RESTRICTION OF PATCHING OF BOUND CONCANAVALIN A AFTER INCORPORATION OF ARACHIDONIC ACID INTO THE PLASMA MEMBRANE OF VIRALLY TRANSFORMED FIBROBLASTS

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ABSTRACT

Topographical distribution of concanavalin A binding sites (CABS) was studied in two lines of virally transformed fibroblasts as a function of fatty acid composition. Fatty acid composition was manipulated by incubating cells in fatty acid, ATP, CoA, and delipidated fetal calf serum (FCS).

VLM cells grown in medium containing 5% FCS have a clustered CABS distribution. Plasma membrane vesicles (PMVs) derived from these cells have an arachidonate content of 1.7%. Elevation of PMV arachidonate to 15.8% results in a marked restriction of CABS patching, while elevation to 6.8% is associated with intermediate restriction of patching. Restriction of patching is associated with increased microviscosity. CABS of Rous sarcoma virus-transformed chicken embryo fibroblasts (RSV-CEF) are also responsive to arachidonate enrichment medium. Whereas untreated cells have a clustered CABS distribution, cells incubated for 24 h in arachidonate enrichment medium have predominantly a dispersed CABS distribution. In both VLM cells and RSV-CEF, ATP, CoA, and delipidated FCS alone have no effect upon CABS mobility.

Inhibition of CABS patching is also observed when aspirin is included in the arachidonate enrichment medium but not when the cells are incubated in prostaglandins, thus suggesting that the restriction of CABS mobility is not mediated by prostaglandins. Other fatty acids (palmitate, oleate, nonadecanoate) failed to restrict CABS movement. The inhibition of CABS mobility is independent of cell shape change.

KEY WORDS arachidonic acid · Con A binding sites · glycoprotein mobility · patching · plasma membrane lipid composition · transformed fibroblasts

Ligand-induced receptor site redistribution may be important in ligand-receptor function and in modulation of cell-cell and cell-microenvironment interactions (2, 7, 10, 16, 19, 33). Transformed fibroblasts provide an excellent tool to study ligand-induced glycoprotein redistribution. In this respect, glycoproteins such as concanavalin A (Con A) binding sites (CABS) are more mobile in the plasma membrane of most transformed fibro-

blasts as compared to parental normal, early passage fibroblasts. Such mobility allows Con A, a tetramer at physiological pH, to cross-link its receptors into patches on most transformed cells (6, 22, 23, 31, 32). With the exception of some clones of BHK-21 and 3T3 cells (29, 32), which are established cell lines and not always suitable as normal prototypes (4), Con A does not induce clustering of its receptors on normal cell surfaces (6, 22, 23, 31, 32). The nature of the forces that control receptor site redistribution is as yet incompletely defined.

Fatty acyl composition affects a number of eukaryotic cell surface properties, including contact inhibition of cell movement (13, 14), the transition temperature of Con A agglutinability (14), cell adhesion (13), and receptor-mediated endocytosis (18). In this regard, there have been many reports demonstrating a consistant decrease in the amount of plasma membrane arachidonic acid after transformation (35, 40, 41). To determine whether fatty acyl composition and cell surface fluidity may modulate ligand-induced receptor redistribution, we have studied patching of CABS on transformed fibroblasts as a function of experimentally induced changes in plasma membrane arachidonate content and microviscosity.

MATERIALS AND METHODS

Cell Culture

VLM cells, a line of SV40-transformed secondary embryonic Balb/c mouse fibroblasts which are injected into the syngeneic host and recloned from the resultant tumor (30), were generously supplied by Dr. Satvir Tevethia (University of Pennsylvania Medical School at Hershey). Stock cultures were grown to confluence in 75-cm² plastic tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). The cells were fed with Dulbecco's Modified Eagle's Medium (DME), supplemented with 5% fetal calf serum (FCS), penicillin (50 U/ml), streptomycin (50 µg/ml), tylocine (60 µg/ml), 3% glutamine (10 µl/ml) and 7.5% sodium bicarbonate (22 µl/ml). All culture media and additions were purchased from Grand Island Biological Co., Grand Island, N.Y., except the penicillin and Versene which were obtained from Microbiological Associates (Walkersville, Md.) and trypsin which was ordered from Calbiochem (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.). For passage, cells were removed from culture flasks with 0.25% trypsin and 0.04% Versene. Cells were fed routinely three times weekly and passed at confluence, about once every week.

Balb/c embryonic fibroblasts were prepared from day-16 mouse embryos under sterile conditions. The embryos were decapitated, eviscerated, and minced into 1-mm³ fragments which were subsequently incubated in 0.25% trypsin on a magnetic stirrer for 10 min. The supernate was then combined with an equal volume of DME with 10% FCS. The cells were pelleted, rinsed in phosphate-buffered saline (PBS), and resuspended in the medium described above containing 10% FCS. Cells were plated into 75-cm² plastic flasks and maintained in DME-10% FCS. All cultures of Balb/c fibroblasts were used before the sixth passage.

Chicken embryo fibroblasts (CEF) and their Rous sarcoma virus-transformed derivates (RSV-CEF) were the generous gift of Dr. Dan Rifkin (New York University School of Medicine) and Dr. John Coffin (Tufts University School of Medicine). The normal cells were grown in Eagle's minimal essential medium (EMEM) supplemented as above for DME, with 10% FCS. RSV-CEF were grown in EMEM-5% FCS.

For experiments in which surface replicas of cells were prepared, cells were removed from culture flasks with trypsin and Versene, and 1 ml of cell suspension was plated onto 24 × 40-mm sterile glass coverslips (Corning Glass Works, Science Products Div., Corning, N.Y.), at a density of 7.0 × 10⁴ cells/ml. The coverslips were maintained in 60 × 15-mm plastic Petri dishes (Falcon) and used 24-48 h after plating. Cells were maintained on completed DME or EMEM as previously described. All cultures were grown at 37°C in a humidified 5% CO_2-95% air atmosphere.

Fatty Acid Incubations

Fatty acids were incorporated into plasma membrane phospholipids according to the method of Curtis et al. (9). Briefly, in short-term experiments, cells were incubated for 30 min in Hanks' Balanced Salt Solution (HBSS) containing sodium ATP (1.25 × 10⁻⁵ M, Sigma Chemical Co., St. Louis, Mo.), sodium coenzyme A (CoA, 5.0 × 10⁻⁶ M, Sigma), and the desired fatty acid (10 µg/ml). In long-term experiments, cells were incubated in DME or EMEM containing 5% delipidated FCS (25), ATP, CoA, the desired fatty acid at the concentrations specified above, and butylated hydroxytoluene (BHT, 10 µg/ml, Calbiochem) to prevent lipid peroxidation. Fatty acids employed were plamitic acid (16:0, Applied Science Labs, Inc., State College, Pa.), nonadecanoic acid (19:0, Applied Science Labs), oleic acid (18:1, Sigma) and arachidonic acid (20:4, Sigma). The latter was judged to be 99% pure by gas liquid chromatography (GLC) before the start of the experiments. Fatty acids were added as sterile ethanolic solutions to the media described above and under nitrogen to further retard lipid peroxidation. Final ethanol concentration never exceeded 0.05%. In control experiments, cells were incubated for 30 min in ATP and CoA (concentrations specified above) or for 24 h in DME containing 5% delipidated FCS, ATP, CoA, BHT, and ethanol. Delipidated FCS was prepared according to the method of Scanu and Edelstein (25).

In another series of experiments, cells were incubated for 30 min in HBSS containing 20:4, ATP, CoA, and aspirin (50 μg/ml, Sigma), or for 24 h in DME containing 5% delipidated FCS, 20:4, ATP, CoA, BHT, and aspirin (50 μg/ml) (34). Controls were incubated as above with the omission of 20:4. The effect of prostaglandin E_2 (PGE$_2$) on CABS topography was accessed by incubating cells for 20 min in HBSS containing either 10^{-7} or 10^{-10} M PGE$_2$.

Visualization of Con A Binding Sites

Con A was visualized by employing the hemocyanin (Hemo) replica technique of Smith and Revel (29). This technique has been described in detail (23). Briefly, coverslips were treated with the appropriate fatty acids and chemicals for the desired time, treated 10 min with Con A (100 μg/ml, Sigma), rinsed three times with HBSS, treated with Hemo (*Busycon caniculatum*, 500 μg/ml), rinsed as above, and fixed in 2% glutaraldehyde (Fisher Scientific Co., Pittsburg, Pa.). Fatty acids and other chemicals were present in the labeling and rinsing solutions at the same concentrations used for the incubation. Cells were postfixed in 2% aqueous OsO$_4$ (Steven Metallugical, New York), dehydrated in a series of graded alcohols, passed through amyl acetate (Fisher), and dried under a warm air stream emitted from a hair dryer. The cells were shadowed with platinum and coated with carbon in a DV-502 evaporator (Denton Vacuum Inc., Cherry Hill, N.J.), and the replicas were examined in a Philips 200 electron microscope. For scoring of binding site topography, the first 55 cells encountered starting from the lower left-hand corner of each grid were examined and sorted into four categories:

CLUSTERED: CABS are present in discrete clusters which are typically absent from cell extensions (pseudopodia, microvilli, and filopodia) and also tend to be withdrawn from the cell periphery (Fig. 1 a).

RETICULATED: CABS are present as a lacy network which may be absent from the cell periphery in some regions of a cell, while extending to the margins in other regions (Fig. 1 b).

DISPERSED: CABS are randomly distributed over the cell surface including microvilli and filopodia. (Fig. 1 c) In some cells, CABS extended to all cell edges. In other cells, the margins were partially cleared of CABS.

INTERMEDIATE: A given cell shows two or more CABS patterns on different regions of its surface.

Plasma Membrane Isolation

Plasma membrane vesicles (PMVs) were prepared according to Scott (26). This technique was chosen because it produces vesicles derived from the dorsal cell surface, the same surface examined in our lectin binding site redistribution studies. It was shown previously that such vesicles are enriched in the plasma membrane marker 5'-nucleotidase and deficient in NADH-cytochrome c reductase, an enzyme typical of the endoplas-

mic reticulum (26). Briefly, cells were incubated for 2 h in PBS containing dithiothreitol (2 mM, Sigma) CaCl$_2$ (0.5 mM, Fisher), and paraformaldehyde (75 mM, Matheson, Coleman & Bell, East Rutherford, N.J.). Although their plasma membranes bleb and vesiculate extensively under these conditons, the cells remain intact and viable as determined by trypan blue dye exclusion and electron microscopy of cells sectioned perpendicular to the substrate. The medium containing vesicles was collected, spun at 2,000 g for 10 min at 4°C, and the supernate spun at 30,000 g for 30 min at 4°C to harvest PMVs. PMVs were dialyzed overnight against PBS before lipid analysis. Purity was assessed by electron microscopy of thin sections of an aliquot of the sample. The aliquot was pelleted, fixed for 15 min at room temperature in 2% glutaraldehyde in 0.1 M cacodylate buffer, postfixed for 15 min in 2% OsO$_4$ in cacodylate, dehydrated in ethanol and propylene oxide, and embedded in Araldite. Thin sections were stained with lead citrate and examined with a Philips 200 electron microscope. To rule out the possibility that the PMVs form by a mechanism that potentially excludes membrane proteins (12), aliquots of the PMV fraction, as well as VLM cells, were freeze fractured in a Balzers' BAF 301 freeze-etch apparatus (Balzers' High Vacuum Corp., Santa Ana, Calif.) to reveal typical intramembrane particles (IMPs) (Fig. 7) The average number of particles per square micrometer was calculated on both PMVs and VLM plasma membrane.

Fatty Acid Analysis

Incorporation of 20:4 into both PMV phospholipids and whole cell lipid extracts was demonstrated by GLC. Lipids were first extracted according to Bligh and Dyer (3) in 1:2 chloroform:methanol. The extracts were then applied in hexane to silicic acid columns (Unisil, 100–200 mesh, Clarkson Chemical Co., Williamsport, Pa.), and phospholipids were eluted according to the procedure of Goodman (11). The phospholipid fraction was then saponified, and the resulting fatty acids were methylated with boron trifluoride (Applied Science Labs) (20). The fatty acid methyl esters were analyzed by GLC using either a 5710A Hewlett-Packard gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a 6-ft column of 10% Silar 10C on Gas-Chrom Q, with a programmed run of 160°–200°C, or a Perkin-Elmer 990 gas chromatograph (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) equiped with a 6 ft column of 15% diethylene glycol succinate (DEGS) on Gas-Chrom P, at 170°C. Peaks were identified by comparing retention times with those of standards (Supelco, Inc., Bellefonte, Pa.; Applied Science Labs). Areas under peaks were quantitated by a Ladd Digitizer planimeter (Ladd Research Industries, Inc. Burlington, Vt.).

Microviscosity Measurements

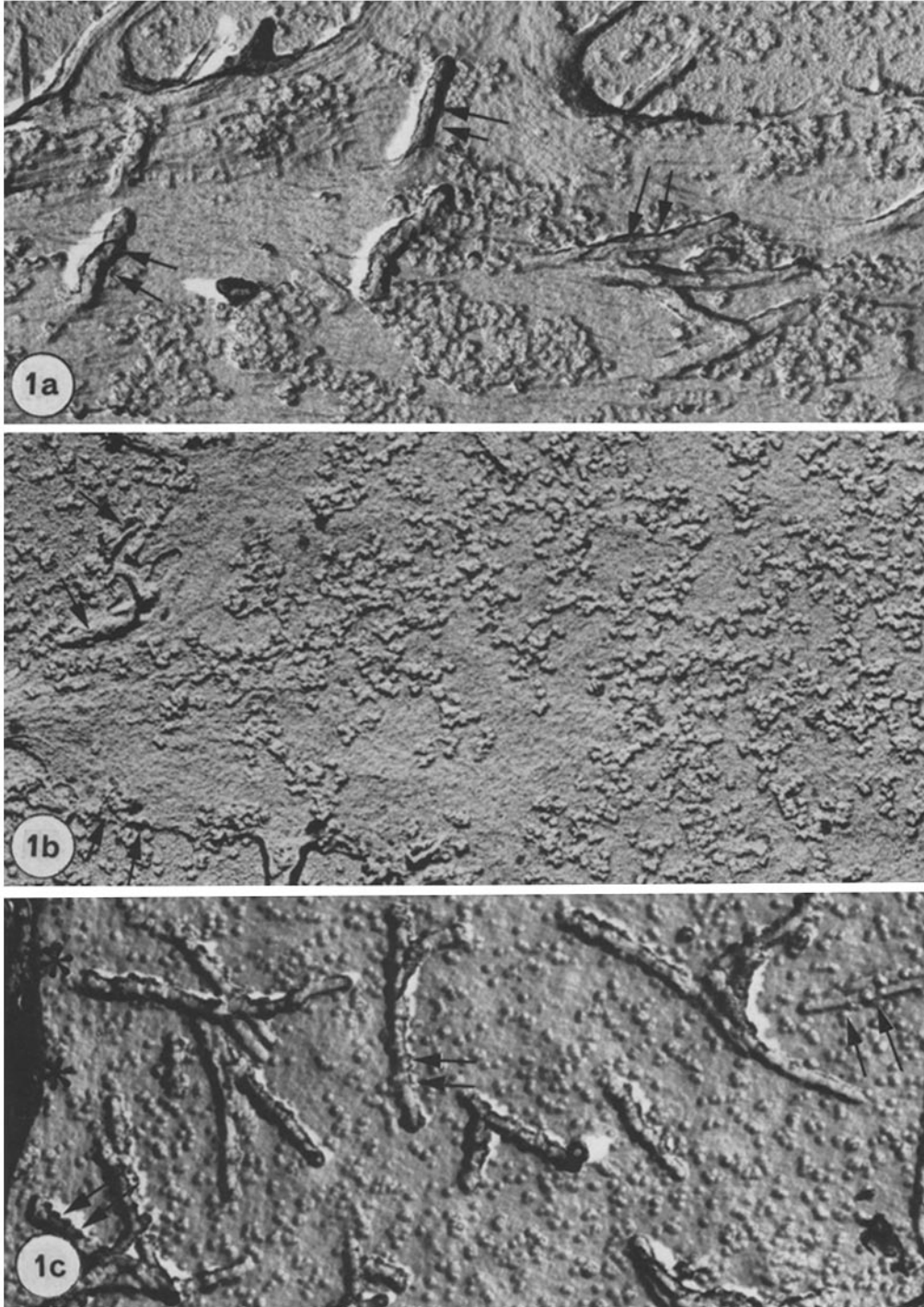PMV microviscosity was measured by means of the

Figure 1 Three examples of CABS topography on surface replicas of VLM cells. Hemo label binds to free valences of surface-bound Con A. (a) Clustered CABS distribution. Note clearing of Hemo label from microvilli (arrows). × 28,300. (b) Reticulated CABS distribution. Note the lacy, interconnected network of Con A/Hemo on the cell surface, and clearing of Con A/Hemo from cell edges (arrows). × 33,200. (c) Dispersed CABS distribution. Observe labeling of microvilli with Con A/Hemo (arrows). Although this cell demonstrated no clearing of Con A from the cell margins (asterisk) some dispersed patterns were also associated with a slight clearing of Con A from cell peripheries. × 23,800.

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fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into the membrane bilayer, according to the method of Shinitzky and Inbar (28). All polarization measurements were obtained at 25°C with an Elscint microviscometer, model MV-1a (Elscint Inc., Hackensack, N. J.).

The degree of fluorescence polarization P, and the degree of fluorescence anisotropy, r, can be calculated from the following equations (28):

$$P = \frac{I_{\parallel}/I_{\perp} - 1}{I_{\parallel}/I_{\perp} + 2} \qquad (1)$$

$$r = \frac{I_{\parallel}/I_{\perp} - 1}{I_{\parallel}/I_{\perp} + 2} \qquad (2)$$

$I_{\parallel}$ is the intensity of emitted light as measured through the polarizer oriented parallel to the polarization plane of the absorbed light. $I_{\perp}$ is the intensity of emitted light as measured through the polarizer oriented perpendicular to the plane of the first polarizer. Microviscosity can be estimated by the following equation (27).

$$\bar{\eta} = \frac{2.4r}{0.362-r} \qquad (3)$$

where $\bar{\eta}$ is the microviscosity preventing the rotation of DPH about its longitudinal axis.

RESULTS

The data below are summarized in Table I and Fig. 2.

Untreated Cells

CELL SHAPE: In an asynchronous population, VLM cells are pleiomorphic in shape. Most cells are fusiform to polygonal with one or more pseudopodia extending from the nuclear area. The pseudopodia are characteristically slender as they leave the cell, flaring out at their terminal ends. Slim filopodia extend from the margins of the perikarya as well as from pseudopodia. The cells are not well spread and are characterized by numerous microvilli (Fig. 3). VLM cells do not show density-dependent inhibition of cell division and routinely form multilayers four to five layers thick if allowed to continue growth after confluence is reached (5).

The parental embryonic Balb/c fibroblasts, on the other hand, are polygonal in shape, flattened onto the substrate, and nearly devoid of microvilli (Fig. 4). These cells show density-dependent inhibition of cell division and movement and form a stable monolayer (5).

TABLE I

Percent of Cells Showing a Given Con A Binding Site Distribution After Experimental Manipulations

Incubation	Cell	Time	CABS Distribution			
			Clustered	Reticu-lated	Dispersed	Interme-diate
			%			
PBS	VLM		96.3	3.7	0	0
20:4, ATP, CoA	VLM	30 min	24.6	36.0	30.7	8.8
20:4, ATP, CoA, aspirin	VLM	30 min	27.8	27.8	37.0	7.4
ATP, CoA	VLM	30 min	96.3	1.85	0	1.85
ATP, CoA, aspirin	VLM	30 min	94.4	6.6	0	0
PGE₂ (10⁻⁷ M)	VLM	20 min	98.15	0	0	1.85
PGE₂ (10⁻¹⁰ M)	VLM	20 min	100.0	0	0	0
20:4	VLM	30 min	98.15	1.85	0	0
20:4, ATP, CoA, delipidated (dlpd FCS), BHT, EtOH	VLM	24 h	0	7.4	87.0	5.6
20:4, ATP, CoA, dlpd FCS, BHT, aspirin, EtOH	VLM	24 h	1.85	3.7	94.4	0
ATP, CoA, dlpd FCS, BHT, EtOH	VLM	24 h	94.4	3.7	1.85	0
ATP, CoA, dlpd FCS, BHT, EtOH, aspirin	VLM	24 h	96.3	3.7	0	0
18:1, ATP, CoA, dlpd FCS, EtOH	VLM	24 h	96.3	0	1.85	1.85
16:0, ATP, CoA, dlpd FCS, EtOH	VLM	24 h	94.4	5.6	0	0
19:0, ATP, CoA, dlpd FCS, EtOH	VLM	24 h	92.6	5.6	1.85	0
20:4, ATP, CoA, dlpd FCS, BHT, EtOH	RSV-CEF	24 h	1.8	5.4	89.3	3.6
ATP, CoA, dlpd FCS, BHT, EtOH	RSV-CEF	24 h	92.6	7.4	0	0

* Represents count of 55 cells for each experiment.

CABS DISTRIBUTION: In agreement with previous observations (6), Con A cross-links its receptors into large clusters (primary CABS distribution), and these clusters are withdrawn from microvilli, filopodia, and other cell margins (secondary CABS distribution) on VLM cells labeled with ConA/Hemo (Fig. 2, Table I). In contrast, the CABS remain completely dispersed after Con A/Hemo labeling on the normal embryonic Balb/c fibroblasts (similar to Fig. 1 c).
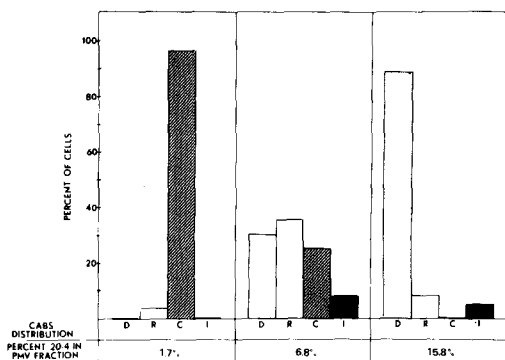

FIGURE 2 CABS distribution as a function of PMV arachidonic acid (20:4) content. Lipid data was generated by GLC. D, dispersed; R, reticulated; C, clustered; I, intermediate. (Descriptions of these CABS topographies are given in the Materials and Methods section).

Effect of a Short-Term Incubation in Arachidonic Acid (20:4), ATP, and CoA

When VLM cells are incubated in a mixture of 20:4, ATP, and CoA for 30 min at 37°C before Con A/Hemo labeling, there is a shift toward the dispersed pattern of Con A binding typical of the untransformed parental cell line (Fig. 2, Table I). While 24.6% of the cells retain the clustered CABS distribution characteristic of VLM cells, 36.0% of the cells demonstrate a reticulated appearance of CABS, and 30.7% attain the dispersed CABS pattern that is characteristic of the normal secondary embryonic fibroblasts (Fig. 5). In all cases, the cells maintain the pleiomorphic shape typical of untreated VLM cells, except for occasional large bulges which stain with oil red 0 and represent intracellular lipid droplets.

Control incubations in ATP and CoA (without 20:4) or in 20:4 (without ATP and CoA) for 30 min at 37°C have no effect on either CABS topography or cell shape (Table I).
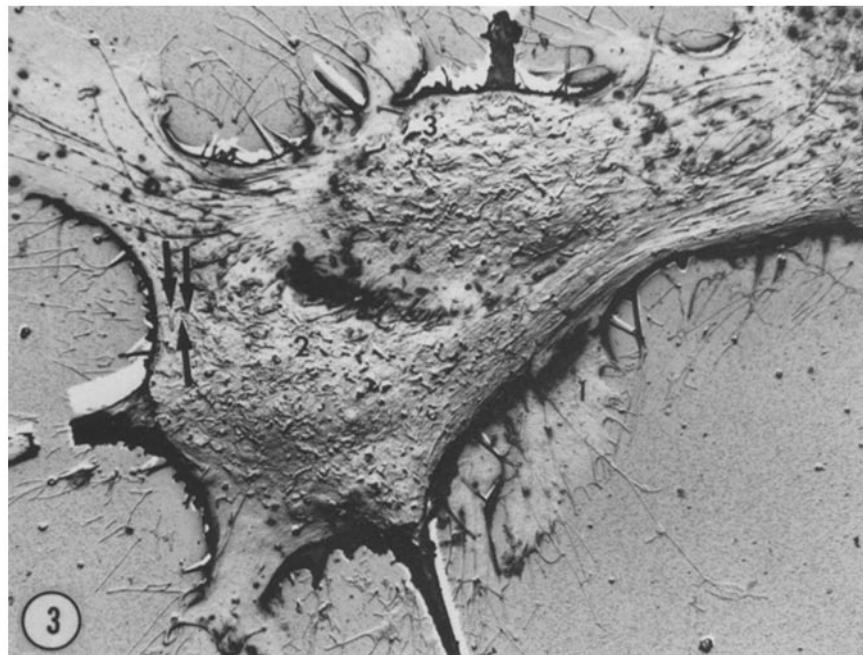

FIGURE 3 Surface replica of untreated VLM cells. Note the underlapping of the three cells (1, 2, and 3) demonstrating a lack of contact inhibition of movement in the VLM line. It can be seen that the cells are pleiomorphic in shape, not well spread, and possess numerous microvilli (arrows). × 2,600.
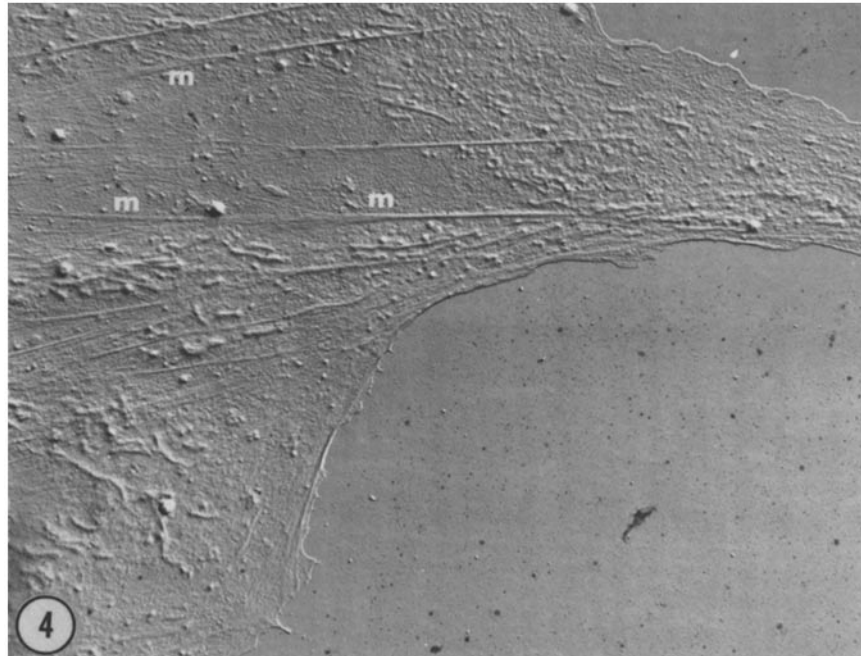
FIGURE 4 Surface replica of a secondary Balb/c embryonic fibroblast. In contrast to the VLM cell, note its flattened morphology and the paucity of microvilli. These cells collapse slightly during the dehydration procedure, revealing an elaborate system of subplasmalemmal microfilament bundles (m). × 2,000.

FIGURE 5 Surface replica of a VLM cell incubated for 30 min with arachidonate, ATP, and CoA. Note dispersal of Con A/Hemo complexes, as well as the presence of many bulges (L) representing intracellular lipid droplets. × 30,600.

Effect of a 24-h Incubation in Arachidonic Acid, ATP, and CoA

When VLM cells are incubated in 20:4, ATP, and CoA for 24 h, the CABS distribution is shifted quite dramatically to the dispersed configuration (Fig. 2, Table I). 87.0% of the cells have the dispersed CABS pattern, while only 7.4% exhibit the reticulated distribution. There is no obvious change in cell shape.

Control cells incubated in ATP, CoA, BHT, and delipidated FCS (in the absence of 20:4) show typical clustering and clearing of CABS on 94.4% of the cells (Table I). Such cells exhibit no shape change.

Effect of Prostaglandin Modulation

When cells are incubated for 30 min in 20:4, ATP, CoA, and aspirin to inhibit the synthesis of prostaglandins from 20:4 (34), the CABS distribution is still shifted toward the dispersed configuration (Table I): 36.9% of the cells attain the dispersed CABS topography characteristic of normal cells, 27.8% show a reticulated CABS distribution, 7.4% an intermediate distribution, and 27.8% a clustered distribution. There is no appar-

ent change in cell shape. Cells incubated for 30 min with aspirin alone show a typical clustered CABS distribution as do cells incubated for 20 min in PGE₂ (Table I).

In a long-term experiment, cells were incubated in 20:4, ATP, CoA, BHT, delipidated FCS, and aspirin. Under these conditions, 94.4% of the cells display a dispersed CABS configuration and retain their characteristic shape. A minority of the cells (3.7%) have a reticulated CABS distribution (Table I). These are essentially the same results obtained when cells were incubated for 24 h in 20:4 without aspirin. CABS of control cells, incubated for 24 h in ATP, CoA, delipidated FCS, and aspirin (no 20:4), were for the most part clustered (94.4% of the cells, Table I).

Lipid Analysis

PMVs were prepared from cells grown in 20:4, ATP, and CoA for 1 h and in 20:4, ATP, CoA, BHT, and delipidated FCS for 24 h. Electron microscopy revealed a pure pellet (Fig. 6). Freeze fracture of the pellet revealed the presence of IMPs (Fig. 7). Importantly, the particle/area ratios of plasma membranes and PMVs are similar. Whereas PMVs had 416.8 IMPs/μm^2, plasma
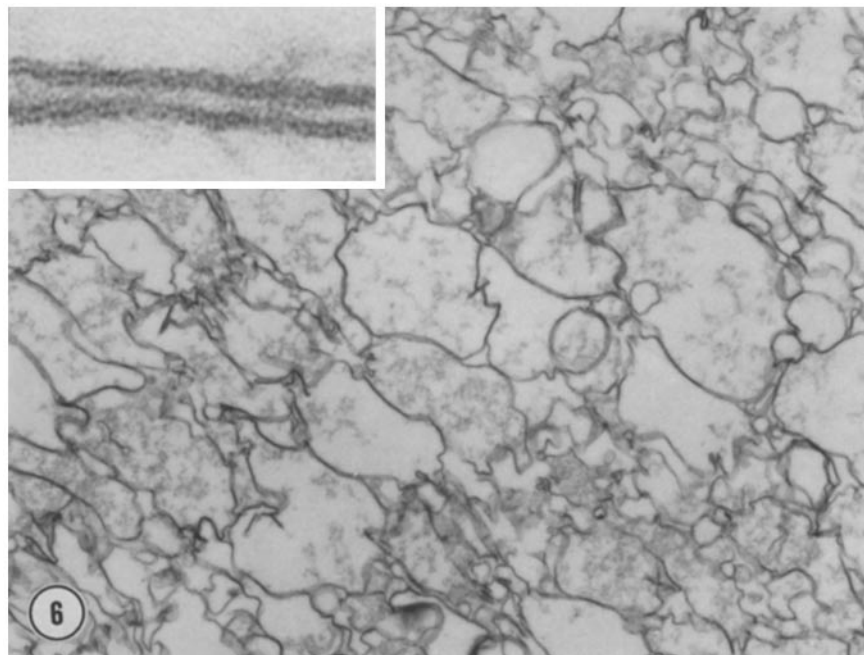

FIGURE 6   Thin section through the top of a pellet of a typical PMV preparation. The bottom of the pellet showed the same degree of purity. × 11,900. (*Inset*) Higher magnification of portion of two adjacent PMVs showing the trilaminar structure of the plasma membrane. × 517,000.
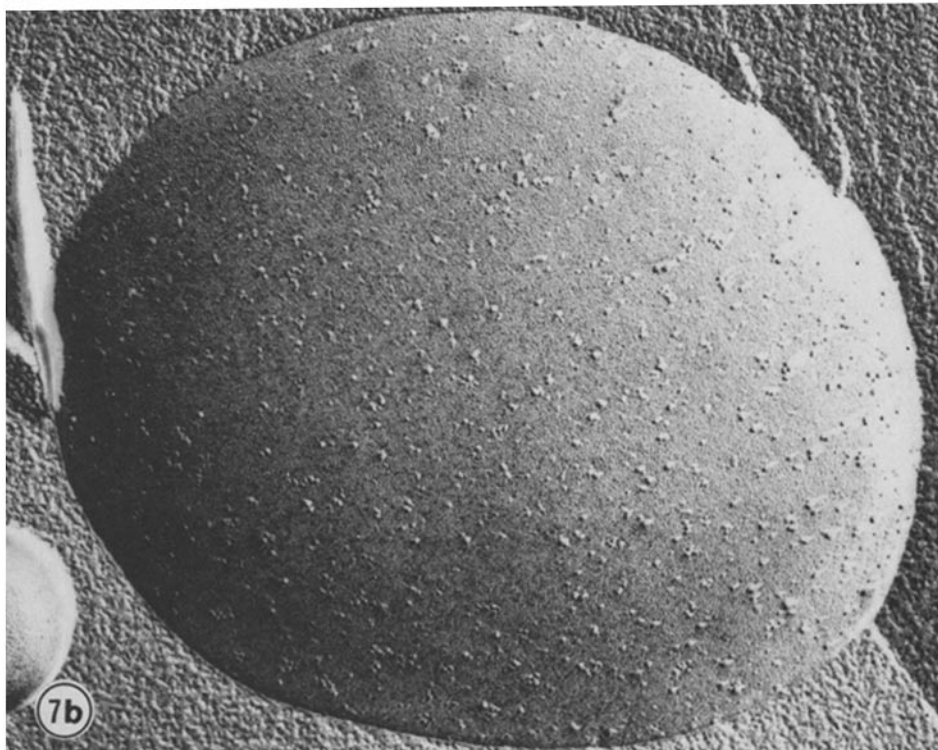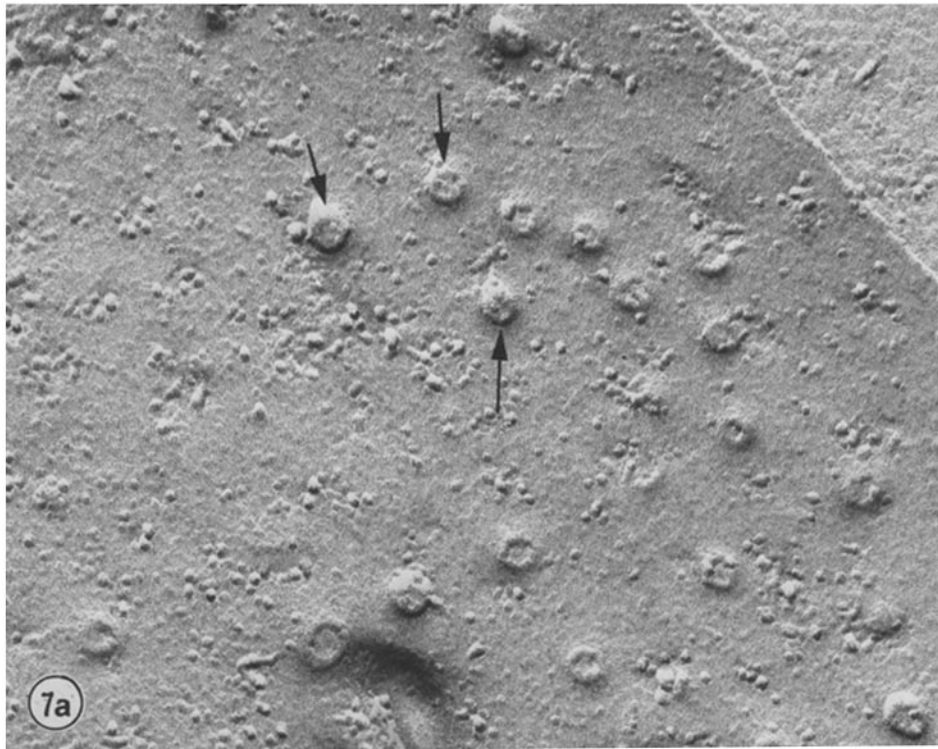
FIGURE 7 Freeze-fracture electron micrographs. (a) Replica of P face of a freeze-fractured surface of a VLM cell showing IMPs. P plasma membranes averaged 348 IMPs/μm^2. m, microvilli. $\times$ 82,500. (b) Replica of the P face of a freeze-fractured PMV showing IMPs which are present at a density of 417 IMPs/μm^2. $\times$ 116,900.

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membranes had 347.8 IMPs μm^2, indicating that PMVs are not selective of protein-poor domains. Fatty acid profiles of PMV phospholipids from cells treated as described above as well as those from control cells incubated in DME-5% FCS are shown in Table II. The salient features of these profiles are as follows: (a) 20:4 accounted for only 1.7% of the phospholipid acyl chains in cells grown in DME with 5% FCS. PMVs from such cells had a microviscosity of 331 cP (Table III), and could redistribute their CABS into patches (Fig. 2, Table I). (b) Following a 1 h incubation in 20:4, ATP, and CoA, PMV 20:4 was elevated to 6.8%. Cells grown under such conditions showed partial restriction of CABS mobility expressed as either a reticulated CABS distribution or a dispersed CABS distribution (Fig. 2, Table I). (c) After a 24-h incubation in 20:4 ATP, CoA, BHT, and delipidated FCS, 20:4 accounted for 15.8% of the phospholipid fatty acid in PMVs. Unexpectedly, enrichment in 20:4 was associated with microviscosity of 404 cP (Table III). Cells with elevated PMV microviscosity and elevated 20:4 in PMVs showed a marked restriction of CABS mobility, with complete dispersal of CABS occurring in 87% of the cells (Fig. 2, Table I).

TABLE II

Relative Fatty Acyl Composition of PMV Phospholipids After 1- and 24-h Incubations of VLM Cells in Arachidonate

Fatty acid	Control*	1 h‡	24 h§
		%	
16:0	33.7 (16.3)‖	15.2	28.2 (21.2)‖
16:1	10.3 (14.8)	7.4	0.4 (8.7)
18:0	11.3 (14.8)	17.7	16.9 (11.8)
18:1	36.2 (39.6)	33.7	19.5 (35.5)¶
18:2	1.7 (5.6)	3.2	3.2
18:3**	— (2.9)	3.5	1.8 (0.9)
20:3	0.3 (1.4)	1.6	0.5 (0.9)
20:4	1.7 (4.2)	6.8	15.8 (10.8)

* Cells were incubated in DME-5% FCS for 2 d.

‡ Cells were treated for 1 h with HBSS containing ATP (1.25 × 10⁻⁵ M), CoA (5.0 × 10⁻⁶ M), and 20:4 (10 μg/ml).

§ Cells were incubated for 24 h in DME containing ATP (1.25 × 10⁻⁵ M), CoA (5.0 × 10⁻⁶ M), 20:4 (10 μg/ml), BHT (10 μg/ml), and 5% delipidated FCS.

‖ Numbers in parentheses represent fatty acyl composition of phospholipid extracts of homogenates of cells after PMV production. Control cells grown in 10% FCS.

¶ Value represents sum of 18:1 and 18:2.

** Represents total of 18:3ω3 and 18:3ω6.

TABLE III

Microviscosity of PMVs of VLM cells Before and After a 24-h Incubation in Arachidonate, ATP, CoA, and Delipidated FCS

	P*	η̄‡
Before incubation	0.286	331
After incubation	0.306	404

* Fluorescence polarization $= \dfrac{\dfrac{I_{\parallel}}{I_{\perp}} - 1}{\dfrac{I_{\parallel}}{I_{\perp}} + 1}$

‡ Microviscosity $\approx \dfrac{2.4r}{0.362 - r}$. Microviscosity is expressed in cP.

Phospholipids of cells were analyzed after PMV production. These whole cell phospholipid extracts also showed enrichment in 20:4 after the 24-h incubation in 20:4, ATP, and CoA (Table II). Thus, there was probably no gross selection of arachidonate-rich domains during the vesiculation process.

Response of RSV-CEF to Arachidonate

CABS of another transformed cell line, RSV-CEF, responded the same as those of VLM cells after cells were incubated for 24 h in 20:4, ATP, CoA, BHT, and delipidated FCS (Table I). Before the 20:4 treatment, 84.4% of the cells showed clustering and clearing of CABS; after the 20:4 treatment, this was shifted to 1.8% with 89.3% cells showing the dispersed CABS topography.

It should be pointed out that, for the most part, untransformed CEF have a dispersed CABS distribution, with clearing from the edges. 64.3% of the cells show a dispersed CABS topography with binding sites incompletely extended to the cell periphery.

Effect of Other Fatty Acids upon CABS Mobility

Incubation of VLM cells in DME containing nonadecanoic acid (19:0), palmitic acid (16:0), or oleic acid (18:1), with ATP, CoA, and delipidated FCS for 24 h had essentially no effect upon CABS mobility. In all three cases, CABS were clustered and cleared in >90% of the cells examined (Table I). However, conclusions concerning the effects of these fatty acids on lectin-binding site redistribution cannot be drawn until the extent of incorporation of these fatty acids into PMV phospholipids is ascertained.

DISCUSSION

A number of reports have indicated that total membrane phospholipids in general, and in some cases plasma membrane phospholipids in particular, of several different types of neoplastic cells are deficient in arachidonic acid (15, 35, 40, 41). We have extended these observations to murine cells, using secondary cultures of Balb/c embryonic fibroblasts, and VLM cells, their SV-40 transformed, tumorigenic derivatives. PMV phospholipids of VLM cells contain 3.0% (±0.3) arachidonate when grown in DME-10% FCS (1.1% when grown in DME-5% FCS) whereas the PMV phospholipids of the parental cells, secondary Balb/c embryonic fibroblasts, contain 8.9% (±0.9) arachidonate ($P = 0.00005$). Interestingly, PMVs of normal fibroblasts are more viscous, even though they are enriched in arachidonate relative to PMVs of transformed cells.[1]

To determine whether such lipid differences might affect the redistribution of membrane glycoproteins, we assessed CABS topography as a function of PMV and whole cell phospholipid arachidonate content. Incubation of both VLM cells and RSV-CEF in arachidonate, ATP, and CoA inhibited the lectin-induced clustering of CABS that is typically observed on these cells. The topological distribution of Con A surface receptors might be affected by several possible mechanisms.

(a) Arachidonic acid is a precursor of and promotes the synthesis of certain prostaglandins (34) which have been shown to stimulate the synthesis of cyclic AMP (cAMP) in many systems including fibroblasts (17). Elevated cAMP levels cause reversion of many plasma membrane properties of transformed cells to those of parental cells including reduction of Con A-mediated agglutination (39), increased cell spreading (6, 21, 39), and inhibition of Con A-induced clustering (6). Thus, arachidonate might exert its effect by elevating cytoplasmic prostaglandin and/or cAMP levels. However, when aspirin (50 μg/ml) was included in both the 30-min and 24-h arachidonate, ATP, CoA incubations, CABS topography remained dispersed and there was no change in cell shape, indicating that the effect is not due to prostaglandin-mediated pathways. Furthermore, incubation

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[1] Hill, D. J., and J. Z. Borysenko. 1979. Lipid and microviscosity differences between normal and transformed cells. Manuscript in preparation.

in PGE_2 itself did not inhibit patching of CABS.

(b) Free fatty acids, including arachidonate, have been shown to stimulate guanylate cyclase in cultured fibroblasts (36). It is unlikely that increases in cyclic GMP (cGMP) levels could account for the inhibition of CABS mobility observed in arachidonate/ATP/CoA-treated VLM cells because it has been demonstrated previously that cGMP not only fails to restrict CABS mobility in VLM cells but actually promotes receptor mobility resulting in more closely packed CABS clusters (6).

(c) Cytoskeletal elements have been widely implicated in topographical control of various surface receptors (2, 7, 10, 31). We have demonstrated, however, that modulation of membrane fatty acyl composition can inhibit ligand-induced patching of CABS without altering cell shape. The independence of a cell shape change from a change in ligand-induced CABS topography is of interest because in most of the cases examined to date, changes in receptor topography have been associated with cell shape changes. These cell shape changes were produced by pharmacologically altering the cytoskeleton (6, 31). For instance, elevation of cAMP in VLM cells causes flattening of the cells in association with a restriction of CABS mobility (6) possibly due to an effect of cAMP upon the cytoskeleton (21, 38). These studies, as well as others, imply that a given cell shape is associated with a particular kind of cytoskeletal organization, and that cytoskeletal organization may control CABS mobility. The absence of a cell shape change in VLM cells and RSV-CEF after incubation in arachidonate ATP CoA suggests that the restriction of lectin-induced patching does not involve gross changes in cytoskeletal organization. However, we can not exclude the possibility that the fatty acid manipulations cause subtle reorganizations in the cytoskeleton, such as changes in the amount of actin or tubulin associated with the plasma membrane. Such subtle changes might not result in recognizable changes in cell shape.

(d) We have demonstrated that after VLM cells are incubated for 1 h in HBSS containing arachidonate, ATP, and CoA, arachidonate comprises 6.8% of PMV phospholipids (1 h was chosen because the addition of arachidonate, ATP, and CoA to the labeling solutions following the original 30-min incubation results in a 60-min total incubation). Furthermore, increasing the incubation time to 24 h results in PMV phospholipids containing

15.8% arachidonate. Thus, the degree of restriction of CABS mobility seems to vary directly with percent elevation in phospholipid arachidonic acid present in the plasma membrane-derived vesicles (Fig. 3, Table II). Elevations in arachidonate and reductions in oleate are also observed when total cell phospholipids are analyzed. Other studies investigating either total cell phospholipids (37) or phospholipids of plasma membranes produced by the procedure of Perdue (cited in 40) have likewise reported a decrease in oleate when polyunsaturated fatty acids are increased. These data suggest that plasma membrane produced by the vesiculation technique of Scott (26) does not select for specific membrane domains rich in arachidonate and poor in oleate. That the vesiculation procedure does not select for protein-poor domains (12) was demonstrated by the presence of the full complement of IMPs in the PMVs when quantitated by freeze-fracture.

Alterations in membrane fatty acyl composition may influence receptor mobility by changing fluidity of the cell surface. Mobility of CABS is. in fact, correlated with fluidity of the cell surface in our model system. Increased microviscosity, both in normal cell and in transformed cell PMVs after arachidonate treatment, is associated with restriction of ligand-induced CABS patching. The mechanism by which elevations in arachidonate may both increase PMV microviscosity and restrict CABS clustering is currently obscure. However, it is possible that the elevated microviscosity and inhibition of receptor site movement may involve interaction between cholesterol and arachidonic acyl chains within the membrane. Cholesterol in a polyunsaturated environment increases the viscosity of artificial membranes (8, 35). It is interesting that some transformed cell lines show increases in plasmalemmal cholesterol upon transformation (1). Although increased plasmalemmal cholesterol has not as yet been demonstrated in VLM cells, it is possible that arachidonate might exert its effect by such an interaction with cholesterol. It is also possible that cholesterol levels may increase in the plasma membrane after arachidonate incorporation in a manner similar to the compensatory increase in oleate and decrease in cholesterol that takes place in *Mycoplasma* membranes when the temperature is lowered (24).

In conclusion, altered plasma membrane fatty acyl composition is associated with both altered membrane microviscosity and altered membrane glycoprotein mobility. These data suggest that plasma membrane fatty acyl composition is an important determinant in the maintenance of receptor site topography.

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