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Subcellular Localization of Forssman Glycolipid in Epithelial MDCK Cells by Immuno-electronmicroscopy after Freeze-Substitution

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Abstract. Forssman antigen, a neutral glycosphingolipid carrying five monosaccharides, was localized in epithelial MDCK cells by the immunogold technique. Labeling with a well defined mAb and protein A–gold after freeze-substitution and low temperature embedding in Lowicryl HM20 of aldehyde-fixed and cryoprotected cells, resulted in high levels of specific labeling and excellent retention of cellular ultrastructure compared to ultra-thin cryosections. No Forssman glycolipid was lost from the cells during freeze-substitution as measured by radio-immunostaining of lipid extracts. Redistribution of the glycolipid between membranes did not occur. Forssman glycolipid, abundantly expressed on the surface of MDCK II cells, did not move to neighboring cell surfaces in cocultures with Forssman negative MDCK I cells, even though they were connected by tight junctions. The labeling density on the apical plasma membrane was 1.4–1.6 times higher than basolateral. Roughly two-thirds of the gold particles were found intracellularly. The Golgi complex was labeled for Forssman as were endosomes, identified by endocytosed albumin-gold, and lysosomes, defined by double labeling for cathepsin D. In most cases, the nuclear envelope was Forssman positive, but the labeling density was 10-fold less than on the plasma membrane. Mitochondria and peroxisomes, the latter identified by catalase, remained free of label, consistent with the notion that they do not receive transport vesicles carrying glycosphingolipids. The present method of lipid immunolabeling holds great potential for the localization of other antigenic lipids.

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Morphological observations have provided significant contributions to our present understanding of the metabolic processing, the intracellular trafficking, and the function of membrane lipids. However, since many such studies have depended on fluorescent lipid analogues as the principal probe (van Meer, 1989; Pagano, 1990), the resolution was mostly limited to that of the light microscope. In contrast to several very successful immunocytochemical localization procedures for proteins (Hayat, 1989; Verkleij and Leunissen, 1989), methods for the reliable and reproducible immunolocalization of lipids at the electron-microscopic level have not been developed. Such methods should meet the same criteria required for protein localization i.e., maintenance of antigenicity, immobilization of antigens, accessibility of antigens, and preservation of overall cellular structure. Proteins can be fixed by aldehydes without severe loss of antigenicity in many cases. The major technical problem with lipids has not been the lack of well-defined antilipid antibodies (Feizi, 1985), but the fact that lipids are not immobilized by routine fixatives. This causes their extraction when the tissue is dehydrated with organic solvents during embedding procedures.

A number of antibodies has been characterized to recognize glycolipids, and the presence of these lipids on the cell surface has allowed the use of pre-embedding methods for their immunolabeling. Pre-embedding labeling in combination with plastic embedding (Stern and Bretscher, 1979; Nichols et al., 1987; Seybold et al., 1989), freeze-etch labeling (Tillack et al., 1983; Rock et al., 1990), and fracture-label (Barbosa and Pinto da Silva, 1983) have provided us with insights in the surface distribution of glycolipids on cells and on liposomes. In contrast, attempts to reveal the intracellular distribution of glycolipids have been largely restricted to the immunofluorescence level. Indirect immunofluorescence staining on semi-thin frozen sections of MDCK cells has shown that the Forssman glycolipid is not restricted to the apical plasma membrane but mainly occurs intracellularly (Hansson et al., 1986). Similar observations have been reported in other cell types (Tanaka and Leduc, 1956) and for other lipids (Symington et al., 1987). Attempts to define the intracellular locations of glycolipids at the electron microscopic level have not been very successful (Boddingius and Dijkman, 1989; Kanai et al., 1990).

To determine the subcellular distribution of the Forssman glycolipid in MDCK cells at the EM level, we have tried to avoid the problem of lipid extraction in two ways. First, we have taken the most obvious route of circumventing dehydration by the use of thawed ultrathin cryosections. For proteins this is presently the most widely applied and successful procedure for immuno-EM localization. In this method, sec-

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tions are cut at low temperature from tissue that has been fixed and infused with a concentrated sucrose solution as a cryoprotectant (Tokuyasu, 1980).

In a second approach we dehydrated the tissue by freeze-substitution. This procedure, when applied to nonfixed tissue, has been shown successful in making embedding compatible with lipid retention (Humbel and Müller, 1984; Weibull and Christiansson, 1986) and preservation of pure lipidic structures (Verkleij et al., 1985). However, tissue and cell fixation by rapid freezing often induces serious freezing damage. Only a few micron at the very surface of the specimen retains a good ultrastructure without visible ice crystals. Larger samples can only be frozen without ice crystal formation after cryoprotection or by the technically rather demanding technique of high pressure freezing (Moor, 1987). Therefore, rather than using fresh-frozen material, we have combined parts of the cryo-ultramicrotomy and the freeze-substitution procedures by using aldehyde-fixed cells infused with a cryoprotectant for freeze-substitution embedding in Lowicryl HM20.

While cryosections were found to suffer from extraction of the Forssman glycolipid (low labeling) and partial relocation from membranes over the section, the novel hybrid technique resulted in excellent preservation of the ultrastructure together with highly efficient and accurate localization of the glycolipid.

Materials and Methods

Cell Culture

MDCK strain I and II cells were grown on 24.5-mm diameter Transwell filters (Costar Data Packaging Corp., Cambridge MA) as described before (van Meer et al., 1987). Instead of performing the cell culture in the original six-well cluster dishes, the filter holders were suspended in 20-mm high polypropylene rings. Six of these were placed into a 150-mm diameter dish containing 100 ml of growth medium, supplying a sixfold larger volume of basal medium per filter. Cells were plated at a density of 3.5×10^5 cells/cm^2 and used after 3–4 d. Under these conditions, a monolayer of MDCK II cells contained $(5.0 \pm 0.5) \times 10^6$ cells ($n = 12$; three experiments) and 129 ± 1 ($n = 2$) nmol phospholipids. This cell density of $(1.1 \pm 0.1) \times 10^6$ cells/cm^2 and the phospholipid composition were identical to those on cellulose ester filters (Hansson et al., 1986). The present cell density in Transwells is twofold higher than when these had been suspended in a six-well cluster dish (van Meer et al., 1987) suggesting that in the latter situation the medium supply on the basolateral aspect of the Transwells was limiting.

Immunoreagents

The antibody against the Forssman glycolipid, 33B12, was a gift of A. Sonnenberg (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and is a rat monoclonal of the IgG2c subclass which binds to protein A-Sepharose. The antibody reacts with the terminal sugar sequence GalNAc α1-3 GalNAc and is specific for Forssman (Sonnenberg et al., 1986). The antibody against cathepsin D was a gift of K. von Figura (Georg August Universität, Göttingen, Germany) (Gieselmann et al., 1983). The anticatalase antibody was raised against a two times crystallized bovine catalase (product number C-100, Sigma Chemical Company, St. Louis, MO) and was affinity purified. BSA coupled to 5-nm gold particles (BSAG)[1] and protein A-gold complexes (PAG) of different size classes were prepared by the tannic acid-citrate method (Slot and Geuze, 1985).

1. *Abbreviations used in this paper*: BSAG, BSA coupled to 5-nm gold; PAG, protein A-gold complexes; TGR, *trans*-Golgi reticulum.

Endocytosis of BSA-Gold

Filter-grown MDCK II cells were washed twice with serum-free medium and incubated for 1 h at 37°C in the medium containing BSAG (OD_{520} ~5) on both sides of the cell monolayer. Cells were rinsed quickly with cold 0.1 M Pipes buffer (pH 7.2) and fixed for freeze-substitution.

Radio-immunolabeling of Forssman Glycolipid on Thin Layer Chromatograms

Cellular lipids were extracted by a one-phase extraction (Rose and Oklander, 1965) to avoid loss of Forssman glycolipid into the water phase of a two-phase extraction (Sonnenberg et al., 1986). Lipid classes were separated by thin layer chromatography (TLC) on aluminum-backed silica sheets (SG 60, Merck, Darmstadt, FRG) in chloroform/methanol/water 60:35:8 (vol/vol), after the plates had been prerun in the same solvent system. The TLC sheet was developed over a distance of 45 mm and dried under a hair-drier (30 min). A band of 20 mm in the middle of the TLC sheet containing the Forssman glycolipid (retention factor of 0.3–0.4, Hansson et al., 1986; Sonnenberg et al., 1986) was cut out and labeled with the anti-Forssman antibody as described by Magnani et al. (1987). The chromatogram was soaked for 1 min into hexane containing 0.4% poly-isobutyl-methacrylate (300K M. W., Aldrich, Brussels, Belgium) diluted from a 2.5% stock in chloroform. After drying under the hair-drier for 15 min, the sheet was blocked in blotto (5% milk powder [wt/vol] in 50 mM Tris/HCl, pH 7.8, 2 mM $CaCl_2$) for 30 min. Subsequently, it was placed onto a glass support slightly smaller than the TLC sheet and then in a petri dish lined with wet filter paper. After careful application to the silica surface of an antibody dilution in blotto (60 μl/cm^2), the petri dish was slowly rocked back and forth horizontally for 60 min at room temperature. The sheet was then washed 4× by dipping into blotto for 1 min, after which it was incubated for 60 min with ^{125}I-protein A. Washes were repeated for 4 × 1 min in blotto and 2× 1 min in ice-cold PBS. The TLC sheet was air-dried. Forssman glycolipid was located on x-ray film, exposed at −70°C. Radioactivity was determined by excising the bands from the aluminum sheet and gamma-counting. The corresponding area from a lane where no lipid extract had been applied served as a blank.

Cryo-ultramicrotomy and Immunogold Labeling

MDCK cells, grown to confluency in 3.4-cm petri dishes, were fixed for 1 h with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, on ice. Cells were scraped from the petri dish and embedded as a pellet in 10% gelatin in 0.1 M phosphate buffer at 37°C. After solidification at 4°C the gelatin was postfixed with 0.5% glutaraldehyde at 4°C. Small blocks of ~1 mm^3 were cut, infiltrated with 2.3 M sucrose, mounted on LKB specimen stubs, and frozen in liquid nitrogen (LKB Instruments, Inc., Gaithersburg, MD). Ultrathin cryosections were cut at −100°C on a Leica/LKB V with cryo-attachment. Frozen sections were transferred to carbon-coated Formvar grids, immunogold labeled and stained as described before (Slot et al., 1988). Thawed sections were incubated for 1 h at room temperature with anti-Forssman and subsequently with 12 nm PAG (OD_{520} = 0.2). Sections were observed and photographed in a Jeol 1200EX electron microscope at 80 kV.

Freeze-Substitution and Low Temperature Embedding

Transwell filters with a confluent monolayer of MDCK cells were rinsed shortly with 0.1 M Pipes buffer (pH 7.2) and fixed for 1 h in 2% paraformaldehyde in 0.1 M Pipes on ice. Cells were cryoprotected by immersing the filters for 30 min in 30% glycerol containing 1% paraformaldehyde in 0.1 M Pipes or 2.3 M sucrose. The filters were cut in small squares of ~1 mm^2. Several squares were mounted on top of a LKB specimen stub. Glycerol-protected samples were frozen in liquid propane (−180°C) and sucrose-protected samples were frozen in liquid nitrogen. Samples were stored in liquid nitrogen before use.

Frozen samples were transferred (under liquid nitrogen) into the Reichert Cs-auto freeze-substitution unit. Freeze-substitution was carried out at −90°C in methanol supplemented with 0.5% uranyl acetate for at least 36 h. After raising the temperature to −45°C at a rate of 5°C/h and several washes with pure methanol, the samples were infiltrated with Lowicryl HM20. Infiltration was done in the following graded series of Lowicryl-methanol mixtures: 1:1 for 2 h, 2:1 for 2 h, pure Lowicryl for 2 h, and pure Lowicryl overnight. The samples were transferred, inside the Reichert Cs-

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auto, to a flat embedding mold filled with pure Lowicryl HM20 and polymerized by UV-light at −45°C for 2 d.

Immunogold Labeling of Lowicryl Sections

Ultrathin Lowicryl sections were cut perpendicular to the filters with a Leica/LKB III ultramicrotome. The sections were placed on carbon-coated Formvar grids and labeled according to the following procedure. Nonspecific binding sites were blocked by incubating the sections for 20 min on a drop of 5% FCS in 20 mM Tris, 130 mM NaCl, pH 8.2. Sections were incubated at room temperature with anti-Forssman diluted in 0.1% BSA in 20 mM Tris, 130 mM NaCl, pH 8.2 (BSA/Tris), for 1 h. After rinsing three times for 10 min with BSA/Tris the sections were incubated with PAG for 1 h and rinsed three times for 10 min with BSA/Tris, two times for 5 min with Tris, and three times for 10 min with bidistilled water. In double-labeling experiments, the last step was followed by fixation of the sections for 10 min with 1% glutaraldehyde in bidistilled water and blocking with 0.02% glycine in Tris buffer before labeling with the second antibody and PAG of a different size. Sections were stained for 7 min with 3% uranyl acetate and for 4 min with Reynolds lead citrate (Reynolds, 1963). Sections were observed and photographed in a Jeol 1200EX electron microscope at 60 kV.

Results

Preservation of the Cellular Forssman Glycolipid during Freeze-Substitution

Radio-immunolabeling of glycosphingolipids after separation by TLC proved to be a reliable method for quantitation of the Forssman antigen in lipid extracts of MDCK II cells (Fig. 1). Only the Forssman band was detected on exposed films of the complete TLC sheet. An accurate standard curve was obtained for the labeling intensity as a function of the amount of cellular lipids applied to the TLC sheet (Fig. 1 *A*). Under the present growth conditions, 10^5 MDCK II cells contain ∼25 pmol of Forssman glycolipid (Hansson et al., 1986). For studying possible loss of cellular lipids during freeze-substitution, three filters with a confluent monolayer of MDCK II cells (3 d) were cut from their holders and subjected to the freeze-substitution procedure. Subsequently, their lipids were extracted and a defined fraction of each extract, equivalent to 2×10^5 cells, was subjected to TLC together with extracts from control filters. Quantitation of the radioactivity after immunostaining showed that the Forssman antigen had been completely retained during the freeze-substitution procedure (103 ± 15%, Fig. 1). Lanes containing the lipid extract from 2×10^5 MDCK I cells yielded blank values. Densitometry of linearly exposed films gave identical results (data not shown).

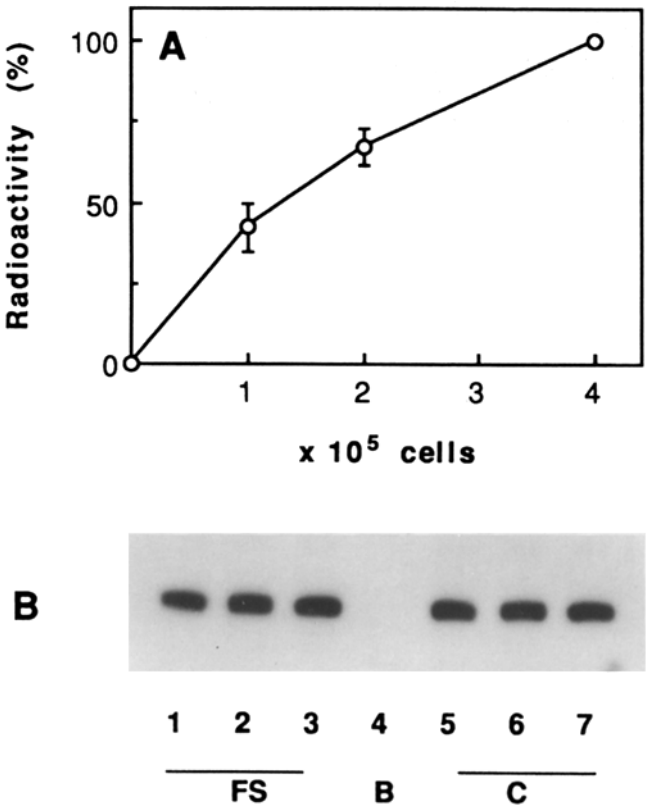

Figure 1. Retention of Forssman glycolipid throughout the freeze-substitution procedure. (*A*) Standard curve of radio-immunolabeling of Forssman glycolipid with anti-Forssman antibody and ^{125}I-protein A on TLC as described under Materials and Methods. Data are the mean of five curves in three experiments. Results could be quantitatively compared on the same TLC sheet only. This was due to variability in the coating procedure of the TLC sheet and to variations in batches of ^{125}I-protein A. The labeling obtained with 4×10^5 cells (1,000–5,000 cpm; blank, 170 cpm) was set to 100%. (*B*) 16 h exposure of a radio-immunolabeled TLC sheet. (lanes *1–3*) Lipid extracts of three sets of 2×10^5 MDCK II cells subjected to the freeze-substitution procedure (*FS*). (lane *4*) Blank, no lipids applied (*B*). (lanes *5–7*) Lipids from three sets of control cells (*C*). Quantitation of the bands gave $(2.5 \pm 0.4) \times 10^3$ cpm for the freeze-substituted samples vs. $(2.4 \pm 0.3) \times 10^3$ cpm for the control cells. These data combined with a second, identical quantitation showed that freeze-substituted monolayers contained 103 ± 15% of the Forssman glycolipid present in the controls.

Morphology

To mimic as closely as possible the morphology of an epithelium in vivo, MDCK cells were grown on permeable supports. The preparation of ultrathin cryosections of MDCK cells grown on filters turned out to be a cumbersome procedure. The cryosectioning often resulted in rupture of the cells from the filters, and wrinkled sections. In addition, large empty vacuoles were often observed. In contrast, prefixed and cryoprotected MDCK cells showed excellent overall morphology after freeze-substitution and low temperature embedding in Lowicryl HM20 (Fig. 2). Extended monolayers of intact cells on the filter were obtained, in which many large and small vacuoles exhibited internal vesicles and membrane structures. The MDCK cells were nicely polarized, expressing microvilli on the apical plasma membrane, tight junctions with neighboring cells, and showing well defined Golgi complexes located primarily at the apical side of the nucleus, which in turn was situated in the basal half of the cell.

Immunogold Labeling

Labeling of ultrathin cryosections with the antibody against Forssman and PAG resulted in a sparse labeling of the apical plasma membrane (Fig. 3 *A*). Beside the plasma membrane many intracellular organelles were labeled (Fig. 3 *B*), including the Golgi complex, electron-lucent, electron-dense, and multivesicular vesicles. However, beside this organelle-associated labeling we often observed gold labeling spread over the cytoplasm and the nucleus (Fig. 3 *B*) on cryosec-

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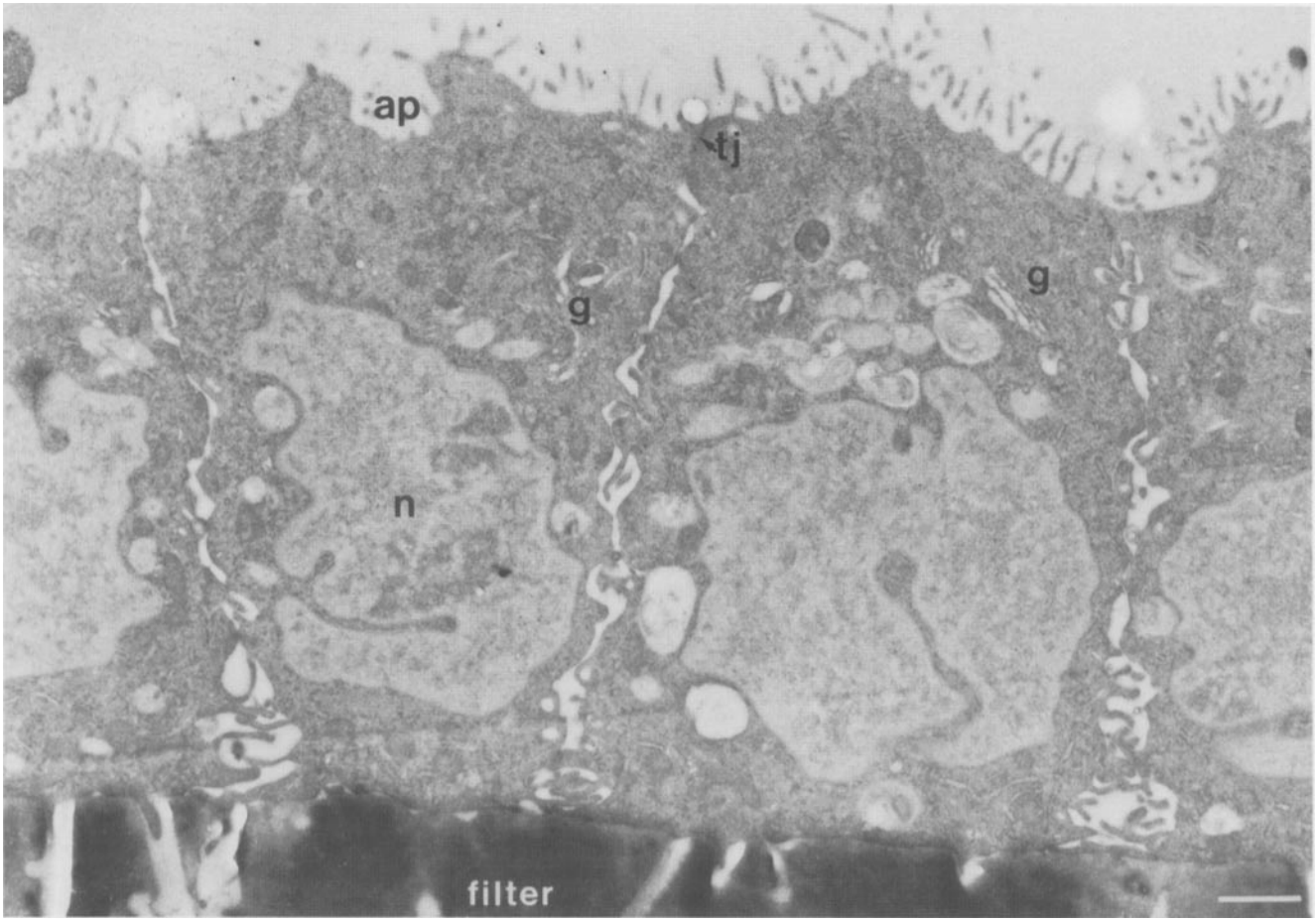

Figure 2. Electron micrograph of freeze-substituted and Lowicryl HM20-embedded MDCK strain II cells grown on Transwell filters. The MDCK cells form a tight monolayer on the filter (*f*) with apical microvilli (*ap*), tight junctions (*tj*) with neighboring cells, the nucleus (*n*) at the basal part of the cell and Golgi complexes (*g*) located predominantly at the apical side of the nucleus. Bar, 1 μm.

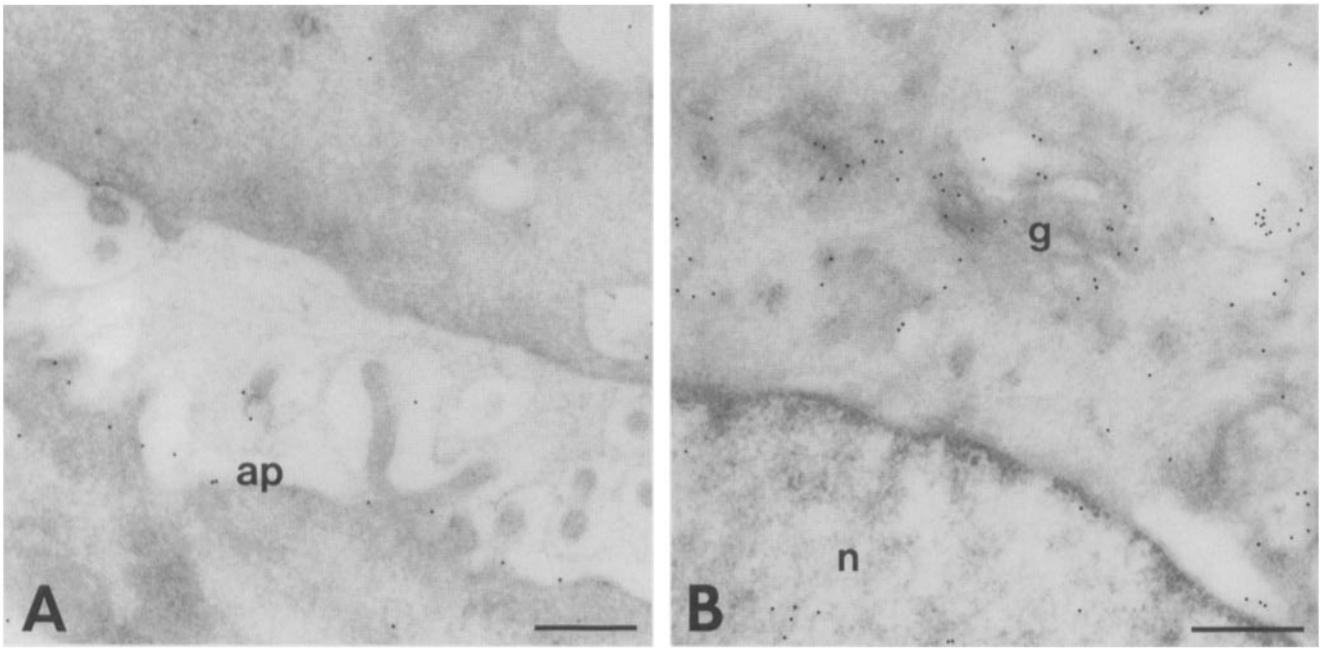

Figure 3. Electron micrographs of ultrathin cryosections of a MDCK strain II cell pellet immunolabeled for the Forssman glycolipid with 12 nm PAG. (*A*) The apical plasma membrane (*ap*) is sparsely labeled. (*B*) Intracellular label is found in the Golgi complex (*g*), electron-lucent, electron-dense, and multivesicular vesicles, the nuclear matrix (*n*), and the cytoplasm. Bars, 0.5 μm.

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tions of MDCK cells. This labeling was probably due to extraction and relocation of the Forssman glycolipid during section thawing and the immuno-incubations, since control sections, labeled with an irrelevant antibody, were negative. Identical observations were made on filter grown cells (data not shown).

Immunogold labeling of the Forssman antigen on Lowicryl sections of freeze-substituted MDCK cells resulted in a dramatic increase of specific label (Fig. 4). This increase was not due to the omission of the glutaraldehyde fixation since postfixation of sections with glutaraldehyde in the double-labeling procedures did not reduce subsequent labeling with anti-Forssman (see Fig. 6). Background labeling over the nuclear matrix was far less than 0.1% of the total number of gold particles per cell. Both the apical and the basolateral plasma membrane were heavily labeled. Quantitation showed that the labeling density, expressed as gold particles per membrane length, was 1.4 times higher at the apical plasma membrane (1.12 ± 0.09) than at the basolateral plasma membrane (0.81 ± 0.03) (Table I *B*). Mitochondria (Figs. 4 and 5 *A*) and peroxisomes (Fig. 5 *B*) were negative for Forssman labeling. An average of <1 gold particle/mitochondrion was found, which could be attributed to background labeling. Peroxisomes were identified by double-labeling with an antibody against the peroxisomal marker catalase. Immuno-double-labeling showed that apart from incidental background labeling, all catalase-positive structures observed ($n > 50$) were negative for Forssman labeling (Fig. 5 *B*). The negative mitochondria and peroxisomes indicate the high specificity of the Forssman labeling. Control experiments in which the primary antibody was omitted, or replaced by an irrelevant antiserum, showed no gold labeling. Intracellular labeling was found in the Golgi complex, in small vesicular and tubular structures underneath the plasma membrane, in vesicular structures throughout the cytoplasm, in larger electron-lucent and electron-dense structures often containing internal membrane structures (Fig. 4), and in the nuclear envelope (Fig. 6). The relative distribution of gold particles over the plasma membrane and intracellular membranes showed that ~66% of the label was intracellular (Table I *A*). The labeling densities in both the Golgi complex (0.38 ± 0.4) and the nuclear envelope (0.10 ± 0.02) were clearly lower than the plasma membrane (Table I *B*). Lysosomes (and late endosomes), which were double labeled for cathepsin D, contained fairly large amounts of the Forssman glycolipid (Fig. 6). To discriminate between the biosynthetic

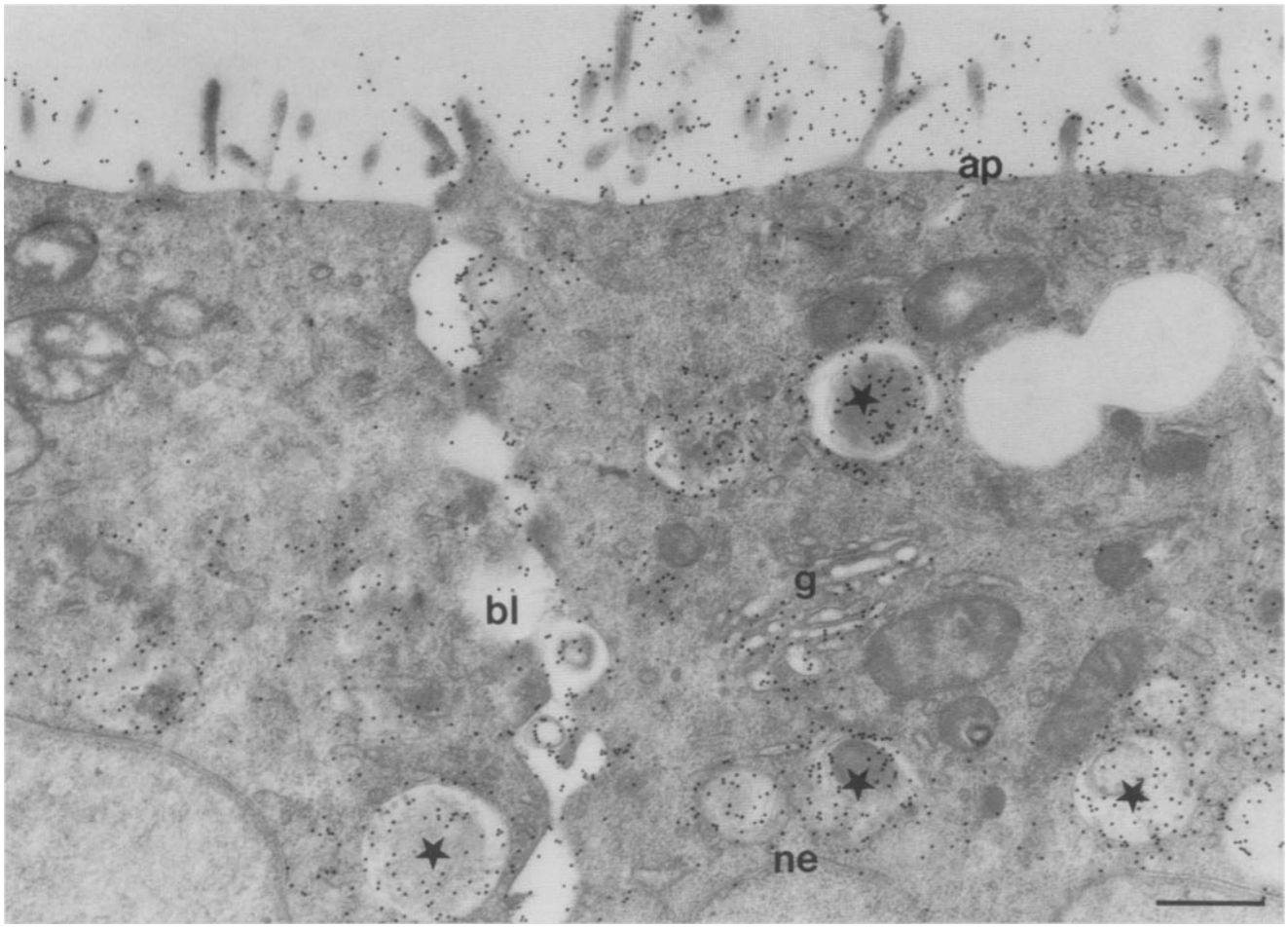

Figure 4. Distribution of the Forssman glycolipid, labeled with 15 nm PAG, in freeze-substituted and Lowicryl HM20–embedded MDCK strain II cells grown on filters. Labeling of the Forssman glycolipid is observed at the apical (*ap*) and basolateral (*bl*) plasma membrane, in the Golgi complex (*g*), in small vesicular and tubular structures throughout the cytoplasm, in electron-lucent, electron-dense, and multivesicular structures (*), and the nuclear envelope (*ne*). Bar, 0.5 μm.

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Table I. Percentual Distribution and Labeling Densities of the Forssman Glycolipid in MDCK Strain II Cells after Freeze-Substitution and Lowicryl HM20 Embedding

A

Compartment	% Gold
Apical plasma membrane	22 ± 1
Basolateral plasma membrane	12 ± 1
Intracellular	66 ± 2

B

Compartment	Labeling density (gold/membrane length)
Apical plasma membrane	1.12 ± 0.09 (n = 14)
Basolateral plasma membrane	0.81 ± 0.03 (n = 9)
Golgi complex	0.38 ± 0.04 (n = 24)
Nuclear envelope	0.10 ± 0.02 (n = 11)

(*A*) The percentual distribution of gold particles over the plasma membrane and cell interior was calculated from Lowicryl HM20 sections which had been labeled with the antibody against the Forssman glycolipid and subsequently with a mixture of 10 and 20 nm PAG in such a ratio that ~5% of the gold label was 20 nm, to facilitate counting. From 14 cells of two different sections 4,304 gold particles of 20 nm were counted directly from the screen in the electron microscope at low magnification. (*B*) The labeling density over membranes in the different compartments was calculated from the number of gold particles per corresponding membrane length. The membrane length is expressed as the number of intersections with a sampling grid in 54,000× micrographs. The spacing between the lines of the grid was 0.36 μm. The number of compartments counted is given between brackets, and 1,399 gold particles were counted. The values in *A* and *B* are expressed as the mean value ± SEM.

and the endocytotic pathway, MDCK cells were allowed to endocytose BSAG continuously for 1 h. After this incubation BSAG was found in small vesicles, in tubular and tubular-vesicular structures, in multivesicular structures, and in lysosomes (Fig. 7). Many BSAG positive small vesicles and tubular-vesicular structures contained the Forssman glycolipid. In addition, vesicular structures positive for BSAG were observed that were negative for Forssman, while the opposite was also true (Fig. 7). In a double-labeling experiment, lysosomes as identified by anticathepsin D, were found to contain both BSAG and Forssman (Fig. 7).

Finally, we tested whether Forssman glycolipid when expressed on one membrane, would be able to equilibrate with membranes exposed to it. For this, MDCK II cells were cocultured with Forssman-negative MDCK I cells. Monolayers of MDCK I cells were indeed free of gold after immunolabeling for Forssman (not shown). In mixed monolayers of MDCK I and II cells, where functional tight junctions are formed between the two cell types (van Meer et al., 1986), Forssman positive cells were found adjacent to negative cells (Fig. 8). In areas of close contact between the cells, gold label could not be unequivocally assigned to the positive cell. However, from areas where the lateral membranes were sufficiently separated, it can be concluded that redistribution of the Forssman glycolipid between apposed membranes during coculture or during the EM preparation procedure had not occurred.

Discussion

Forssman Antigen: Structure and Antigenicity

The present study describes the immunolocalization of Forssman antigen in MDCK II cells. After its first definition as an antigen (Forssman, 1911), the molecule was chemically characterized 60 yr later as the five-sugar glycosphingolipid IV^3-GalNAcα-Gb$_4$Cer (IUPAC-IUB nomenclature, 1977) or GalNAc α1-3 GalNAc β1-3 Gal α1-4 Gal β1-4 Glc β1-1 Cer (Siddiqui and Hakomori, 1971). Although crossreactions of

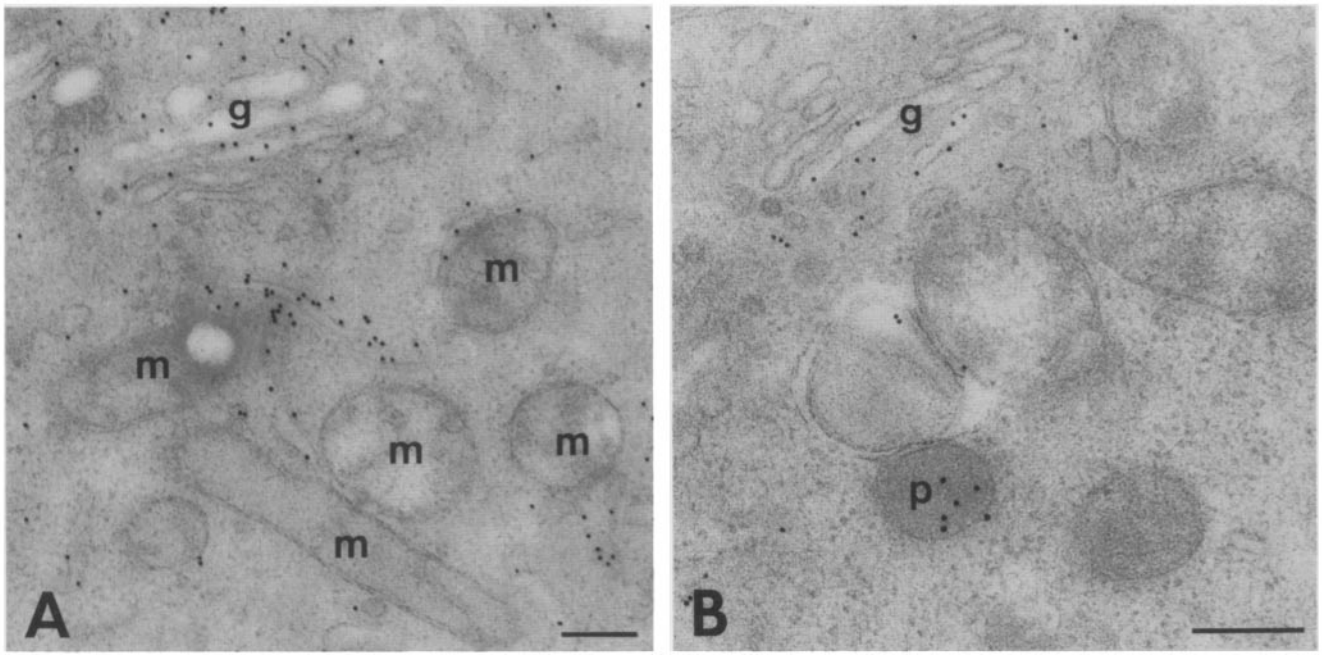

Figure 5. Electron micrographs of substitution-embedded MDCK strain II cells labeled for the Forssman glycolipid with 10 nm PAG. (*A*) Mitochondria (*m*), close to a positive Golgi complex (*g*) show no labeling. (*B*) Double labeling with anticatalase (15 nm PAG). A catalase-containing peroxisome (*p*) is negative for Forssman labeling in contrast to the Golgi complex (*g*). Bars, 0.2 μm.

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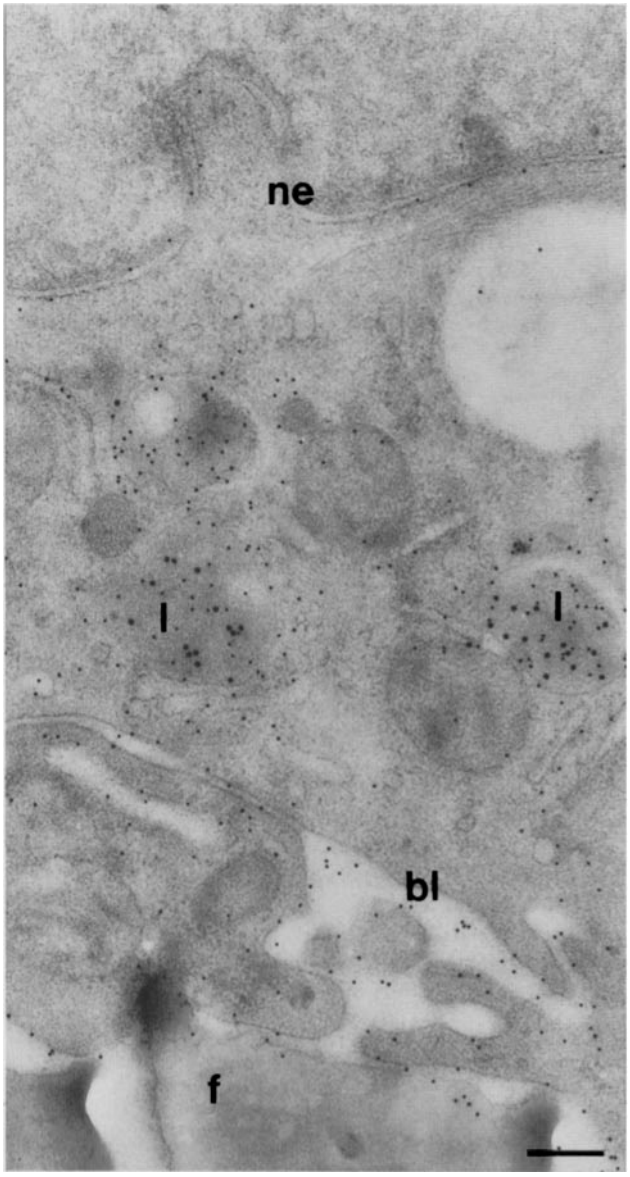

Figure 6. Substitution-embedded MDCK strain II cells double labeled for cathepsin D (15 nm PAG) and the Forssman glycolipid (10 nm PAG). The same results were obtained when changing the order of the antibodies or the sizes of the gold. Cathepsin D is present in lysosomes (*l*) together with the Forssman glycolipid. Small vesicular and tubular structures, the nuclear envelope (*ne*) and the basolateral membrane (*bl*) next to the filter (*f*) also contain the Forssman glycolipid, but no cathepsin D. Bar, 0.2 μm.

anti-Forssman antibodies with other glycolipids have been reported, our antibody in MDCK cells most likely reacted with Forssman glycolipid exclusively. (*a*) The present 33B12 monoclonal only recognized Forssman glycolipid in a lipid extract in which one other anti-Forssman antibody also reacted with shorter and longer glycolipid species (Sonnenberg et al., 1986). (*b*) In MDCK II lipid extracts run on full length (100 mm) TLC sheets, 33B12 stained Forssman glycolipid exclusively. (*c*) Neither of the three potentially crossreacting glycosphingolipids, a ceramide carrying four monosaccharides (Gahmberg and Hakomori, 1975), one with seven and one with eight monosaccharides (Slomiany and Slomiany, 1978) were chemically detected in MDCK II cells, whereas Forssman glycolipid constituted 20–40 mol % of their neutral glycosphingolipids (Hansson et al., 1986; Nichols et al., 1986*a*; Niimura and Ishizuka, 1986). (*d*) The closely related MDCK I cells, which lack Forssman glycolipid (Hansson et al., 1986), showed no immunolabeling on TLC or sections (Fig. 8). (*e*) Finally, the presence of Forssman activity on glycoproteins (Slomiany et al., 1982) was unlikely in MDCK II, as immunostaining of a protein blot of MDCK II cells was negative (not shown).

Validity of the Preparation and Labeling Procedure

Our results demonstrate for the first time a reliable intracellular localization of a glycosphingolipid at the electron-microscopic level. The immunolocalization of the Forssman antigen combined a high specificity with an excellent morphological preservation. Freeze-substitution has been shown previously to result in the retention of most lipids and proteins (Humbel and Müller, 1984; Weibull and Christiansson, 1986). This could have been different for lipids containing hydrophilic sugar chains. However, it is demonstrated in the present study that during the preparation procedure the Forssman glycosphingolipid was quantitatively retained (Fig. 1). The increased labeling density on the plasma membrane, as compared to cryosections, points out that the freeze-substitution procedure preserves this glycolipid much better than the cryo-ultramicrotomy method. Morphological studies have also shown that freeze-substitution is capable of preserving pure lipidic structures (Verkleij et al., 1985) and lipid-rich organelles (Ikeda, 1985). Here we show that the Forssman glycolipid remained membrane associated, as it did not redistribute during the preparation and labeling procedures. Mitochondria and peroxisomes, Forssman negative membranes according to chemical criteria (van Meer, 1989), indeed remained free of label.

In Figs. 4, 6, and 7 the gold label is clearly observed on both sides of the membranes. An interpretation that Forssman glycolipid would be present in both bilayer leaflets does not fit the accepted view that glycosphingolipids are confined to the non-cytoplasmic leaflets of the membrane bilayers of cellular organelles (Hakomori, 1981, 1990; Thompson and Tillack, 1985). This view is based on the observations that glycosphingolipids are expressed on the surface but not the cytoplasmic side of the plasma membrane, and on the localization of a number of glycosylation reactions in the lumen of the Golgi and of glycosphingolipid degradation in the lumen of the lysosomes. Most likely, therefore, the random transmembrane gold distribution is due to folding of the primary antibody or the protein A–gold.

The fact that Forsman antigen is expressed on the outside of MDCK II cells (van Meer et al., 1986; Nichols et al., 1986*b*, 1987), while the surface of MDCK I cells within submicrometer distance from a positive MDCK II cell surface (Fig. 8) remained Forssman negative, implies that during freeze-substitution the lipid not even exchanged between closely apposed surfaces.

On Tight Junction Structure

The absence of Forssman from the apical surface of an MDCK I cell that was connected to an MDCK II cell by tight

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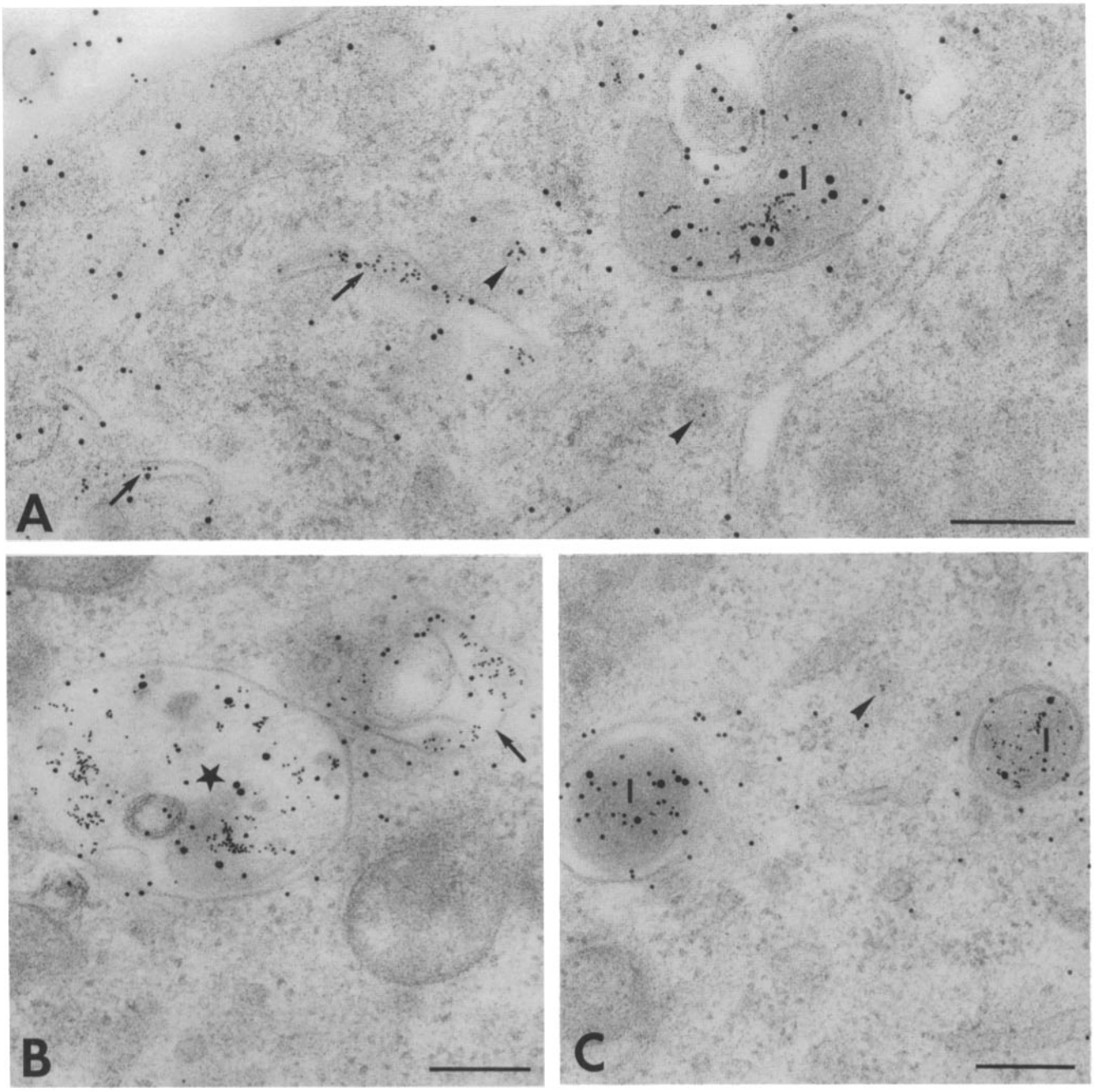

Figure 7. Substitution-embedded MDCK strain II cells which had endocytosed 5 nm BSAG for 1 h at 37°C. Sections were double-labeled for the Forssman glycolipid (10 nm PAG) and cathepsin D (15 nm PAG). BSAG-containing endocytotic profiles often contain the Forssman glycolipid (*arrows* in *A* and *B*) but some BSAG positive vesicles are devoid of the Forssman glycolipid (*arrowheads* in *A* and *C*). Dense lysosomes (*l*) and more electron-lucent lysosomes (*), lysosomes defined by the presence of cathepsin D, contain BSAG and are labeled for the Forssman glycolipid. Bars, 0.2 μm.

junctions, confirms at the electron-microscopic level the notion from earlier light microscopic observations that lipids are unable to diffuse from the apical surface of one epithelial cell to that of the next, even at a time scale of days (Nichols et al., 1986*b*; van Meer et al., 1986). The novel observation that also the lateral surface of the MDCK I cell remained free of Forssman under these conditions demonstrates that a barrier for lipid diffusion between neighboring cells exists on both the apical and basolateral face of the tight junction. Together, these observations argue against the lipid model of tight junctions in its simple form (Kachar and Reese, 1982; Pinto da Silva and Kachar, 1982).

Forssman Glycolipid on the Cell Surface

Forssman antigen is abundant on the MDCK II cell surface (van Meer et al., 1986; Nichols et al., 1986*b*; 1987; Figs. 4 and 8). Glycosphingolipids on the cell surface have been assigned a variety of functions in growth and differentiation,

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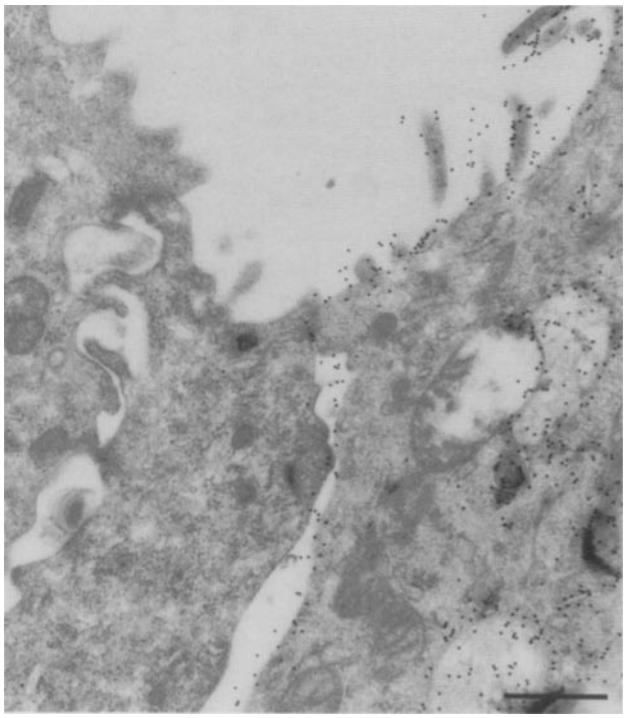

Figure 8. Mixed monolayer of MDCK strain I and strain II cells after 3 d of coculture, immunolabeled for the Forssman glycolipid after substitution embedding. Positive and negative cells are found juxtaposed. Bar, 0.5 μm.

cellular interactions, and signal transduction. In each case, discrete glycosphingolipids would interact with ligands in solution or on apposed cell surfaces (Curatolo, 1987*b*; Hakomori, 1981, 1990). A specific function of Forssman antigen is suggested by the fact that it is expressed at defined stages of mouse embryogenesis and human cancer (Kanai et al., 1990, and refs. therein).

When present in significant amounts, glycosphingolipids change the physico-chemical properties of the membrane (exoplasmic leaflet). Membrane rigidity increases due to intermolecular hydrogen bonding between the glycosphingolipids, a capacity absent from most phospholipids (Pascher, 1976; Thompson and Tillack, 1985; Curatolo, 1987*a*, *b*). The effect is fully expressed by the one-sugar ceramides, glucosylceramide and galactosylceramide. Prime examples are found in plasma membranes displaying extreme concentrations of glycosphingolipids, like myelin (Morell et al., 1989) and apical plasma membranes of epithelia (Simons and van Meer, 1988), where these lipids account for roughly 30–35 mol % of the total lipids. The apical surface of intestinal cells, and also MDCK cells, appears to be covered by glycosphingolipids (Forstner et al., 1968; Simons and van Meer, 1988).

Although the enrichment of (glyco)sphingolipids in the apical plasma membrane, accompanied by a reduced percentage of the phospholipid phosphatidylcholine, seems to be the most general feature of epithelial lipid polarity, the basolateral plasma membrane is by no means devoid of glycosphingolipids. The surface density of the species that constitute the bulk of the epithelial glycosphingolipids, notably glucosylceramide, is three- to fourfold reduced on the basolateral surface (Kawai et al., 1974; Simons and van Meer, 1988). Similarly, Forssman antigen, a bulk glycosphingolipid of MDCK II (Hansson et al., 1986; Nichols et al., 1986*a*; Niimura and Ishizuka, 1986), has been located on both surfaces of MDCK II cells at the electron-microscopic level by preembedding immunogold labeling (Nichols et al., 1987), which is now confirmed by immunogold labeling on sections of freeze-substituted MDCK II cells (Figs. 4 and 8).

Chemical analysis of radiolabeled glycosphingolipids that had been shed from the two cell surfaces of MDCK II cells showed that Forssman glycolipid and glucosylceramide displayed a twofold apical/basolateral enrichment relative to galactosylceramide. However, this work did not provide absolute numbers for the apical/basolateral ratio of the surface densities of either lipid (Nichols et al., 1988). In the present study, we have found 1.8 times more gold particles on the apical than on the basolateral cell surface (Table I *A*). The relative surface area of the two domains in MDCK II cells grown under the present conditions has been measured to be 1.1 (apical/basolateral; Parton, R., personal communication). From these numbers the density of gold particles on the apical surface as compared to that on the basolateral surface can be calculated to be 1.6. This is in fair agreement with the relative surface density of 1.4 that was measured independently by a direct count of gold particles per membrane length (Table I *B*), and suggests an enrichment of Forssman glycolipid on the apical cell surface. However, it should be realized that extrapolation of the measured surface densities of gold particles to surface densities of the Forssman glycolipid requires a linear relationship between labeling and Forssman density, for the moment an unproven assumption. One of the potential pitfalls is that Forssman glycolipid tends to organize in small clusters in the membrane and that the size of these clusters may depend on the membrane type (see Rock et al., 1990 and refs. therein). The quantitative potential of the method is one of the objects of our present studies.

Intracellular Distribution of Forssman Glycolipid

Two-thirds of the Forssman glycolipid was assigned to defined intracellular organelles (Table I *A*). This refutes the general idea that glycosphingolipids would be typical plasma membrane components (Hakomori, 1981; 1990; Thompson and Tillack, 1985; Curatolo, 1987b). Evidence for large intracellular pools of individual glycosphingolipids has already been provided by immunofluorescence studies (Tanaka and Leduc, 1956; Hansson et al., 1986; Symington et al., 1987) and biochemical studies (Matyas and Morré, 1987).

The intracellular compartment in which the presence of Forssman glycolipid is most readily understood is the Golgi apparatus, for this is where the terminal glycosyltransferases are situated (Paulson and Colley, 1989). Forssman glycolipid was found in the Golgi stacks and in the *trans*-Golgi reticulum (TGR). The TGR has been suggested to be the site of epithelial glycosphingolipid sorting (Simons and van Meer, 1988). Apical vesicles would bud from areas of the TGR membrane containing microdomains of glucosylceramide and other apically enriched glycosphingolipids, whereas vesicles destined for the basolateral domain would bud from glycolipid poor areas. Our present results do not provide a direct test for this hypothesis. More efficient labeling will be required for a statement on possible microdomains of Forssman glycolipid in the TGR.

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The localization of Forssman in organelles along the endocytic route as defined by the presence of endocytosed BSAG is consistent with what is known about lipid transport. The endosomal lipid composition generally seems very similar to that of the plasma membrane, suggesting that endocytosis does not select for or against specific lipids (van Meer, 1989). Some vesicles containing endocytosed BSAG were not labeled for Forssman (Fig. 7). This does not necessarily imply that these vesicles do not contain Forssman glycolipid. Even if a vesicle has the same Forssman content as the plasma membrane, there is a statistical chance that it remains unlabeled due to its small size (see Figs. 4 and 7). Small Forssman-containing structures, negative for BSAG, may be part of the biosynthetic pathway or may lack BSAG due to its inefficient uptake. The abundance of Forssman glycolipid in the internal membranes of multivesicular endosomes and lysosomes (as defined by colocalization with cathepsin D, Figs. 6 and 7) may imply that a large fraction of the intracellular Forssman does not participate in membrane recycling between endosomes and the plasma membrane.

A significant level of Forssman labeling was observed in the nuclear envelope (Figs. 4 and 6, Table I *B*), implying the presence of Forssman in the ER. Recently we have observed significant and specific labeling of the nuclear envelope by an antibody against the glycosphingolipid galactosylceramide as well. The chemical presence of glycosphingolipids in the nuclear envelope and ER has been suggested by cell fractionation work (discussed by Matyas and Morré, 1987), but such results have always been subject to the criticism of possible cross-contamination of the fractions. The presence of glycosphingolipids in these membranes is rather unexpected, because the general idea is that glycosphingolipids are transported by carrier vesicles only, and because a vesicular pathway between their site of synthesis, the Golgi, and the ER has not been described. Two possibilities for this pathway are vesicular transport back through the Golgi, or alternatively transport to the plasma membrane and via endocytic membranes to the ER. Evidence for the latter process has been reported. After endocytosis of Simian Virus 40, one third of the virus eventually reached the ER lumen without ever being apparent in the Golgi (Kartenbeck et al., 1989).

No Forssman labeling was observed in mitochondria and peroxisomes. This nicely illustrates the specificity of the labeling and the fact that lipid redistribution did not occur during the present study. This observation strongly supports the idea that glycosphingolipids are transported in the luminal leaflet of carrier vesicles only and that mitochondria and peroxisomes do not take part in such vesicular exchange processes. Although mitochondrial fractions have been found to contain glycosphingolipids (e.g., Matyas and Morré, 1987), only the present method may be capable of discriminating between real content and contamination by other membranes.

Future Prospects

The present study demonstrates that it is now possible to localize antigenic lipid molecules in a reliable way even when they contain a hydrophilic sugar chain. This provides us with a new technique for addressing the intracellular distribution and traffic of lipids. Preliminary studies have shown that the method is applicable to antigenic lipids with short sugar chains. Also galactosylceramide has been labeled, which will now allow for double-labeling of different lipids and the determination of their relative distribution.

The first type of information obtained from lipid immunolabeling is a qualitative discrimination between membranes which may or may not contain a certain lipid. With the present level of accuracy it will be possible to firmly establish whether or not the unexpected presence of a certain lipid in an organelle after cell fractionation was due to contamination by other membranes. Examples of this are the proposed presence of glycosphingolipids in mitochondria (Matyas and Morré, 1987), and cardiolipin in the outer mitochondrial membrane (Hovius et al., 1990). Secondly, as apparent from the different labeling densities for the various organelles (Table I *B*) the method has quantitative potential. Obviously, the linearity of the labeling will have to be tested before the labeling density data can be translated to surface densities of the antigen. Eventually, it will be very important to test whether the method can be extended to study lateral heterogeneities in lipid distribution within a single organelle. These have been shown for proteins, which has been crucial in testing models of protein sorting (Geuze et al., 1987). All in all, the present method of lipid immunolabeling appears highly promising for providing new insights into the cell biology of lipids.

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