Specializations in Filopodial Membranes at Points of Attachment to the Substrate

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ABSTRACT A mouse cell line (LM), which grows predominantly as spindle-shaped cells with numerous filopodia, was employed in this study. These filopodial projections appear to be important as sites of attachment to the substratum in LM cells. Morphologically the filopodia are slender projections from the cell body which usually attach to the substrate at their distal ends (filopodial footpads). Freeze-fracture of monolayer cultures *in situ* preserves the spatial relationship of filopodial processes to that of the cell body. Examination of these freeze-fracture preparations reveals a striking difference in the density of intramembrane particles (IMP) in the filopodial-footpad plasmalemma compared with the plasmalemma of the cell body (number of IMP in footpad > cell body). Additionally, there is a marked difference in the number of filipin-sterol complexes on the cell body, compared with the filopodial footpad, implying a difference in the cholesterol content in these regions (filipin-sterol complexes in footpad < cell body). These data suggest a structural and functional specialization of the filopodial-footpad plasma membrane which may be related to cell adhesion.

Many cultured cells have specialized microprojections on their cell surface. One such category of microprojection is the filopodium (32). Filopodia generally project from the cell body and make contact with the substratum. The role of the filopodia in cell adhesion and spreading has not been clearly defined; however, Albrecht-Buehler (1) has presented evidence that these structures have a substrate-exploring function. Several studies have attempted to characterize the sites of adhesion between cells and the substratum on which they reside (for example, see references 1, 2, 6, 11–13, 16, 17, 24, 25, 28, 31, and 33). The nature of adhesion sites, from both a chemical and morphological point of view, remains to be fully defined.

In this study we examine filopodia of a mouse fibroblast cell line. Our findings illustrate that the filopodial plasmalemma in contact with the substrate shows some striking morphological differences when compared with the plasmalemma of the cell body. These differences may reflect specializations of the plasma membrane which play a role in cell-substrate adhesion.

MATERIALS AND METHODS

Cells and Culture Conditions

The mouse cell line LM (TK^-) clone ID (referred to herein as LM) was used in this study (cells were kindly provided by Dr. Richard Davidson, Children's Hospital, Boston, Mass.). Asynchronous cultures were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were plated in 35- or 60-mm Falcon plastic tissue culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) containing round glass coverslips (see below).

Transmission Electron Microscopy

Cells were grown on 25-mm coverslips (Corning Glass Works, Science Products Div., Corning, N. Y., No. 2 thickness). Coverslips were rinsed by gently dipping into phosphate-buffered saline (PBS) at room temperature. Cells were then fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) with 5% sucrose for 1 h at room temperature. After several washes in buffer, the coverslip preparations were postfixed in 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.3), washed in 0.05 M sodium maleate buffer (pH 5.2), and en bloc stained with 2% uranyl acetate in sodium maleate (18). After dehydration and infiltration, the cells were embedded in Epon 812 by inverting BEEM capsules (Better Equipment for Electron Microscopy, Inc. (Bronx, N. Y.) over the coverslips. After polymerization, blocks were removed from the coverslips by transferring directly from 60°C to liquid nitrogen. Sections perpendicular to the substratum were obtained after reembedding small pieces of Epon as described by Connelly (10). Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 200 electron microscope operated at 60 kV.

Scanning Electron Microscopy

Cells were grown on 13-mm coverslips (Gold Seal, No. 1 thickness). Coverslip preparations were gently washed in PBS at 37°C, then fixed in 1% glutaraldehyde in PBS for 15 min at 37°C. The initial fixative was replaced by 3% glutaraldehyde in PBS and fixed for an additional 30 min at 37°C before being transferred to room temperature. Cells were washed in PBS and postfixed in 2% OsO_4 in PBS, then dehydrated in a graded ethanol series. After 100% ethanol, the coverslips were incubated in three changes of 100% acetone. Cells were critical point dried

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out of acetone in a Samdri PVT-3 (Tousimis Research Corp., Rockville, Md.) and coated with gold-palladium in a Hummer sputtering device (Technics Inc., Alexandria, Va.). Samples were examined in an Etec autoscan operated at 20 kV.

Freeze-Fracture

Cell monolayers were freeze-fractured according to the procedure of Yee et al. (34). Cells were grown on small coverslips (Gold Seal, 4 mm diameter, No. 0 thickness) as previously described (26). The coverslips were mounted on special gold-plated specimen carriers with the cells in contact with the carrier. Coverslip and specimen carrier were frozen as a unit in Freon 22 and liquid nitrogen. The assembly was then placed into a double replica device and fractured in a Balzers BA360 M freeze-fracture apparatus (Balzers Corp., Hudson, N. H.). Fractured specimens were shadowed with platinum and coated with carbon. The replica on the coverslip was removed from the glass with hydrofluoric acid while the complimentary replica was teased away from the specimen carrier into distilled water. Replicas were cleaned with commercial bleach and dimethylformamide and picked up on Formvar-coated grids.

Cholesterol Localization

The distribution of cholesterol within LM cell plasma membrane was studied by using the polyene antibiotic filipin as an ultrastructural probe for membrane sterol (3, 4, 14, 19, 20, 29). Cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3 with 5% sucrose), washed in cacodylate buffer, and incubated for 30 min to 4 h in 50 μg/ml unfractionated filipin (Upjohn Co.,) in 0.1 M cacodylate buffer with 5% sucrose (27). Samples were then processed for freeze-fracture analysis or thin-section electron microscopy (as above).

Preparation of "Substrate Attached Material"

Cells grown on small coverslips were rinsed in Ca^{++}-Mg^{++}-free PBS then incubated with 1 mM EDTA in Ca^{++}-Mg^{++}-free PBS. This medium was gently aspirated over the coverslips until all the cells were removed. The coverslips, with "substrate-attached material" but no cells, were fixed and processed for standard freeze-fracture and for cholesterol localization (as above).

Morphometry

Size and density measurements of intramembrane particles (IMP) on the P-fracture face of plasma membrane from the cell body and the filopodial footpad were compared. Micrographs at a final magnification of 87,000 were used for all measurements. A clear plastic overlay, with a grid pattern corresponding to membrane areas 1/16 μm^2 in size, was placed over the micrograph, and all IMP within the grid area were counted. Size of IMP was determined by measuring the long and short axis (IMP were seldom exactly spherical) to the nearest 0.1 mm with a X7 loupe containing a micrometer. The average of the long and short axis was taken as the IMP diameter.

RESULTS

Nonconfluent cultures of asynchronously grown LM cells contain a mixture of cell shapes; however, most cells are spindle shaped rather than being highly flattened (Table I). The appearance of filopodia associated with typical spindle-shaped cells show filopodial structures that generally project down at an angle from the cell body to contact the substratum (Fig. 1 *A* and *B*). Based upon SEM observations, the distal portions of filopodia represent important points of cell-substratum contact in LM cells.

Cells grown on glass coverslips and then freeze-fractured *in situ* in such a way that the fracture plane passed through the bottom of the cell (i.e., the plasma membrane adjacent to the substratum) revealed a pronounced difference in the distribution and number of IMP in the filopodial footpads compared with the cell body (Fig. 2). Quantitation of the density of IMP shows that the filopodial footpad membrane contains more than twice as many IMP as the adjacent cell body plasma membrane (Table II). Furthermore, the mean diameter of IMP from the filopodial footpad region differed from that of the cell body and the entire population of footpad IMP was shifted to the left, relative to the cell body IMP (Fig. 3).

TABLE I

Number of Cells Belonging to Different Shape Categories as Determined by SEM

	Shape category		
SEM preparations	Round	Spindle shaped	Flat
No. 1	34	351	45
No. 2	41	284	27
No. 3	45	438	43

Another difference in the plasmalemma of the filopodial-footpad and the cell body was detected after filipin treatment. Freeze-fracture images of filipin-treated cells revealed randomly distributed filipin-sterol complexes in the cell body plasmalemma while the filopodial-footpad plasmalemma was virtually devoid of these structures. Only occasionally were the characteristic filipin-sterol complexes present in freeze-fractured filopodial footpad membrane (Fig. 4).

Treatment of coverslip cultures of LM cells with EDTA in CA^{++}-Mg^{++}-free PBS causes the cells to round up. Complete detachment of cells was hastened by gentle aspiration. Examination of freeze-fracture replicas from such coverslips revealed that even in the absence of cells, certain cell-derived material remains on the coverslip. Based upon morphological criteria (i.e., the presence of IMP), this substrate-attached material appears to be membranous in nature. Additionally, few filipin-sterol complexes are found in filipin-treated substrate-attached material. The overall morphology of the freeze-fractured substrate-attached material appears to be very similar to intact filopodial footpads both in general size and shape and in number and distribution of IMP and filipin-sterol complexes (Fig. 5).

DISCUSSION

The morphology of filopodia and filopodial footpads has been described at the SEM level by several workers. There appears to be a basic similarity in filopodial-like structures in fibroblasts that are in the process of attaching to the substratum (1, 28) and cells detaching from a substratum (25). The filopodia seen in this study appear similar to those mentioned above; however, it should be noted that the LM cells observed herein were under normal culture conditions and not in the process of attachment or detachment. The fact that the LM cells were not highly flattened (see Table I) probably accounts for the presence of large numbers of filopodia under normal culture conditions just as rounded attaching and detaching cells have numerous filopodia. The filopodial footpads appear to be major points of attachment to the serum-coated glass on which the LM cells were grown. This was evident from examination of SEM samples at high tilt angles where one can observe the relationship of the cell to its substratum, at least at the cell periphery.

Further examination of the filopodial attachment sites was undertaken by freeze-fracture of monolayer cultures *in situ*. It is important that cultures be fractured *in situ* to preserve the spatial relationship of the cell body with the delicate filopodia. While there are several procedures for freeze-fracture of monolayer cultures, the method of Yee et al. (34) was chosen, as this technique appears to be the most straightforward and easiest

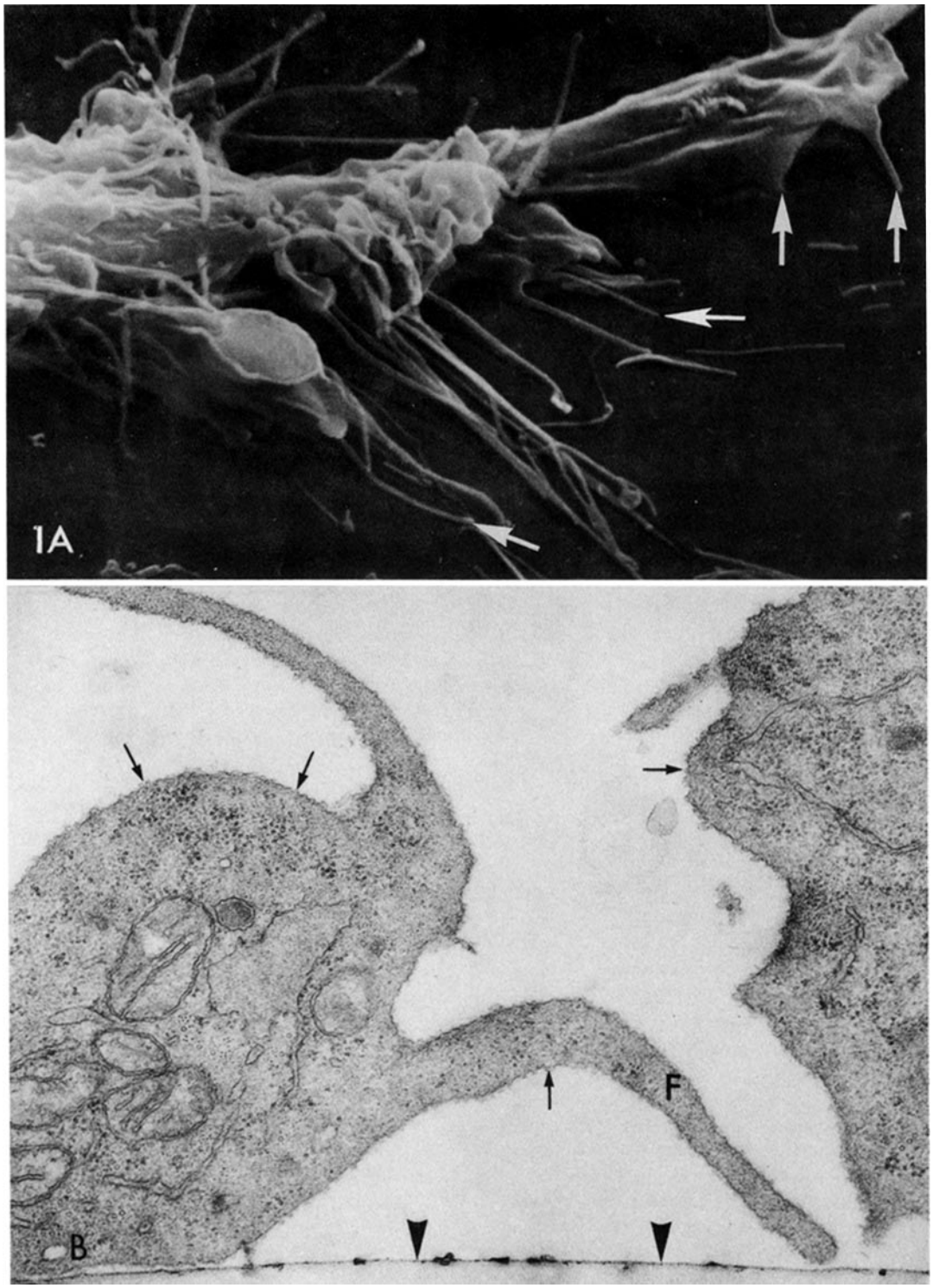

FIGURE 1 (*A*) SEM preparation of LM cell from subconfluent culture. This spindle-shaped cell has numerous filopodia, most of which project from the cell body at an angle and attach to the substratum at their distal ends (arrows). × 10,000. (*B*) Thin section of filipin-treated LM cells, which were sectioned perpendicular to the substratum to study the relationship of filopodia to the cell body. Note that the filopodium (*F*) does not touch the substrate (arrowheads) along its entire length but approaches it only at the distal portion. Also note the characteristic corregated appearance of the plasma membrane (arrows) induced by filipin. × 52,000.

of those presently available. Cells can be grown directly on glass coverslips and do not require special mounts (22) or special coatings (8, 9, 23); also this technique provides complimentary double replicas unlike the method of Pauli et al. (21). Furthermore, in our hands ≥50% of the cells fractured through their bottoms; thus the fracture plane passed through numerous points of cell-substratum interaction. Replicas of LM cells fractured through their bottoms revealed broad areas of cell body plasma membrane with apparently randomly distributed IMPs. Adjacent to, but disjunct from the cell body were smaller patches of plasmalemma. These smaller areas of membrane were distinctly different from the cell body in having a much

FIGURE 2 Freeze-fracture preparation of monolayer culture of LM cells. The cell body (*CB*) has numerous IMP that appear to be randomly distributed within the plane of the membrane (arrows). Note the filipodial footpad regions that have a much higher density of IMP (arrowheads). × 43,000.

TABLE II

Density of Particles on P Face of Plasma Membrane

Region of cell	Particles per 1/16 μm^2*	Particle per μm^2
Cell body	61.9 ± 2.12	990
Filopodial footpads	128.9 ± 6.68	2,063

* Mean ± standard error.

higher density of IMPs. These small densely particulated areas of membrane are thought to be the footpads of filopodia. The morphometric data on IMP density and size distribution suggest a compositional difference in proteins in the filopodial footpad membrane relative to the cell body plasmalemma. However, as Fisher and Stoeckenius (15) point out, caution should be exercised in interpretations based solely on morphological evidence.

While it is possible that they are vesicles shed from the cells, this seems unlikely. In the first place, such vesicular structures were rarely seen in SEM preparations and when present may merely have been footpads with the intervening filopodia broken away during preparation. Secondly, these structures were often irregular in shape (as were footpads seen with SEM), unlike vesicles which would probably be spherical or circular in replicas. To explain why the filopodial footpads are not connected to the cell body in these replicas, a scheme for how the cells fracture through the cell bottom is given in Fig. 6. This interpretation is based upon a combination of our SEM, TEM, and freeze-fracture observations.

A second interesting feature that distinguished the filopodial footpad from the cell body was the difference in the effect of the polyene antibiotic filipin. Filipin binds specifically to certain sterols, such as cholesterol, inducing the formation of

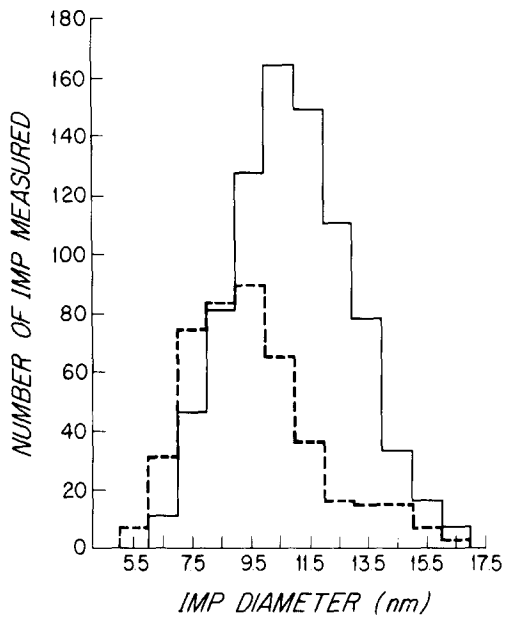

FIGURE 3 Histogram of the size distribution of IMP from the P-fracture face of LM cells (—, cell body; - - -, filopodial footpad). IMP measurements for the cell body were: $n = 822$, $\bar{x} = 10.97$ nm, $\sigma_{\bar{x}} = 2.014$ nm, $\sigma_n = 0.07$ nm. IMP measurements for the filopodial footpad region were: $n = 441$, $\bar{x} = 9.57$ nm, $\sigma_{\bar{x}} = 2.20$ nm, $\sigma_n = 0.11$ nm. The means for IMP size were significantly different at the 0.001 level as determined by the Student's t test.

filipin-sterol complexes which are readily identifiable in freeze-fracture (for review see reference 4). Filipin-sterol complexes were numerous and apparently randomly distributed within the plasma membrane of the cell body of LM cells, but virtually

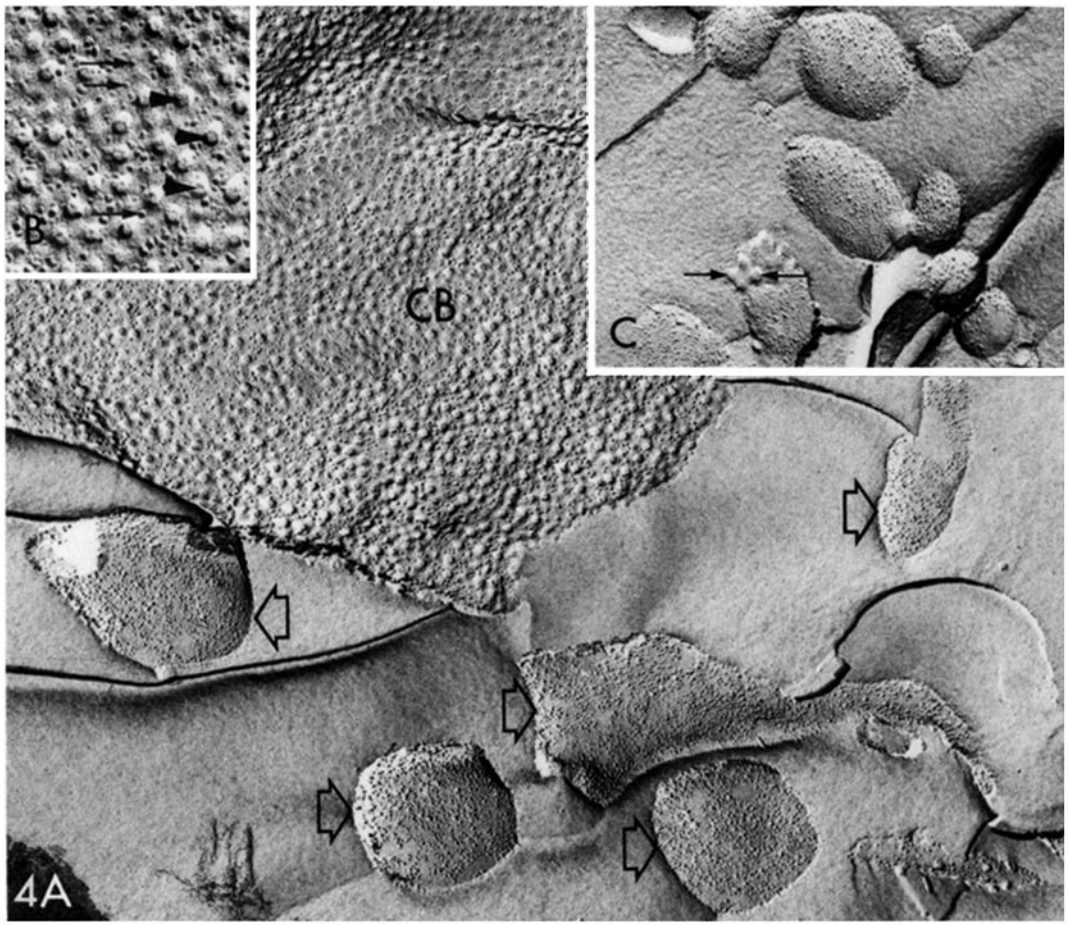

FIGURE 4 Freeze-fracture preparation of monolayer cultures of LM cells that were fixed in glutaraldehyde then treated with filipin before fracturing. (*A*) Filipin-sterol complexes are randomly distributed over the cell body (*CB*) while filopodial footpad membranes in this cell are devoid of the filipin-sterol complexes (open arrows). × 38,800. (*B*) Higher magnification of a portion of the cell body showing the filipin-sterol complexes (arrowheads) as well as the IMP (arrows). × 77,600. (*C*) Only occasionally were filipin-sterol complexes seen in filopodial-footpad regions (arrows). × 33,000.

FIGURE 5 Freeze-fracture preparation of EDTA-resistant substrate-attached material that was fixed in glutaraldehyde then treated with filipin before fracturing. The membranes have numerous IMP that in some cases are tightly aggregated with intervening particle-free regions (*). Note the presence of only a few filipin-sterol complexes (arrows). × 32,000.

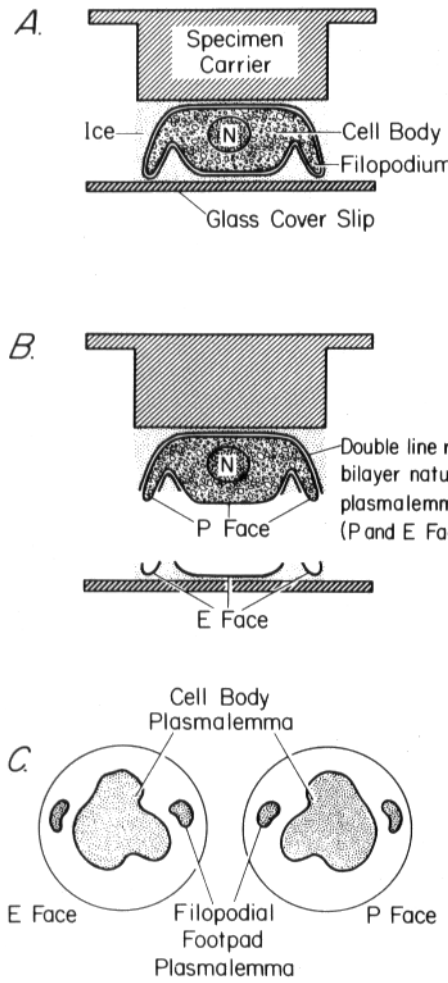

FIGURE 6 Diagram illustrating the *in situ* freeze-fracture of monolayer cultures. This diagram also explains how the filopodial footpads appear disjunct from the cell body in freeze-fracture replicas. (*A*) The complete sandwich-type preparation before fracturing. (*B*) A fracture through the cell bottom (substratum side). (*C*) The complementary fracture faces viewed from above, show the filopodial footpads separated from the cell body proper.

absent from the filopodial footpad membrane. This finding presumably represents a difference in the amount of cholesterol in different regions of the cell plasma membrane, with the footpad regions having less than the cell body. Regional differences in filipin-cholesterol complexes, and thus cholesterol, have been previously reported in guinea pig sperm (14), smooth muscle cells (20), basal disks, and retinal rod outer segments (3), and coated pits on the surface of cultured cells (19). Another possibility that our data do not rule out is that equal amounts of cholesterol are present in all parts of the plasma membrane, but that which is in the footpads is under some local constraints and therefore cannot be aggregated by filipin.

The results presented here suggest that there are local differences in both protein and lipid components of the plasma membrane of LM cells. Furthermore, these differences are related to a portion of the cell involved in adhesion to the substratum. Other workers have reported differences in the chemical composition of the substrate adhesion sites. Culp (13) describes differences in the protein composition, and Cathcart and Culp (7) report differences in phospholipid composition of adhesion sites compared with the surface membrane. In the present study, the similarity between the freeze-fracture images of filopodial footpad membrane and the EDTA-resistant substrate-attached material suggests that they are the same structures.

These observations represent a preliminary characterization of cell surface differences in LM cells which may have functional significance. It is interesting to speculate that the increased number of IMP at sites of filopodium-substratum interaction may represent an accumulation of proteins involved in adhesion. Alternatively, the IMP may represent specialized sites for association of the plasmalemma and cytoskeletal components which are related to adhesion. Adhesion-related membrane sites have been suggested from the interference-reflection and immunofluorescence microscopy observations of Bradley et al. (5) and Wehland et al. (33) and the high voltage electron microscopy observations of Heath and Dunn (17). The sites of increased IMP density observed at points of filopodium-substratum interaction may be equivalent to the "fibronexus" (30), an electron-dense zone on specific regions of the plasmalemma of certain fibroblasts. Additionally, the altered protein composition of the filopodial-footpad plasmalemma may require an altered lipid environment for proper function as suggested by the filipin data. It is our hope that these findings may provide some insight into regional specialization of the cell surface and the cell-substratum interaction.

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