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.

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% OsO4 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% OsO4 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...
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 dimethylylformamide
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 0.1M
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 mm² 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 con-
tain a mixture of cell shapes; however, most cells are spindle
shaped rather than being highly flattened (Table I). The ap-
pearance of filopodia associated with typical spindle-shaped
cells show filipodial 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 distribu-
tion 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).

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 ran-
domly distributed filipin-sterol complexes in the cell body
plasmalemma whereas the filopodial-footpad plasmalemma was
virtually devoid of these structures. Only occasionally were
the characteristic filipin-sterol complexes present in freeze-frac-
tured filopodial footpad membrane (Fig. 4).

Treatment of coverslip cultures of LM cells with EDTA in
Ca**+-Ms**+-free PBS causes the cells to round up. Complete
detachment of cells was hastened by gentle aspiration. Exam-
ination 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 sub-
strate-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,
the fact 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 pres-
ence of large numbers of filopodia under normal culture con-
ditions 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 mono-
layer cultures, the method of Yee et al. (34) was chosen, as this
technique appears to be the most straightforward and easiest

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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
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 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.
The present study suggests that there are local differences in both protein and lipid components of the plasma membrane. The authors wish to thank Dr. Richard L. Hoover for reading the manuscript and providing several helpful suggestions. Excellent technical assistance was provided by Jane Christiansen and Robert Rubin.

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