Localization of Cell Surface Glycoproteins in Membrane Domains Associated with the Underlying Filament Network

E. ROOS,* H. SPIELE,* C. A. FELTKAMP,* H. HUISMAN,* F. A. C. WIEGANT,* J. TRAAS,* and D. A. M. MESLAND*

*Division of Cell Biology, The Netherlands Cancer Institute (Antoni van Leeuwenhoek Huis), 1066 CX Amsterdam, The Netherlands, and *Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Dr. Wiegant’s present address is Laboratory for Molecular Cell Biology, University of Utrecht, The Netherlands. Dr. Traas’s present address is Botanic Laboratory, Catholic University of Nijmegen, The Netherlands. Dr. Mesland’s present address is European Space Technology and Research Center, Division TB, Noordwijk, The Netherlands.

ABSTRACT To visualize the localization of cell surface constituents in relation to the plasma membrane–associated filament network, we developed a method based on a combination of immunogold labeling and dry-cleaving. For labeling we used trinitrophenyl-derivatized ligand, anti-TNP antibodies, and protein A-coated colloidal gold. Dry-cleaving (Mesland, D. A. M., H. Spiele, and E. Roos, 1981, Exp. Cell Res., 132:169-184) involves cleavage of lightly fixed critical point-dried cells by means of adhesive tape. Since cells cleave close to the cell surface, the remaining layer is thin enough to be examined in transmission electron microscopy.

Using this method, we studied concanavalin A-binding constituents on the medium-facing surface of H35 hepatoma cells. The distribution of the gold particles, which was partly dispersed and partly patchy, coincided strikingly with membrane-associated filaments, and label was virtually absent from areas overlying openings in the filament network. In stereo pairs we observed the label to be localized to areas of somewhat enhanced electron density at the plane of the membrane. These areas were interconnected in a pattern congruent with the filament network.

Preliminary observations on wheat germ agglutinin receptors on the hepatoma cells as well as concanavalin A receptors on isolated hepatocytes yielded comparable results. It thus appears that surface glycoproteins, although seemingly randomly distributed as observed in thin sections, may actually be localized to particular membrane domains associated with underlying filaments.
and its submembranous localization (32-34), suggests that such a network is a general feature of cells, although its function is not necessarily comparable to that of the erythrocyte (35). Indeed, in membrane "blebs," which lack an associated cytoskeleton, the diffusion rate of surface proteins is much higher than in other parts of the cell (36). Apparently, the constraints are due to transient interactions with the cytoskeleton. It may then be assumed that such interactions keep even mobile proteins localized preferentially in areas where the network is attached to the membrane rather than homogeneously distributed. We present evidence supporting this notion in the case of concanavalin A (Con A)-binding proteins1 of hepatoma cells.

This evidence was obtained with the use of dry-cleaving, a method developed by us to visualize submembranous structures in the electron microscope (3). Lightly fixed, critical point-dried cells on a grid covered with a carbon-over-Formvar film, are attached to adhesive tape and subsequently removed, which leads to cleavage of the cells close to the Formvar-attached membrane (see Fig. 1). The layer remaining on the grid is thin enough to be studied in a transmission electron microscope. The method was first applied to cells that had been cultured on the grids, exposing the substrate-facing membrane and associated network (37). Later we showed that also cells that had been fixed in suspension could be cleaved, after attachment to poly-L-lysine-coated, Formvar-covered grids (38). Thus, we demonstrated the presence of membrane-associated filament networks, of distinct morphology, in several different cell types (38).

We now show that also the medium-facing surface can be studied, after inversion of a fixed monolayer on a poly-L-lysine-coated grid (see Fig. 1). This allowed us to examine cells of which the medium-facing surface had been labeled immunocytochemically to visualize membrane-associated filaments and Con A-binding membrane proteins simultaneously. Thus, we observed that these membrane proteins were localized in areas overlying the network. Moreover, the label defined the plane of the membrane in stereo electron micrographs and highlighted within this plane areas of somewhat enhanced electron density to which all of the label was localized. These membrane areas were interconnected in a pattern congruent with the filament network. These observations suggested that plasma membranes contain protein-rich microdomains in areas associated with cytoskeletal filaments.

MATERIALS AND METHODS

Cell Preparation: H35 hepatoma cells were cultured as previously described (38), except that they were grown in 16-mm multiwell plates on round plastic or glass coverslips. Preparation of cells was as described before (37-39). Briefly, cells were fixed in 0.1% glutaraldehyde, inverted on a grid (see below), treated with 1% tannic acid, postfixed with 0.5% OsO4, stained with uranyl acetate, dehydrated, and critical point dried. Rat hepatocytes were isolated and cultured in Petriperm dishes as detailed previously (37), and treated again, and subsequently dialyzed for 48 h against three changes of 1 liter of PBS. The extinction of the resulting solution was measured at 280 nm and the Con A concentration adjusted to 1 mg/ml, using underivatized Con A as standard. Subsequently, the extinction at 338 nm was measured and the degree of substitution calculated based on a molecular extinction coefficient of 11,100 for TNP (40). The molar ratio of TNP and Con A was approximately 2, and could not be increased by prolonged incubation or with higher concentrations down, on the sticky side of a clean piece of adhesive tape and gently pressed. Subsequently, the grid was removed, cleaving the cells and leaving the largest part of each cell on the tape. The grid was immediately viewed in the electron microscope, or stored for short periods in a dry environment. Further details are given in references 37-39. The whole procedure is illustrated in Fig. 1.

**Trinitrophenyl (TNP)-Con A:** 5 mg Con A and 100 mg a-methylmannoside (both from Sigma Chemical Co., St. Louis, MO) were dissolved in 4 ml phosphate-buffered saline (PBS). At room temperature, 3 mg trinitrobenzene-sulphonic acid in 1 ml of PBS was added dropwise under vigorous vortexing. The reaction was allowed to proceed for 30 min and then stopped by addition of 0.2 M glycine (40 ml). The mixture was concentrated under pressure in an Amicon filter unit (cut off: 10,000), diluted with PBS, concentrated again, and subsequently dialyzed for 48 h against three changes of 1 liter of PBS. The extinction of the resulting solution was measured at 280 nm and the Con A concentration adjusted to 1 mg/ml, using underivatized Con A as standard. Subsequently, the extinction at 338 nm was measured and the degree of substitution calculated based on a molecular extinction coefficient of 11,100 for TNP (40). The molar ratio of TNP and Con A was approximately 2, and could not be increased by prolonged incubation or with higher concentrations.

---

1. Abbreviations used in this paper: Con A, concanavalin A; TNP, trinitrophenyl.

**FIGURE 1** Schematic illustration of the dry-cleaving method as applied in this study. A piece of a glutaraldehyde-fixed monolayer of H35 hepatoma cells (a) is cut loose (b) and inverted onto a carbon-over-Formvar (Formvar/C) film-covered grid, coated with poly-L-lysine (c), treated with tannic acid and osmium tetroxide, dehydrated, and critical point dried. The grid is then placed cell-side down on adhesive tape (d), gently pressed, and removed (e), which leads to cleavage close to the grid (e). The resulting preparation (f) consists of the plasma membrane and a thin layer of cytoplasmic material.
of trinitrobenzene-sulphonic acid. Wheat germ agglutinin–TNP was prepared similarly, using N-acetylgalactosamine (Sigma Chemical Co.) instead of α-methylmannoside. In this case the molar ratio was approximately 1.

**Anti-TNP Antibodies:** TNP-derivatized bovine serum albumin (BSA) was prepared essentially as described above. Anti-TNP antibody was raised in a sheep by immunization with 1 mg TNP-derivatized BSA in complete Freund’s adjuvant, followed after 4 wk by a second injection with 0.5 mg TNP-BSA in incomplete Freund’s adjuvant. 1 wk later, citrated plasma was obtained. The plasma was defibrinated and inactivated by heating for 1 h at 56°C.

**Protein A–Gold Conjugate:** Protein A-coated gold particles with a diameter of 4–6 nm were prepared according to Slot and Geuze (41).

**Reaction with Lectins:** Cells were usually prefixed with 0.1% glutaraldehyde in HMK buffer (0.1 M KCl, 3 mM MgCl2, 10 mM HEPES, pH 6.9) for at least 15 min and then treated for at least 5 min with 20 mM glycine in PBS to quench unreacted aldehyde groups. Next, the cells were incubated at room temperature with 10–500 μg/ml Con A–TNP in PBS for 30 min. Controls were treated with Con A–TNP + 0.1 M α-methylmannoside or with PBS only. After three washes with PBS, anti-TNP antiserum was added in a 1:10 dilution, determined separately for each batch. Prior to use, this solution was centrifuged in a Beckman minifuge for at least 15 min and then treated for at least 5 min with 20 mM glycine in PBS to quench unreacted aldehyde groups. Next, the cells were incubated at room temperature with 10–500 μg/ml Con A–TNP in PBS for 30 min. Controls were treated with Con A–TNP + 0.1 M α-methylmannoside or with PBS only. After three washes with PBS, anti-TNP antiserum was added in a 1:10 dilution, previously determined to be optimal. After 30 min, the cells were washed three times with 0.1% BSA in PBS, and then incubated with an optimal dilution of the protein A–gold suspension in 0.1% BSA in PBS, determined separately for each batch. Prior to use, this solution was centrifuged in a Beckman minifuge to remove aggregates. After three washes in PBS, with intervals of 10 min, and 1 wash in HMK buffer (37), the cells were postfixed in 0.1% glutaraldehyde in HMK buffer, and further treated as described under "cell preparation." Part of the dry-cleaved cells were embedded in Epon to be able to observe in thin sections whether the cell membrane and the label had remained intact during treatment and critical point drying. As a result, the medium-facing rather than the substrate-facing plasma membrane and associated cytoplasmic material remained on the grid (Fig. 1f). This layer was thin enough to be examined in transmission electron microscopy.

In the present study we modified the method to visualize the medium-facing rather than the substrate-facing plasma membrane and associated material. A piece of a glutaraldehyde-fixed monolayer was cut out (Fig. 1b), inverted, and placed on a poly-L-lysine-coated grid (Fig. 1c) before further treatment and critical point drying. As a result, the medium-facing membrane was attached to the grid, and cells tended to cleave close to this membrane. Fig. 2 is a low power micrograph of such a preparation, showing both cleaved and uncleaved parts of a cell.

**Immunogold Labeling**

Using this method we examined cells of which the medium-facing surface had first been labeled to localize particular cell surface constituents. We describe here the visualization of Con A-binding molecules on H35 hepatoma cells with the use of a novel cytochemical method, based on TNP-derivatized ligand, anti-TNP antibodies, and protein A-coated colloidal gold. Optimal conditions were first established using thin sections of embedded cells. Fig. 3 shows the number of particles per μm as a function of Con A–TNP concentration, using optimal concentrations of anti-TNP antiserum and protein A–gold. Fig. 4a shows a cell treated with 500 μg Con A–TNP per ml. Cells incubated with Con A–TNP in the presence of the hapten sugar α-methylmannoside (Fig. 4b), as well as cells incubated with PBS only before treatment with antiserum and protein A–gold, were virtually free of label.
**Immunogold-labeled, Dry-cleaved Preparations**

Fig. 5 shows a stereo view of a dry-cleaved preparation of a labeled H35 hepatoma cell. The stereo pair is oriented such that the point of view is from within the cell through, first, the cortical filaments, second, the medium-facing membrane and, finally, the extracellularly located label. It can easily be seen that all of the label is located at approximately the same plane, which therefore should be a plane close to the extracellular face of the plasma membrane.

Closest to the viewer, i.e., at some distance intracellularly from the membrane, a dense array of knobby filaments with many associated proteins was seen to be present. These are the black areas in Fig. 5. Long, straight filaments were seen at this level (Fig. 5) as well as an occasional microtubule (not shown). The plane of cleavage was apparently within this layer and was irregular, so that in many areas this dense filament layer appeared to have been torn away (Fig. 5). Between this layer and the plasma membrane a network of various kinds of filaments was present, which could be most

---

**Figure 4** (Top) Part of a cell treated with 500 μg/ml Con A–TNP, followed by anti-TNP antibodies and protein A–gold. (Bottom) Same as a but treatment with Con A–TNP in the presence of 100 mM α-methylmannoside. × 47,500.

**Figure 5** Stereo view of a dry-cleaved preparation of the medium-facing surface of a cell treated as in top panel of Fig. 4. The view is from inside the cell. The black areas constitute a dense network some distance from the membrane. Note long, straight filaments (open arrows). In some parts, e.g., the framed area, this layer appears to have been torn away. The gold particles are located at sites of enhanced electron density at the membrane (e.g., arrows). × 90,000.
easily seen in the areas where overlying filaments had been torn away. Figs. 6–8 show photographical enlargements of parts of Fig. 5. Short thin filaments (~2 nm) can be seen (Fig. 6), as well as short strands of ~6 nm diameter (Figs. 7 and 8) which in some areas appear to be arranged in a hexagonal pattern with a ~28-nm center-to-center distance (Fig. 8). This network appeared to terminate at a plane located at a small but distinct distance from the gold label.

**Distribution of the Label**

As can be seen in Figs. 5–8, the gold label was partly dispersed and partly located in clusters of up to ~25 particles. The label was not randomly distributed but located preferentially where many filaments were present, and strikingly absent from membrane areas overlying openings in the network. Upon inspection of the stereo pairs, the label was found to be localized to areas of somewhat higher electron density. This was particularly evident in case of clustered particles. Filaments of the cortical network often terminated at or near such electron-dense areas (Fig. 6). The areas were interconnected and thus appeared to form a network of membrane domains.

The electron-dense membrane areas were also seen in dry-cleaved cells that had not been treated with Con A-TNP although less readily because these were not highlighted by the presence of label (Fig. 9). The empty membrane areas were not simply holes in the membrane, since such holes could be readily distinguished (Fig. 9). Furthermore, holes were not usually seen in thin sections of embedded, dry-cleaved cells (Fig. 10). The distribution of label was not different from that seen on non-cleaved cells. On replicas of the upper surface of dried but not cleaved cells (Fig. 11) label was seen in particular areas in a pattern similar to that observed in dry-cleaved cells. In cells fixed in ice-cold glutaraldehyde, and treated at 0°C up to and including osmium tetroxide fixation, the same distribution was seen as in cells treated similarly but at room temperature (not shown).

**Non-pre-fixed Cells**

In some experiments, cells were incubated with Con A-TNP for 5, 10, or 30 min at 37°C, before fixation. Fig. 12 shows the number of gold particles per μm², counted on dry-cleaved preparations. Numbers per μm as counted on thin sections were converted to numbers per μm² assuming a section thickness of 90 nm. The latter data were in good agreement with those counted on dry-cleaved preparations. In both cases, the amount of label rapidly declined between 0 and 10 min after Con A-TNP application, and remained constant thereafter. At all times, the label remained dispersed as well as clustered, and an increase in clustering was not apparent.

**WGA on Hepatoma Cells and Con A on Hepatocytes**

Results obtained with wheat germ agglutinin–TNP were quite similar to those described for Con A–TNP. Again, label was at the electron-dense membrane areas, dispersed as well as clustered. Label was virtually absent when the lectin was applied together with the hapten sugar N-acetylglucosamine. In preliminary experiments with isolated hepatocytes, the membrane-associated network was found to be denser than in the hepatoma cells. Nevertheless, all label was again localized in areas associated with the filaments, and absent from the small areas overlying openings in the network (not shown).
FIGURE 7  Enlargement of a part of the same preparation as in Fig. 5. Note strands of ~6 nm (arrows) and high electron density at the membrane where gold particles are clustered (open arrows). × 225,000.

FIGURE 8  Enlargement of a part of Fig. 5. Note 6-nm strands in a hexagonal pattern (arrows), and labeled membrane (open arrows). × 225,000.
DISCUSSION

To visualize the topographical relation between membrane-associated filaments and membrane constituents, we combined immunogold labeling and dry-cleaving. To study the applicability of the method, we selected a particularly suitable model. The epithelial cell monolayers used had the advantage that they could be easily detached and inverted, as was necessary to cleave the cells close to the medium-facing surface. Furthermore, we chose to visualize lectin-binding surface molecules, because they are numerous and because they can be labeled after fixation. However, preliminary results indicate that the method can also be applied to adherent fibroblasts as well as to suspended cells, and to the visualization of less
distribution was an artifact of our method. For instance, one
protein-rich microdomains exist in the plasma membrane of
those areas. This observation suggested that protein-poor and
to a comparatively high concentration of surface proteins at
erythrocyte membrane (43). This density may have been due
localized to membrane areas of enhanced electron density,
hexagonal pattern suggested it to be composed of clathrin
membrane was usually intact, and the location and distribu-
the dry-cleaving procedure. Finally, in thin sections of embedded dry-cleaved preparations, the cell
membrane usually was intact, and the location and distribu-
of label not different from uncleaved cells (Fig. 10).
An alternative possibility is redistribution due to inadequate
fixation. Cell surface proteins are not necessarily completely
immobilized by glutaraldehyde fixation (45) and one might
imagine that solitary, non-cross-linked proteins are still able
to move after fixation until they collide with cytoskeleton-
associated proteins, to which they might then be cross-linked.
This movement should be severely restricted, if not annihil-
ated, at 0°C (46, 47). However, when all treatments from
available electron density fixation were performed at 0°C, the same distribution of label
was found, rendering this possibility less likely. Furthermore,
free aldehyde groups were reacted with glycine and this should
have prevented further cross-linking. A last possibility to be
considered is redistribution of cell surface proteins induced,
even after fixation, by attachment to the poly-L-lysine or by
the dry-cleaving procedure. The observation that the distri-
bution of label on replicas of the upper surface of cells that
had not been inverted and not dry-cleaved (Fig. 11) was
comparable to that in dry-cleaved cells, appears to exclude
this possible artifact.
The concept of membrane microdomains has been postu-
lated before, based on fluorescence polarization measure-
ments of lipid fluidity (48). The present observations suggest
a possible explanation for the existence of such membrane
domains. If membrane proteins interact with membrane-
associated filaments they would tend to be preferentially
localized to sites where such filaments are present, even if the
interactions are weak and transient. This would lead to pro-
rich and protein-poor regions in the membrane. Differ-
ences in lipid fluidity might then be caused by interactions of
lipids with proteins (49) or even by a different lipid compo-
sition of protein-rich areas, due to preferential association of
proteins with particular lipids (50).
The localization of proteins to particular membrane areas
is not necessarily in contrast to their observed mobility. The
protein-rich areas were finely dispersed and interconnected so
that proteins might readily diffuse through such areas albeit
constrained by their interactions with the filaments. Their
localization near membrane-associated filaments would allow
instantaneous immobilization upon alteration of the affinity
for those filaments after interaction with ligand (7–10).
The method described might be particularly useful to study
alterations in membrane-associated filaments upon patching
and capping of surface molecules. Con A–binding molecules
on H35 hepatoma cells did not extensively redistribute after
binding to Con A, so that this model is evidently not the most
interesting in this respect. The only apparent change observed
was a reduction in label within the first 10 min after exposure
to Con A–TNP (Fig. 12). We have not extensively studied the
reasons for this reduction. Internalization does not seem a
likely explanation because, as previously described (38), these
cells do not contain many coated vesicles. Alternatively, Con

Figure 12. Number of gold particles counted on cells incubated
with 500 μg/ml Con A–TNP at 37°C for various periods before
fixation, ●, dry-cleaved preparations; ★, thin sections. In the latter
case, numbers per μm² were calculated assuming a section thick-
ness of 90 nm. Shown are the results of a representative experiment.

The presence of the gold label at the extracellular face of
the membrane allowed us to localize that face in stereo
micrographs. Many filaments appeared to terminate at a plane
at some distance from the label. It seems likely that this plane
was the intracellular face of the membrane. Different types of
such filaments were observed, with an approximate thickness
of either 2 or 6 nm, the latter sometimes arranged in a
hexagonal pattern. The nature of the protein constituents of
these filaments is not known. However, the dimensions of the
hexagonal pattern suggested it to be composed of clathrin (42).
Anyhow, it appears that membrane connections are not
formed by a single type of filament.
The most striking result of this study was the observed
distribution of lectin-binding surface constituents as revealed
by our novel TNP-anti-TNP/protein A-gold labeling
method. The label was not homogeneously distributed as
might be expected based on observation of thin sections, but
strikingly coincident with the pattern of the filament network.
In stereo micrographs, virtually all label was seen to be
localized to membrane areas of enhanced electron density,
possibly equivalent to the "granular" patches seen at the
erythrocyte membrane (43). This density may have been due
to a comparatively high concentration of surface proteins at
those areas. This observation suggested that protein-poor and
protein-rich microdomains exist in the plasma membrane of
the hepatoma cells as well as of hepatocytes. These microdo-
mains were dispersed so finely that the divergence from
homogeneous distribution was not observed in thin sections,
and may therefore have gone undetected so far.
The possibility should be considered that the observed
distribution was an artifact of our method. For instance, one
might imagine that upon dry-cleaving, part of the label was
selectively removed from certain membrane areas. This pos-
sibility seems remote because it would require the label to be
pulled through the membrane and the cell cortex, which
would evidently result in a perforated membrane. In damaged
preparations, holes in the membrane can readily be seen (Fig.
9) and such holes were not observed at sites where label was
lacking. Furthermore, the amount of label was not different
in dry-cleaved preparations compared to thin sections, assum-
ing the latter to have a thickness of 90 nm, a reasonable
assumption for sections with a light yellow interference color
(44). Although admittedly section thickness cannot be exactly
measured, this would seem to indicate that not a large part of
the label is lost during the dry-cleaving procedure. Finally, in
thin sections of embedded dry-cleaved preparations, the cell
membrane was usually intact, and the location and distribu-
tion of the label not different from uncleaved cells (Fig. 10).

The method described might be particularly useful to study
alterations in membrane-associated filaments upon patching
and capping of surface molecules. Con A–binding molecules
on H35 hepatoma cells did not extensively redistribute after
binding to Con A, so that this model is evidently not the most
interesting in this respect. The only apparent change observed
was a reduction in label within the first 10 min after exposure
to Con A–TNP (Fig. 12). We have not extensively studied the
reasons for this reduction. Internalization does not seem a
likely explanation because, as previously described (38), these
cells do not contain many coated vesicles. Alternatively, Con
A–TNP may have been shed from the cell. Our favored explanation is illustrated in Fig. 13. Upon limited cross-linking of proteins by ConA, part of the determinants may become unaccessible to anti-TNP antibodies. In addition, several ConA–binding determinants, each able to bind a single lectin molecule on prefixed cells, are bound to the same lectin molecule after cross-linking, again leading to decreased overall binding. Thus, one should realize that there is not necessarily a strict relation between the number of ConA–binding determinants present and the amount of label.

In summary, we have developed a method potentially useful for the study of membrane–cytoskeleton interactions in relation to cell surface protein mobility. Furthermore, we have observed an inhomogeneous distribution of surface glycoproteins and an apparent association of these proteins with membrane-associated filaments which, if found to be generally applicable, should be taken into account in models of membrane phenomena.

We thank Nico Ong for photography and Gea Meijerink for secretarial assistance.

Received for publication 19 December 1984, and in revised form 11 June 1985.