	27

TABLE III

PROTEOLYTIC SENSITIVITY OF [^{3}H]FUCOSE-LABELED GLYCOPROTEINS AND ^{125}I-LACTOPEROXIDASE-LABELED PROTEINS IN CELLS BEFORE AND AFTER ADHESION TO A GELATIN SUBSTRATE

^{125}I-lactoperoxidase-labeled samples were derived from cells labeled with ^{125}I-lactoperoxidase as described in the Methods, and then either directly proteolysed, or allowed to attach to gelatin substrates for 1 h at 37°C prior to proteolytic treatment. [^{3}H]Fucose-labeled samples were derived from cells labeled with 0.7 μCi/ml [^{3}H]fucose for 24 h prior to attachment to gelatin substrates for 1 h at 37°C (see Methods). Percent label remaining in each fraction was determined by dividing the cell-associated trichloroacetic acid-precipitable label remaining after proteolysis by the trichloroacetic acid-precipitable label present in untreated cell suspensions or monolayers.

Proteolytic condition	^{125}I remaining in freshly labeled cells (%)	^{125}I remaining in attached cells (%)	[^{3}H]Fucose-label remaining in attached cells (%)
2 mg/ml trypsin for 20 m at 37°C	8	34	59
2 mg/ml pronase for 20 m at 37°C	9	27	53

lactoperoxidase) prior to cell adhesion. A maximum of 72% of the total cellular label was recovered in the the external plasma membrane fractions obtained from these cells. These results are consistent with other studies on the rate of plasma membrane internalization [2,13], and were supported by proteolysis studies (Table III). The concentrations of Pronase and trypsin used in the experiments in Table III released the cells from the gelatin substrate without reducing cell viability, thus exposing the entire cell surface to the proteinases. These results (Table III) show that the two proteinases could remove over 90% of the ^{125}I label from freshly labeled cells, but only about 70% of the label from cells which had been incubated at 37°C for 1 h following labeling, suggesting that 20–30% of this cell surface label had been internalized during the 1 h of adhesion to the substrate.

The last column in Table II contains the re-

TABLE IV

RECOVERY AND PURIFICATION OF ^{125}I-LACTOPEROXIDASE-LABELED PROTEINS AND [^{3}H]FUCOSE-CONTAINING GLYCOPROTEINS IN FRACTIONS OBTAINED USING THE SILICA MICROBEAD PROCEDURE

Samples were derived from cells labeled with ^{125}I using lactoperoxidase and then allowed to attach to a gelatin substrate for 1 h at 37°C or from cells labeled for 24 h in the presence of 15 μCi/ml of [^{3}H]fucose prior to attachment to a gelatin substrate. Protein content was determined by the Lowry assay and radiolabel content was determined by trichloroacetic acid-precipitation (see Methods). Values from ^{125}I-labeling experiments are shown ± S.E. with the number of experiments included the parentheses. Percent recoveries were based on the radio-label present in the coated monolayer samples. Fold enrichment was calculated by dividing the specific activity (radio-label/mg protein) of each fraction by the specific activity of the coated monolayer samples.

Sample	^{125}I-lactoperoxidase labeled proteins		[^{3}H]Fucose glycoproteins	
	^{125}I-label recovered (%)	fold enrichment	^{3}H-label recovered (%)	fold enrichment
Coated monolayer (H)	(100)	(1)	(100)	(1)
Ventral plasma membrane fraction (V)	30 ± 2.3 (3)	6.0 ± 0.98 (3)[b]	23	3.6[b]
Dorsal plasma membrane fraction (D)	29 ± 1.4 (3)[a]	3.8 ± 0.55 (3)[a]	26	2.0
Particulate cytoplasmic fraction (P)	20 ± 5 (2)	1.7 ± 0.35 (2)	23	2.2
Soluble cytoplasmic fraction (S)	11 ± 2 (2)	0.3 ± 0 (2)	26	0.27
Combined fractions	94 ± 8.4 (3)	–	98	–

[a] In these particular experiments, the dorsal plasma membrane fraction was obtained by a lower-speed centrifugation (670 × g) for 4 min.

[b] These values were corrected for the consistent 2-fold overestimate of protein content determined by the Lowry assay that occurred for the ventral membrane fraction (See Results, and Table I).

covery data of proteins labeled with [^{3}H]fucose, a sugar which has been reported to preferentially label HeLa cell plasma membrane glycoproteins [30]. Less than 50% of the [^{3}H]fucose-labeled glycoproteins present in HeLa cells were recovered in the external plasma membrane fractions generated by the silica microbead technique. The implication that less than half of this label was present on the cell surface was supported by the proteolytic-sensitivity data in Table III which showed that only about 45% of the fucose label could be removed by proteolysis.

Purification of plasma membrane labels

Table IV shows the plasma membrane fractions isolated by the silica microbead procedure were enriched with ^{125}I-lactoperoxidase-labeled proteins and [^{3}H]fucose-labeled glycoproteins. The fold enrichments of [^{3}H]fucose label in the external plasma membrane fraction were lower than those obtained for the ^{125}I-lactoperoxidase-labeled proteins, consistent with the lower percent of the [^{3}H]fucose-labeled glycoproteins present on the cell surface. The recovery results showed that, unlike the recovery of total cellular proteins, all of the [^{3}H]fucose-labeled glycoproteins and ^{125}I-lactoperoxidase labeled proteins were recovered in the combined fractions.

Although a portion of each label was recovered in the soluble cytoplasmic fraction, more than half of this labeled materials could be sedimented by centrifugation in a Beckman Airfuge at 150 000 $\times$ g for 10 min (data not shown), suggesting that it was in the form of small membrane vesicles. In addition, direct analysis showed that no [^{3}H]fucose-labeled glycoproteins were released by the pre-lysis glycerol incubation step or imidazole rinse, offering further evidence that all of this label was in a membrane-bound form.

Discussion

The plasma membrane is a complex organelle responsible for many cellular functions. In culture the ventral region of the plasma membrane anchors the cell to a solid substratum [9,10,31], the dorsal plasma membrane may serve as a selective biological barrier [1], and an internal pool contains plasma membrane components in equilibrium with the cell surface [2]. A technique which permits the isolation of ventral and dorsal plasma membrane fractions of HeLa cells attached to a solid substrate has been developed. This isolation technique consists of coating the cell's dorsal surface with colloidal silica and polyacrylic acid, hypotonically lysing the cell and shearing off the dorsal plasma membrane sheets. Under the conditions employed, the ventral plasma membrane fraction was then isolated by virtue of its tight association with the substrate, and the dorsal plasma membrane fraction and cytoplasmic fractions which contained plasma membrane components were obtained by differential-centrifugation of the cell lysate (see Fig. 1).

Scanning electron microscopy (Fig. 2e and f) and direct fluorescent staining of plasma membrane proteins (Fig. 3a) demonstrated that intact ventral plasma membrane domains free of large dorsal plasma membrane fragments were obtained using this procedure. The microscopic techniques also revealed vesicular structures associated with the ventral plasma membrane domain similar to those seen in intact HeLa cells [27] which could be involved with plasma membrane differentiation, endo- or exocytosis. In addition to the vesicular structures, scanning electron micrographs of the isolated ventral plasma membranes (Fig. 2e and f) contain what appear to be cytoskeletal structures. Staining of ventral membranes and whole cells with the F-actin specific probe rhodamine-phalloidin (Fig. 4) confirmed that a specific subset of the F-actin-containing structures was present in the ventral plasma membrane domains isolated by the silica microbead technique, consistent with results obtained using other techniques [3–8,20].

Two iodine labeling techniques were used to mark different domains of the HeLa cell plasma membrane. ^{125}I-labeled wheat germ agglutinin, a lectin which binds to *N*-acetylglucosamine residues of cell surface glycoproteins, was used to indirectly label the dorsal and ventral plasma membrane domains of attached cells. Since this lectin was added following cell adhesion, it provided an excellent label for the dorsal plasma membrane domain, a partial label for the ventral plasma membrane domain, and no labeling of the intracellular plasma membrane pool. Proteins present in the external plasma membrane domain of HeLa cells

were directly labeled with ^{125}I using lactoperoxidase. Since this cell surface label was added prior to cell adhesion, it should have been equally distributed between the dorsal and ventral plasma membrane domains during cell adhesion. In addition to the ^{125}I-lactoperoxidase label in the external plasma membrane domains, a portion of this label should have entered in the intracellular plasma membrane pool via the endocytic pathway [2]. Proteolytic digestion (Table III) confirmed that the procedure used for lactoperoxidase-catalyzed iodination resulted in HeLa cell surface labeling, and that 20–30% of this label became resistant to proteolysis following cell adhesion. This measured endocytic rate is well within the range of rates measured in many cultured cells [2], although it was about twice as fast as the rate determined by Fishman and Cook [13] for HeLa cells growing in suspension culture. One possibile explanation for the differences in these endocytic rates may be a stimulation of membrane shuttling during cell adhesion.

Metabolic incorporation of [^{3}H]fucose was used to achieve a steady-state labeling of all plasma membrane components, since the iodination techniques utilized did not provide uniform labeling of the entire plasma membrane. This sugar is especially useful for labeling the HeLa cell plasma membrane because it is preferentially incorporated in to plasma membrane glycoproteins with little transformation into other sugars, glycogen or amino acids [30]. Proteolytic digestion of HeLa cells labeled with [^{3}H]fucose showed that over half of the [^{3}H]fucose labeled glycoproteins were resistant to proteolysis, suggesting that the intracellular plasma membrane domain represented over 50% of the total HeLa cell plasma membrane. These results agree well with data which show that the intracellular plasma membrane domain of HeLa cells is twice the size of the external plasma membrane domain [13].

Quantitative analysis of the plasma membrane component present in the isolated HeLa cell fractions indicated that high-yield fractions containing the dorsal, ventral and intracellular plasma membrane domains were obtained using the silica microbead technique. The recovery of only 5% of the surface-bound ^{125}I-labeled wheat germ agglutinin in the cytoplasmic fractions (Table II) showed that components of the external plasma membrane did not significantly contaminate the cytoplasmic fractions isolated by this technique. In the absence of labels which specifically marked the intracellular plasma membrane domain, the cellular distribution and recovery of plasma membrane labels was used to assess the removal of intracellular plasma membrane components from the dorsal and ventral plasma membrane fractions. As shown in Table II, the recoveries of ^{125}I-lactoperoxidase-labeled proteins and [^{3}H]fucose-labeled glycoproteins in the cytoplasmic fractions were in near perfect agreement with the intracellular distribution of these labels shown by proteolytic digestion (Table III), providing strong evidence that the intracellular plasma membrane components were separated from the external plasma membrane domains. The data presented in Table II indicate that the ^{125}I-lactoperoxidase label and [^{3}H]fucose label present in the external plasma membrane fractions were equally divided between the dorsal and ventral plasma membrane fractions, consistent with the observed surface area of these two plasma membrane domains in the spread cell. Resolution of individual labeled protein components in these fractions indicated that several proteins were unequally distributed between these two plasma membrane domains (Mason, P.M. and Jacobson, B.S., unpublished data). These results, which were consistent with the morphological data shown in Figs. 2 and 3, provide further evidence that the silica microbead technique completely separated the dorsal and ventral plasma membrane domains of the attached cells.

Preliminary results suggest that the cytoplasmic fraction obtained from the cell lysate following removal of the silica-coated dorsal plasma membrane domain may serve as a valuable source of easily separable intracellular membrane vesicles. The labeled plasma membrane material present in the cytoplasmic fractions could be fractionated by centrifugation. As shown in Table II, half of the ^{125}I-lactoperoxidase-labeled cell surface proteins and [^{3}H]fucose-labeled glycoproteins present in the cytoplasmic fraction could be pelleted at moderate centrifugal forces, yielding a particulate (P) and a soluble (S) cytoplasmic fraction. Furthermore, over 50% of the labeled material present in the soluble cytoplasmic fraction(s) could be sedi-

mented by higher-speed centrifugation (see Results).

The external plasma membrane fractions isolated with the silica microbead technique were biochemically enriched with ^{125}I-lactoperoxidase-labeled cell surface proteins and [^{3}H]fucose-labeled glycoproteins (Table IV). The enrichment obtained for the dorsal plasma membrane fraction was 2–4-fold, while the purification achieved for the ventral plasma membrane fraction was 4–6-fold based on the expected protein content of this fraction (see Table I and IV). It seems likely that the extensive purification of external plasma membrane components may be prevented by the existence of the surface lamina, identified by Ben-Ze'ev et al. [32]. Despite the limitation to the purification of components, the silica microbead technique will be useful for studying the redistributioin of plasma membrane components between the dorsal and ventral domains of the cell surface and the intracellular plasma membrane pool during cell adhesion. Preliminary evidence has shown that specific plasma membrane components segregate between these plasma membrane domains during HeLa cell adhesion to a gelatin substrate. It is hoped that this approach to the problem of intracellular shuttling of plasma membrane components will be useful in the biochemical study of transendocytosis by vascular endothelial cells and the directionality of protein secretion by secretory cells.

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References

1 Rodriguez-Boulan, E. (1983) in Modern Cell Biology, Vol. 1. (Satir, B.H., ed.), pp. 119–170, Alan R. Liss, New York
2 Steinman, R.M., Mellman, L.S., Muller, W.A. and Cohn, Z.A. (1983) J. Cell Biol. 96, 1–27
3 Boyles, T. and Bainton, D.F. (1981) Cell 24, 905–914
4 Clarke, M., Schatten, G. Mazia, D. and Spudich, J.A. (1975) Proc. Natl. Acad. Sci. USA 72, 1758–1762
5 Avnur, Z. and Geiger. B. (1981) J. Mol. Biol. 153, 361–379
6 Avnur, Z., Small, J.V. and Geiger, B. (1983) J. Cell Biol. 96, 1622–1630
7 Geiger, B., Avnur, Z. and Schlessinger, J. (1982) J. Cell Biol. 93, 495–500
8 Aggeler, J. and Werb, Z. (1982) J. Cell Biol. 94, 613–623
9 Cathcart, M.K. and Culp, L.A. (1979) Biochim. Biophys. Acta 556, 331–343
10 Laterra, J., Lark, M.W. and Culp, L.A. (1982) in Extracellular Matrix (Hawks, S. and Wang, J.L., ed.), pp. 121–125, Academic Press, New York
11 Shriver, K. and Rohrschneider, L. (1981) J. Cell Biol. 89, 525–535
12 Steinman, R.M., Brodie, S.E. and Cohn, Z.A. (1976) J. Cell Biol. 68, 665–687
13 Fishman, J.B. and Cook, J.S. (1982) J. Biol. Chem. 257, 8122–8129
14 Widnell, C.C., Schneider, Y.-J., Pierre, B., Baudhuin, P. and Trouet, A. (1982) Cell 28, 61–70
15 Hubbard, A.L. and Cohn, Z.A. (1975) J. Cell Biol. 64, 461–479
16 Mellman, I.S., Steinman, R.M., Unkeless, J.C. and Cohn, Z.A. (1980) J. Cell Biol. 86, 712–722
17 Muller, W.A., Steinman, R.M. and Cohn, Z.A. (1980) J. Cell Biol. 86, 292–303
18 Chaney, L.K. and Jacobson, B.S. (1983) J. Biol. Chem. 258, 10062–10072
19 Jacobson, B.S. and Ryan, U.S. (1982) Tissue Cell 14, 69–83
20 Aggeler, J., Takemura, R. and Werb, Z. (1983) J. Cell Biol. 97, 1452–1458
21 Fairman, K. and Jacobson, B.S. (1983) Tissue Cell 15, 167–180
22 Giloh, H. and Sedat, J.W. (1982) Science 217, 1252–1255
23 Morrison, M. (1974) Methods Enzymol. 32, 103–109
24 Cuatrecasas, P. (1973) Biochemistry 12, 1312–1323
25 Kissane, J.M. and Robins, E. (1958) J. Biol. Chem. 233, 184–188
26 Lowry, O.H., Rosebrough, N.J., Farr A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
27 Maupin, P. and Pollard, T.D. (1983) J. Cell Biol. 96, 51–62
28 Barak, L.S., Yocum, R.R., Nothnagel, E.A. and Webb, W.W. (1980) Proc. Natl. Acad. Sci. USA 77, 980–984
29 Carley, W.W., Barak, L.S. and Webb, W.W. (1981) J. Cell Biol. 90, 797–802
30 Atkinson, P.A. and Summers, D.F. (1971) J. Biol. Chem. 246, 5162–5175
31 Rodriguez-Boulan, E., Paskiet, K.T. and Sabatini, D.D. (1983) J. Cell Biol. 96, 866–874
32 Ben-Ze'ev, A., Duerr, A., Solomon, F. and Penman, S. (1979) Cell 17, 859–865