Glycosphingolipid-Enriched, Detergent-Insoluble Complexes in Protein Sorting in Epithelial Cells†

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Received February 24, 1993

ABSTRACT: In simple epithelial cells, the delivery of apical and basolateral proteins to the cell surface is mediated by sorting in the *trans*-Golgi network and transport via separate vesicular carriers. In order to identify the molecular machinery involved in protein sorting, we have recently studied a detergent-insoluble complex in Madin-Darby canine kidney (MDCK) cells, following CHAPS extraction of exocytic carrier vesicles, specifically including the apical marker protein influenza hemagglutinin (HA). Previously, a Triton X-100 insoluble membrane residue that was enriched in glycosylphosphatidylinositol-anchored (GPI) proteins and glycolipids was characterized and implicated in transport to the apical cell surface [Brown, D., & Rose, J. (1991) *Cell 68*, 533–544]. In this report, the protein compositions of the CHAPS and Triton complexes have been compared by two-dimensional gel analysis. Only a few major membrane proteins are found in the complexes. The protein compositions are qualitatively similar, but differ quantitatively in the individual components. The CHAPS complex is depleted of GPI-linked proteins and retains a minor fraction of lipids similar in composition to that of the Triton X-100 insoluble complex. We propose that *in vivo* the complexes form part of a sorting platform that mediates protein segregation and delivery to the apical cell surface.

Epithelial cells form a boundary between the external and internal environments and perform vectorial functions in secretion, absorption, and ion transport. To fulfill this task, the cell surface is differentiated into apical and basolateral membrane domains with distinct protein and lipid compositions. Intermixing of components is prevented by the tight junctions [for reviews, see Simons and Fuller (1985), Rodriguez-Boulan and Nelson (1989), and Wandinger-Ness and Simons (1991)].

The processes involved in the generation and maintenance of epithelial cell polarity, such as protein sorting and targeting, have been studied extensively using dog kidney (MDCK)[1] cells as a model system (McRoberts et al., 1981; Rodriguez-Boulan, 1983). Apical and basolateral proteins in transit to the cell surface were found to be sorted from each other in the *trans*-Golgi network (TGN) and packaged into different vesicular carriers (Griffiths & Simons, 1986). Likewise, glycosphingolipids, which are highly enriched in the exoplasmic leaflet of the apical membrane domain, are segregated from bulk lipids presumably in the TGN and preferentially transported to the apical cell surface by a vesicular carrier mechanism (van Meer et al., 1987; Kobayashi et al., 1992; van Meer & Burger, 1992). This leads us to propose that protein and lipid sorting is mediated by a coclustering of apically sorted proteins and glycosphingolipid microdomains in the TGN (Simons & van Meer, 1988; Simons & Wandinger-Ness, 1990).

Experimental evidence supporting this view has recently been provided by Brown and Rose (1992). They found that, in MDCK cells, a class of apically targeted proteins anchored to the membrane by a glycosylphosphatidylinositol (GPI) tail (Low, 1989) associates with glycolipids during transit through the Golgi. Association was assayed by measuring the TX-100 solubility of placental alkaline phosphatase (PLAP) and was shown to depend not on protein–protein interactions or interaction with cytoskeletal elements, but rather on the lipid environment encountered by PLAP on its itinerary to the cell surface. The incorporation of apical integral membrane proteins into similar structures is implied by earlier studies of Skibbens et al. (1989), where newly synthesized influenza hemagglutinin (HA) was observed to become TX-100 insoluble late in the Golgi but before arrival at the cell surface.

Our recent approach to identification of the molecular machinery involved in sorting and targeting of proteins to the apical membrane domain focused on analyzing interactions of a cargo protein, influenza HA, with other vesicular components (Kurzchalia et al., 1992). Solubilization of exocytic TGN-derived carrier vesicles, isolated from virally infected, perforated MDCK cells (Simons & Virta, 1987; Bennett et al., 1988), with the zwitterionic detergent CHAPS led to the extraction of a high molecular weight complex of influenza HA associated with a subset of the vesicular proteins. The basolateral marker VSV G protein was excluded from this complex. A detergent extraction protocol allowed the isolation of one of these proteins, VIP21, from a total cellular membrane fraction. The cDNA encoding VIP21 has been cloned and sequenced, and as expected for a component that may be involved in protein sorting and targeting, VIP21 was found to be localized to the Golgi and the cell surface (Kurzchalia et al., 1992).

† K.F. was supported by the Verband der Chemischen Industrie and Boehringer Ingelheim Fonds; T.V.K. was supported by a long-term EMBO fellowship. This work was supported by SFB 352 of the DFG.

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[1] Abbreviations: 2-D, two-dimensional; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonate; endoH *endo-β-N*-acetylglucosaminidase H; gD1–DAF, herpes simplex virus gD1 fused to human decay accelerating factor; GPI, glycosylphosphatidylinositol; HA, influenza hemagglutinin; INA, iodonaphthyl azide; MDCK cell, Madin-Darby canine kidney cell; PLAP, placental alkaline phosphatase; PNS, postnuclear supernatant; TGN, *trans*-Golgi network; TX-100, Triton X-100; TX-114, Triton X-114; VSV, vesicular stomatitis virus.

0006-2960/93/0432-6365$04.00/0

In this study, we provide a link between the Triton-insoluble, glycolipid-enriched membrane residue (Brown & Rose, 1992) and the CHAPS-insoluble high molecular weight complex by comparing their compositions. Both complexes are shown to contain a qualitatively similar set of glycolipid and protein components, but quantitatively they differ dramatically in composition. These findings support our proposal that glycosphingolipid-enriched membrane domains, together with a proteinaceous sorting machinery, are involved in the segregation of proteins and in their delivery to the apical cell surface.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise indicated, all chemicals were obtained from the sources described previously (Bennett et al., 1988; Wandinger-Ness et al., 1990). CHAPS was purchased from Serva (Heidelberg, Germany), triolein was from Sigma, HPTLC plates were from Merck (Darmstadt, Germany), DEAE Sephadex A-25 were from Pharmacia (Uppsala, Sweden), and Iatrobeads were from Iatron Laboratories Inc. (Tokyo, Japan). PI-PLC from *Bacillus cereus* was a kind gift of D. Brown (SUNY, Stony Brook, NY). All glycolipid standards were a gift of Y. Hirabayashi (RIKEN, Saitaina, Japan).

Cell Culture and Viruses. MDCK strain II cells were grown and passaged using media (growth medium, infection medium, labeling medium, chase medium, water-bath medium) previously described (Matlin et al., 1981; Bennett et al., 1988; Wandinger-Ness et al., 1990). For pulse–chase experiments, cells from one confluent 75 cm^2 flask were seeded onto 12 24-mm diameter and 0.4-μm pore size, premounted Transwell polycarbonate filters (a kind gift from Hank Lane, Costar, Cambridge, MA). The filters were transferred to holders in a Petri dish containing 80 mL of growth medium. For preparation of total cellular membrane fractions, cells from one confluent 75 cm^2 flask were seeded onto three 150 cm^2 dishes. Cultures were maintained at 37 °C and 5% CO_2 for 2–3 days. Influenza N virus (A/chick/Germany/49, Hav2 Neq1) was grown, purified, and titred on MDCK cells as previously described (Matlin et al., 1981; Matlin & Simons, 1983).

Pulse–Chase Experiments. Confluent MDCK cell monolayers, grown on 24-mm Transwell filters for 3 days, were rinsed twice in infection medium and placed into a six-well culture dish. Infection medium (200 μL) containing 20 pfu/cell of influenza N virus was added to the apical side and adsorbed to the cells for 1 h. The inoculum was removed; 1 mL of infection medium was added to the apical side and 2.5 mL was added to the basolateral side of the filter. The infection was continued for an additional 2.5 h at 37 °C, 5% CO_2. Pulse-labeling with [^{35}S]methionine was performed for 4 min as described before (Bennett et al., 1988). After various times of chase, the filters were transferred to ice-cold chase medium, washed twice in PBS, and extracted with 600 μL of extraction buffer containing 20 mM CHAPS, 20 mM Tris-HCl (pH 7.4), 2 mM DTT, 2 mM EGTA, and a protease inhibitor cocktail (CLAP: 20 μg/mL each of chymostatin, leupeptin, antipain, and pepstatin). The extracts were transferred to 1.5-mL Eppendorf tubes and incubated with rotation at 4 °C for 30 min. After a 5-min centrifugation at 13 000 rpm and 4 °C in an Eppendorf microfuge, the supernatant fraction was removed (Skibbens et al., 1989). The pellets were recentrifuged to remove the remaining soluble material, and 600 μL of extraction buffer was added to the pellet. Pellets and supernatants were precipitated with 10% TCA, and equal fractions of the precipitates were subjected to SDS–PAGE on 7% gels. The gels were quantitated using the Molecular Dynamics Phosphor-Imager.

Preparation of a Total Cellular Membrane Fraction. Cells grown on 150 cm^2 dishes for 2 days were washed twice with PBS and incubated with 13 mL of metabolic labeling medium containing 1 mCi of [^{35}S]methionine for 12 h at 37 °C, 5% CO_2. The medium was aspirated and stored at 37 °C. After two washes with infection medium, 2 mL of infection medium containing 5 pfu/cell of influenza N virus was added to the dish and incubated for 1 h at 37 °C. Following aspiration, the metabolic labeling medium saved, supplemented with 0.1 mCi of fresh [^{35}S]methionine, was readded to the dish and incubated for 3 h at 37 °C. During this 4-h period, noninfected cells were continously kept in the labeling medium at 37 °C, 5% CO_2. Infected and noninfected cells were washed twice in water-bath medium, 25 mL of water-bath medium containing 150 μg/mL nonlabeled methionine was added to the dish, and they were incubated for 2 h on a 20 °C water bath. After they were warmed to 37 °C for 20 min, the cells were washed twice with ice-cold PBS, scraped with a rubber policeman in 4 mL of PBS, and transferred to a centrifuge tube. A PNS was prepared as described before (Kurzchalia et al., 1992), except that the imidazole buffer was replaced with 10 mM Hepes (pH 7.4) and 2 mM EGTA. Sucrose (~1.2 g) was added to the pooled PNS fractions (~2 mL) of three dishes and dissolved at 4 °C with rotation. DTT was added to a final concentration of 1 mM, and the suspension was transferred to a centrifuge tube. The sample was overlayed with 7.5 mL of 1.2 M and 3 mL of 0.8 M sucrose in 10 mM Hepes (pH 7.4) and 2 mM EGTA and centrifuged for 20 h at 38 000 rpm and 4 °C in a SW40 rotor (Beckman Instruments, Inc., Palo Alto, CA). After collection of 0.5-mL fractions, the peak fractions recovered from the 0.8/1.2 M sucrose interface (total cellular membrane fraction) were pooled.

Detergent Extractions. For the preparation of detergent-insoluble complexes, a total cellular membrane fraction (~2.5 mL) derived from three 150 cm^2 dishes was used. Half of the membranes was extracted with 20 mM CHAPS, 50 mM Tris-HCl (pH 7.4), and CLAP in a total volume of 4 mL for 30 min on ice. This results in a protein/detergent ratio similar to that obtained before (Kurzchalia et al., 1992). The extract was overlayed onto 8 mL of 0.9 M sucrose in 10 mM CHAPS and 50 mM Tris-HCl (pH 7.4) in a SW40 centrifuge tube and centrifuged for 4 h at 38 000 rpm and 4 °C. The pellet was solubilized in 100 μL of 1% (w/v) precondensed TX-114 (Bordier, 1981), 50 mM Tris-HCl (pH 7.4), 2 mM EGTA, 1 mM DTT, and CLAP. In contrast to the protocol described earlier (Kurzchalia et al., 1992), a chelator was included to parallel the procedure of Brown and Rose (1992). In the presence of this calcium chelator, more proteins were partitioning into the detergent phase at 37 °C than was observed in its absence (cf. Kurzchalia et al. (1992)). The sample was kept on ice for 1 h with periodical vortexing.

The other half of the membranes was extracted with 1% (w/v) TX-114 in TNE (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA) and CLAP in a total volume of 2.5 mL for 30 min on ice. Membranes extracted by this procedure resulted in an insoluble complex with a lower protein/detergent ratio than that obtained by Brown and Rose (1992) using whole MDCK cells. The extract was brought to 1.2 M sucrose with ice-cold 2.4 M sucrose in TNE in a SW40 centrifuge tube, overlayed with 6 mL of 1.1 M and 2 mL of 0.15 M sucrose in TNE, and centrifuged for 18 h at 39 000 rpm at

4 °C. After the collection of 0.5-mL fractions, the peak fractions recovered from the 0.15/1.1 M sucrose interface were pooled, diluted 1:4 with TNE, and pelleted for 8 h at 39 000 rpm in a SW40 rotor. The pellet was solubilized in 100 μL of 1% (w/v) TX-114 in TNE and CLAP and was kept on ice for 1 h with periodical vortexing. After warming to 37 °C for 10 min, the sample was cooled on ice.

The CHAPS/TX-114 and TX-114/TX-114 extracts were centrifuged for 15 min at 13 000 rpm and 4 °C in an Eppendorf centrifuge. The supernatants were incubated at 37 °C for 10 min and separated into detergent and aqueous phases by centrifugation for 4 min at room temperature. After an additional phase separation, the detergent phases were analyzed with the BioRad Mini 2-D cell.

Detection of GPI-Anchored Proteins. The TX-114 insoluble complexes from whole MDCK II cells were prepared as described by Brown and Rose (1992). GPI-anchored proteins were isolated as described by Lisanti et al. (1988, 1990) and analyzed with the BioRad Mini 2-D cell.

Lipid Analysis. Lipids were isolated from MDCK cells from six 500 cm^2 dishes or from a CHAPS-insoluble pellet from 12 500 cm^2 dishes prepared as described previously (Kurzchalia et al., 1992), but using centrifugation through a 17-mL cushion of 0.9 M sucrose in 10 mM CHAPS and 50 mM Tris-HCl (pH 7.4) and omitting the TX-114 extraction step. Each 500 cm^2 dish contained 1.3×10^8 cells. Lipids were purified and separated according to Brown and Rose (1992) with the following modification. Gangliosides were desalted by dialysis against water instead of using Sep-Pak C18. Neutral glycolipid species were separated by TLC using chloroform/methanol/water (60:35:8). Phospholipids were quantified by phosphorus (Rouser et al., 1966) after separation by 2-D HPTLC (Kobayashi et al., 1984). Cholesterol was determined by an enzymatic assay kit (Boehringer Mannheim Yamanouchi, Tokyo, Japan). The amounts of cerebrosides, lactosylceramide, Forssman antigen, and GM3 were estimated from TLC plates by densitometric scanning and comparison with standards. Triglyceride was quantified from TLC plates using a triolein standard curve with detection by charring with cupric acetate. The percent (w/w) of lipid in the CHAPS-insoluble pellet and the Triton-insoluble membrane fraction (Brown & Rose, 1992) was estimated using their buoyant densities.

Iodonaphthyl Azide Labeling. [5-^{125}I]-5-Iodonaphthyl 1-azide (INA) was synthesized from 5-aminonaphthyl azide and iodinated according to the procedure of Berovici and Gitler (1978). TGN-derived exocytic carrier vesicles were obtained as described previously (Bennett et al., 1988; Wandinger-Ness et al., 1990). Vesicles derived from one 10-cm Transwell filter (~300 μL) were diluted twice with PBS/4 mM β-mercaptoethanol and supplemented with ~1 μL of INA (10^6 cpm). MDCK cells grown on one 24-mm Transwell filter were washed twice with PBS, scraped from the filter, and centrifuged. The pellet was resuspended in 200 μL of PBS/2 mM β-mercaptoethanol and supplemented with ~1 μL of INA (10^6 cpm). Both samples were then equilibrated at 4 °C for 30 min. Irradiation was carried out for 1.5 min with a black light lamp equipped with a 100-W mercury bulb and a filter cutting wavelengths below 310 nm (Spectroline Model B-100/F, Spectronics Corp., Westbury, NY) in a 10-cm distance. The veiscles were diluted with PBS and pelleted for 3 h in a TLS55 rotor in a Beckman TL100 ultracentrifuge at 55 000 rpm and 4 °C. The cells were centrifuged, and both samples were subjected to 2-D gel electrophoresis as described before (Wandinger-Ness et al., 1990).

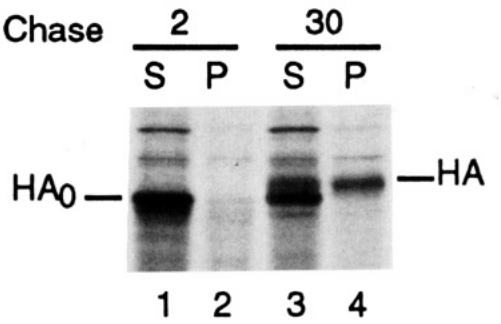

FIGURE 1: HA acquisition of CHAPS insolubility in the late Golgi. MDCK cells grown on Transwell filters were infected with influenza N virus, pulse-labeled with [^{35}S]methionine for 4 min, and chased for the indicated times (minutes). Extraction of cells with 20 mM CHAPS produced a soluble (S) and insoluble pellet (P) fraction. HA_0 denotes the core-glycosylated ER form, and HA indicates the endoH-resistant Golgi form of the influenza hemagglutinin.

SDS–PAGE and 2-D Gel Electrophoresis. Samples to be analyzed by PAGE were dissolved in sample buffer (50 mM Tris-HCl (pH 6.8), 2.5 mM EDTA, 2% SDS (w/v), 5% (w/v) glycerol, and 5% β-mercaptoethanol) and resolved on 7% or 12% polyacrylamide gels using the BioRad Mini-PROTEAN II electrophoresis cell. Resolution of proteins in two dimensions by IEF and SDS–PAGE, based on the method of Bravo (1984), was performed according to Wandinger-Ness et al. (1990). Whenever indicated, the BioRad Mini-PROTEAN II 2-D cell was used instead according to the manufacturer's recommendations, except for the IEF tube gel composition that corresponded to the gel mixture of Bravo (1984). Fluorography was performed with Entensify (DuPont).

RESULTS

Inclusion of Influenza HA into the CHAPS-Insoluble Complex Occurs in the Late Golgi. Brown and Rose (1992) have previously reported that the newly synthesized GPI-linked protein PLAP starts to become insoluble in TX-100 in the medial Golgi, i.e., before and/or during the acquisition of endoH resistance. N-Linked glycans become resistant to enzymatic endoH digestion by the concerted action of *N*-acetylglucosamine transferase I and mannosidase II in the medial Golgi apparatus (Kornfeld & Kornfeld, 1985). On the other hand, Skibbens et al. (1989) showed that the transmembrane apical marker protein influenza HA became TX-100 insoluble in the late Golgi, after acquisition of endoH resistance but before arrival at the cell surface. In order to determine when influenza HA becomes insoluble in CHAPS, we performed a pulse–chase experiment. MDCK cells grown on permeable filter supports were infected with influenza N virus for 4 h. The cells then synthesize predominantly viral proteins (Matlin & Simons, 1983). After a 4-min pulse with [^{35}S]methionine, the cells were incubated in chase medium with excess unlabeled methionine for 2 or 30 min. After extraction with buffer containing 20 mM CHAPS, they were separated into soluble and insoluble fractions. The pulse-labeled HA migrated immediately after synthesis at an apparent molecular mass of 68 kDa (HA_0, Figure 1, lane 1). After 30 min of chase, a second band of lower mobility was visible (HA, Figure 1, lane 3), corresponding to the complex glycosylated, endoH-resistant form (Matlin & Simons, 1983). At 30 min of chase the majority of this form had not yet reached the cell surface (Matlin & Simons, 1984). Directly after synthesis HA_0 was almost completely soluble (Figure 1, lanes 1 and 2), whereas after 30 min of chase more than 55%

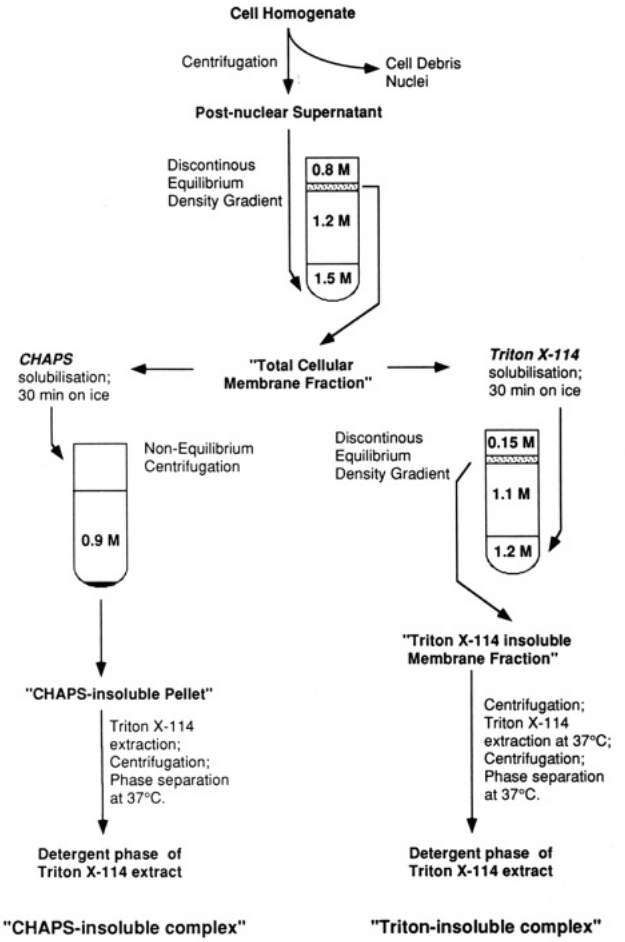

FIGURE 2: Experimental outline of the preparation of a total cellular membrane fraction and of the CHAPS and TX-114 extraction procedures. For details see the Experimental Procedures.

HA, but no HA_0, was found in the CHAPS-insoluble fraction (lanes 3 and 4). HA_0 was also found in the supernatant after shorter times of chase (data not shown). These results indicate that HA has become insoluble in CHAPS by the time it reaches the late Golgi.

Protein Composition of the CHAPS and Triton X-114 Insoluble Complexes. In order to compare the protein composition of the CHAPS-insoluble complex and the TX-100 insoluble membrane fraction isolated by Brown and Rose (1992), we used the protocol shown in Figure 2. As a starting material, a total cellular membrane fraction derived from metabolically ^{35}S-labeled influenza N virus infected MDCK cells was used. Half of the membranes was solubilized with 20 mM CHAPS on ice and centrifuged through a 0.9 M sucrose cushion. The other half was solubilized with 1% TX-114 on ice, and the insoluble membrane raction was floated to the interface of 1.1/0.15 M sucrose. Following an extraction with 1% TX-114 and a temperature-induced phase separation at 37 °C, the detergent phases were analyzed in the BioRad Mini 2-D cell (Figure 3).

The protein composition of the detergent-insoluble complexes (Figure 3, panels B and C) was very simple compared to that of the total membrane fraction (panel A). Both complexes had similar compositions. However, there were large quantitative differences for individual proteins. Influenza HA was present in both complexes, but was more enriched in the CHAPS-insoluble material. With the exception of HA, electrophoretic patterns identical to those in Figure 3 (panels B and C) were observed for the CHAPS and TX-114 complexes

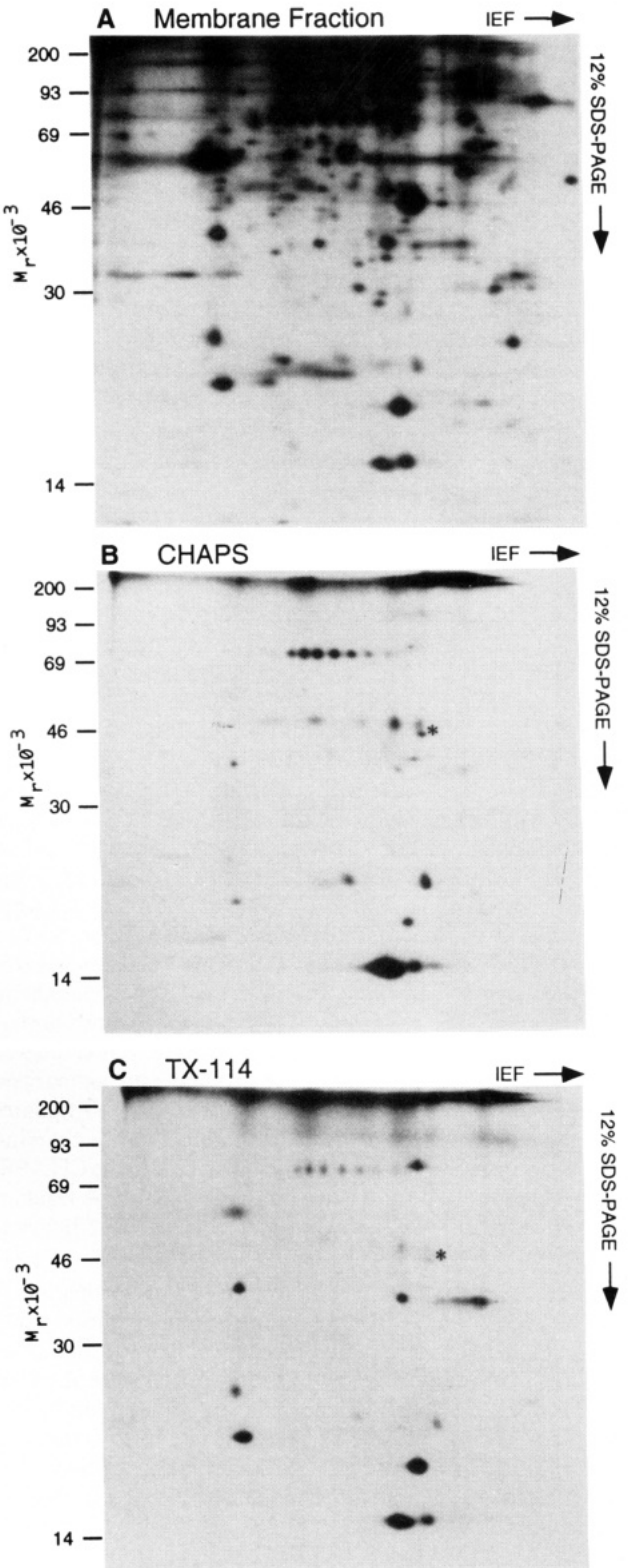

FIGURE 3: Protein composition of the CHAPS and Triton X-114 insoluble complexes. A total cellular membrane fraction (panel A) derived from metabolically labeled [^{35}S]methionine, influenza N virus infected MDCK cells was extracted with either 20 mM CHAPS (panel B) or 1% TX-114 (panel C) on ice and separated into a soluble and an insoluble fraction by sucrose gradient centrifugations (see Figure 2). The insoluble fractions were solubilized with 1% TX-114 and subjected to temperature-induced phase separation. The detergent phases were analyzed with the BioRad Mini 2-D cell and 12% SDS–PAGE. An asterisk marks the position of actin.

derived from noninfected MDCK cells (see Figure 5). Moreover, a very similar electrophoretic pattern for the TX-114 insoluble complex could be observed even after preparation

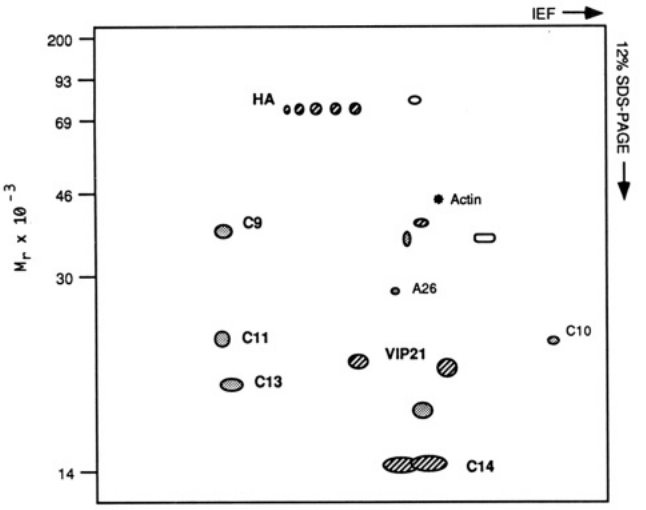

FIGURE 4: Schematic view of the protein composition of the CHAPS and Triton X-114 insoluble complexes. Striped forms indicate proteins relatively enriched in the CHAPS-insoluble complex, shaded forms denote other vesicular proteins, and unfilled forms represent unknown proteins. An asterisk marks the position of actin.

from whole MDCK cells, omitting membrane fractionation (data not shown).

To identify the proteins, the samples were mixed with exocytic carrier vesicles (Bennett et al., 1988) which allowed comparison with the 2-D gel patterns obtained after immunoisolation (Wandinger-Ness et al., 1990) and the assignment of the protein as specifically apical or common to both vesicle types (designated in Figure 4 as A and C, respectively). The CHAPS complex was enriched relative to the TX-114 complex for VIP21, C14, and an unidentified protein (striped forms) and contained small quantities of several other vesicular integral proteins such as C9, C11, and C13 (shaded forms; Figure 4). In contrast, C9, C11, and C13 were very abundant in the TX-114 insoluble material. In addition, A26 and C10 were visible in both complexes after longer exposures of the autoradiograms. No basolateral specific proteins could be detected. Calcium ions may play a role in keeping the vesicular proteins associated in the CHAPS-insoluble pellet, because only VIP21 and C14 were present in the TX-114 detergent phase when the extraction of the CHAPS-insoluble pellet with TX-114 was performed in the absence of a chelator [cf. Figure 3B in this article with Figure 6, B in Kurzchalia et al. 1992)].

GPI-Linked Proteins Are Relatively Depleted from the CHAPS-Insoluble Complex. To detect GPI-anchored proteins, we used a phosphatidylinositol-specific phospholipase C (PI-PLC). Upon cleavage of the GPI anchors by PI-PLC and loss of their hydrophobic moieties, GPI-anchored proteins generally change their partitioning from the detergent to the aqueous phase in a TX-114 phase separation experiment, which allows their isolation (Lisanti et al., 1988). A TX-114 insoluble complex prepared from whole MDCK cells as described by Brown and Rose (1992) served as a starting material. This was incubated with PI-PLC, and following a temperature-induced phase separation the aqueous phase was analyzed in the BioRad Mini 2-D cell. Proteins specifically released into the aqueous phase (Figure 5, panel A) as compared to the control (panel B) are marked with arrows. The molecular masses of these proteins corresponded to 38, 50, 80, and 115 kDa, which are similar to those reported previously (Lisanti et al., 1988; Brown & Rose, 1992). Since cleavage of the GPI anchor produces a cyclic phosphate (Ferguson et al., 1985) and does not change the p*I* of the protein, alignment of panel A with the TX-114 insoluble complex (panel C) and the

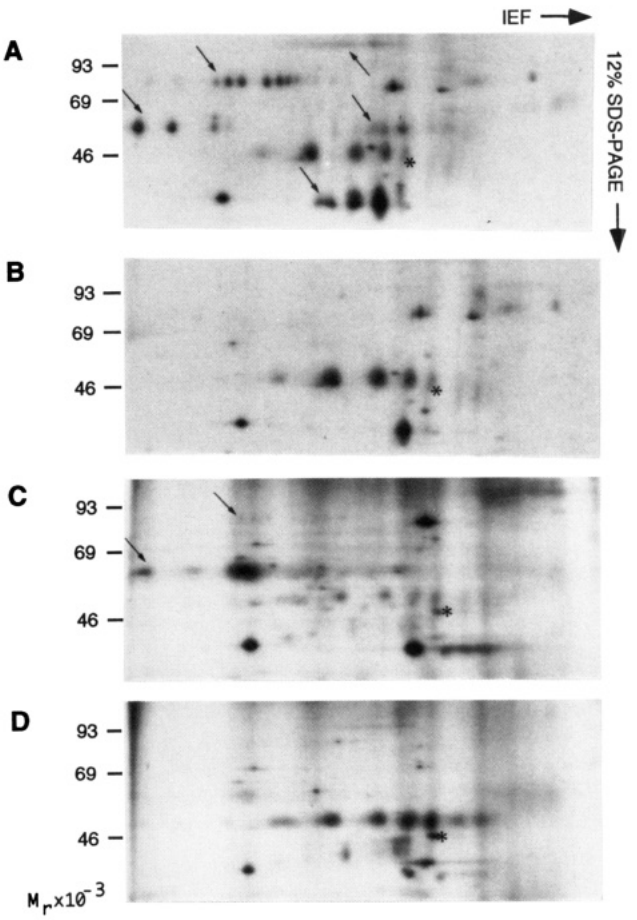

FIGURE 5: GPI-linked proteins present in the TX-114 insoluble complex. The TX-114 insoluble residue (4 °C) from whole MDCK cells was extracted with 1% TX-114 at 37 °C and subjected to teperature-induced phase separation. Equal amounts of the detergent phase were incubated with (panel A) or without (panel B) PI-PLC. Following a second phase separation, the aqueous phases were acetone-precipitated and analyzed with the BioRad Mini 2-D cell and 12% SDS–PAGE. Proteins specifically released into the aqueous phase are labeled with arrows. A TX-114 insoluble complex (panel C) and CHAPS-insoluble complex (panel D) were prepared from total cellular membrane fractions derived from noninfected MDCK cells. Equal amounts of radioactivity were loaded. GPI-linked proteins are labeled. An asterisk marks the position of actin.

CHAPS-insoluble complex (panel D), prepared from a total cellular membrane fraction, was possible (not shown). The GPI-linked proteins were relatively enriched in the TX-114 complex as compared to the CHAPS complex. None of the major proteins shown in Figure 3 corresponded to GPI-linked proteins. Thus, these proteins are minor constituents of the complexes.

A similar depletion of GPI-linked proteins from the CHAPS complex was observed when the CHAPS-insoluble pellet was directly analyzed on 2-D gels without TX-114 phase separation (data not shown), demonstrating that the relative depletion or enrichment was an effect of the initial CHAPS or TX-114 solubilization conditions.

The CHAPS-Insoluble Pellet Contains a Minor, but Selective, Fraction of Lipids. The lipid content was investigated to determine whether glycolipids were enriched in the CHAPS-insoluble pellet, as reported for the Triton-insoluble material (Brown & Rose, 1992). The lipid composition of MDCK cells, shown in Figure 6 and Table I, was comparable to previous reports (Hansson et al., 1986). The CHAPS-insoluble pellet had a strikingly lower content of lipids compared to that of the Triton-insoluble membrane fraction

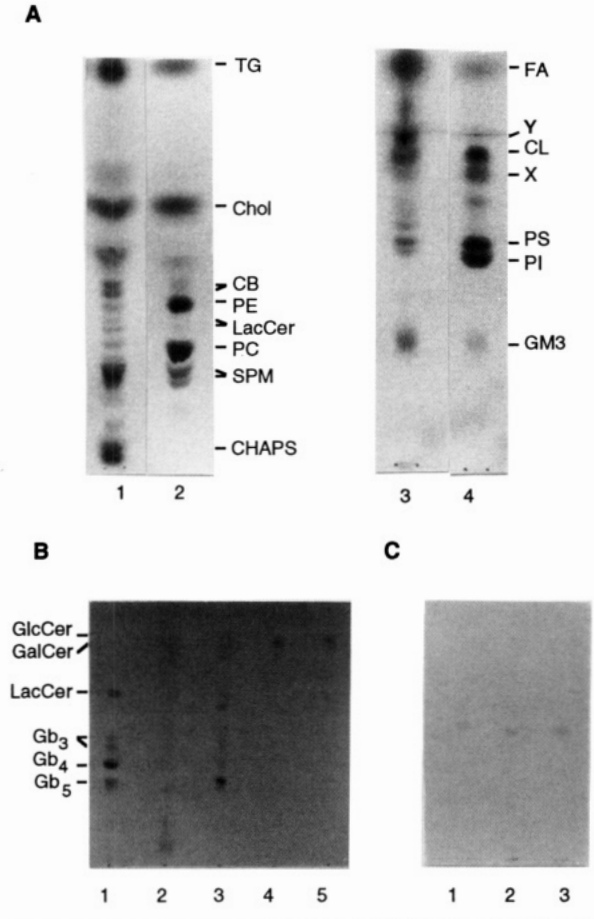

FIGURE 6: (Panel A) Total neutral (lanes 1 and 2) or acidic (lanes 3 and 4) lipids from 1.1×10^6 (neutral) or 6×10^6 (acidic) whole cells (lanes 2 and 4) or from a CHAPS-insoluble pellet from 2.3×10^8 (neutral) or 7×10^8 (acidic) cells (lanes 1 and 3). The lipids were extracted and analyzed as described in the Experimental Procedures. (Panel B) Neutral glycolipids from 5×10^6 cells (lane 3) or from a CHAPS-insoluble pellet from 2.6×10^8 cells (lane 2): lane 1, 1 μg each of mixed glycolipid standards; lane 4, galactosylceramide (1 μg); lane 5, glucosylceramide (1 μg). (Panel C) Gangliosides from 7.8×10^6 cells (lane 3) or from a CHAPS-insoluble pellet from 5.3×10^8 cells (lane 2): lane 1, 1 μg of bovine brain GM3. Abbreviations are as follows: TG, triglyceride; Chol, cholesterol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPM, sphingomyelin; FA, fatty acid; PS, phosphatidylserine; PI, phosphatidylinositol; GlcCer, glucosylceramide; GalCer, galactosylceramide; LacCer, lactosylceramide; Gb_3, ceramide trihexoside; Gb_4, globoside; Gb_5, Forssman antigen; X and Y, unknown.

(one representative experiment is shown; the analysis was performed independently on two CHAPS-insoluble pellets). This difference is in agreement with a buoyant density of 1.21 g/cm^3 for the CHAPS-insoluble pellet, determined by equilibrium density gradient centrifugation (data not shown), versus 1.08 g/cm^3 for the Triton-insoluble material (Brown & Rose, 1992). On the other hand, the lipid composition of the CHAPS-insoluble pellet was very similar to that of the Triton-insoluble membrane fraction; glycerophospholipids were depleted and sphingomyelin was enriched. Cerebrosides and the ganglioside GM3 were similarly enriched, but in contrast, the Forssman antigen and lactosylceramide, which were enriched in the Triton-insoluble fraction, were depleted from the CHAPS-insoluble pellet.

A Subset of Detergent-Insoluble, Vesicular Proteins Is Selectively Labeled with Iodonaphthyl Azide. [5-^{125}I]-5-Iodonaphthyl 1-azide (INA) has been previously used to label integral membrane proteins selectively (Berovici & Gitler, 1978) or, in combination with fluorescent lipid analogs, to label compartment-specific proteins (Rosenwald et al., 1991).

Table I: Lipid Composition of MDCK Cells and the CHAPS-Insoluble Complex[a]

	MDCK cells		CHAPS complex		
lipid	nmol/10^8 cells	%	nmol/10^8 cells	%	percent insoluble
phospholipids	2735		5.8		
phosphatidylcholine		44.6		9.1	<0.1
phosphatidylethanol amine		25.8		7.7	<0.1
phosphatidylserine		8.5		7.8	<0.2
phosphatidylinositol		6.0		0.5	<0.1
cardiolipin		3.0		2.0	<0.2
sphingomyelin		12.1		72.9	1.3
cholesterol	652		5.1		0.8
triglyceride	63		0.47		0.8
cerebrosides	22		0.58		2.6
lactosylceramide	17		0.01		<0.1
Forssman antigen	19		0.02		<0.1
GM3	19		0.23		1.2

[a] Lipids were purified and analyzed as described in the Experimental Procedures.

Our experiments were originally aimed toward confirming the presence of transmembrane proteins in the apical and basolateral carrier vesicles (Wandinger-Ness et al., 1990). However, the experiments revealed another unexpected and interesting finding.

Exocytic carrier vesicles were isolated and iodinated with INA. The samples were then directly analyzed by using large 2-D gels (Wandinger-Ness et al., 1990). The result is shown in Figure 7. Only three proteins were selectively labeled in the exocytic carrier vesicles (panel A). This was in striking contrast to the high number of proteins partitioning into the detergent phase of a TX-114 phase separation (Wandinger-Ness et al., 1990). By aligning the gel with the starting material, these were identified as VIP21, C9, and B7 [see Wandinger-Ness et al. (1990), for comparison]. When, as a control, whole MDCK cells were mechanically scraped from a Transwell filter and labeled with INA using identical conditions (panel B), the pattern was strikingly simple: VIP21, C9, and B7 still represented the major fraction of the labeled proteins. VIP21 is known to represent less than 0.05% of total cellular protein (Kurzchalia et al., 1992; K. Fiedler, & K. Simons, unpublished results). Our data suggest that INA preferentially labels the CHAPS and TX-114 insoluble proteins VIP21 and C9, but also labels B7, a protein enriched in basolateral vesicles and present at the basolateral cell surface (data now shown).

DISCUSSION

A key feature of our working model of protein sorting in epithelial cells is the cooperative sorting of apically destined proteins and lipids (Simons & van Meer, 1988; Simons & Wandinger-Ness, 1990). This model proposes that clusters of glycosphingolipid-enriched membrane microdomains, i.e., rafts, a loose association of glycosphingolipids within the fluid bilayer, putative sorting receptors, and vesicular machinery in the TGN mediate the inclusion of apical proteins, and simultaneous exclusion of basolaterally destined cargo, into sorting vesicles that bud off to be targeted to the apical cell surface.

A prediction of this model is that the onset of glycolipid clustering and the inclusion of apically destined cargo should occur in the Golgi. The synthesis of sphingolipids takes place throughout the Golgi complex (Schwarzmann & Sandhoff, 1990; van Meer & Burger, 1992), and their lateral association may occur as soon as their concentration within the luminal/

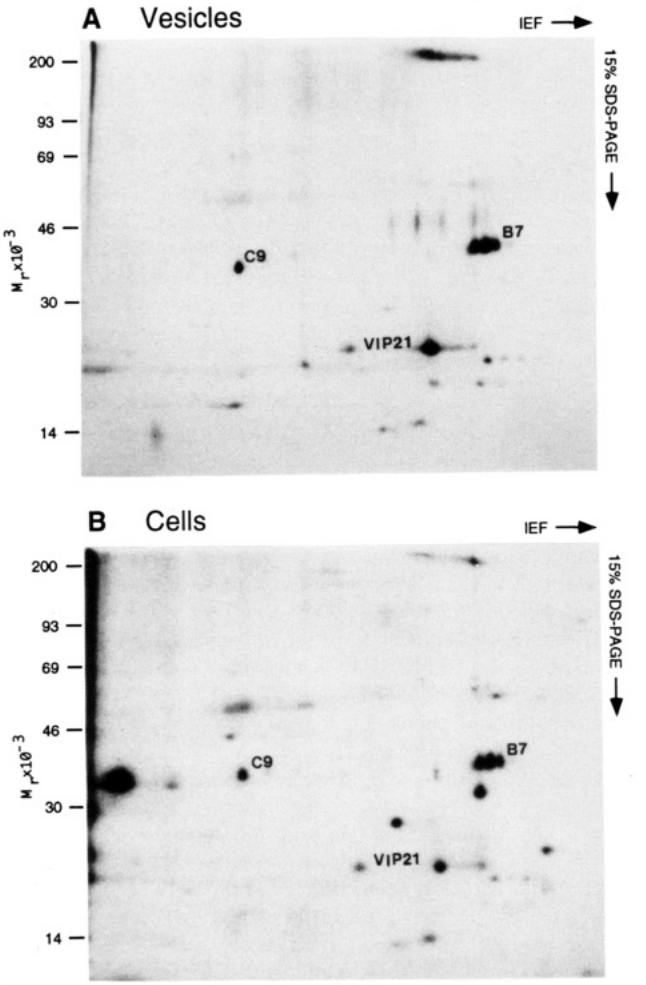

FIGURE 7: Subset of detergent-insoluble, vesicular proteins selectively labeled with iodonaphthyl azide. TGN-derived exocytic carrier vesicles (panel A) isolated from perforated MDCK cells or mechanically scraped MDCK cells (panel B) were iodinated with INA and separated by 2-D IEF/15% SDS–PAGE.

exoplasmic leaflet of the bilayer is sufficiently high. In agreement with this prediction, the newly synthesized GPI-anchored protein PLAP was shown to be incorporated into Triton-insoluble complexes during transit through the Golgi (Brown & Rose, 1992). Influenza HA became Triton-insoluble after the acquisition of complex carbohydrates, but before arrival at the cell surface (Skibbens et al., 1989), and as shown in this work, HA acquired CHAPS insolubility in the Golgi. There was a slight difference in the kinetics of acquisition of detergent insolubility by these proteins: PLAP became insoluble while still in the process of becoming resistant to endoH, while influenza HA was first included in the complex after having acquired endoH resistance. Therefore, PLAP may be incorporated into the glycosphingolipid clusters as soon as they are formed in the Golgi complex, perhaps associating with the raft directly through its glycophospholipid anchor carbohydrate moieties; the influenza HA, on the other hand, may be included as cargo in the rafts first in the TGN, perhaps not directly associating with the sphingolipids but rather binding through its putative luminal sorting signal (Roth et al., 1987; McQueen et al., 1986) to a sorting receptor interacting directly with the raft.

Following transport to the cell surface, clusters of glycolipids and apically sorted proteins must dissociate to reach their equilibrium distribution. In agreement with this, the GPI-anchored fusion protein gD1–DAF arrives at the cell surface as an immobilized cluster which equilibrates within ~2 h with resident mobile gD1–DAF (Hannan et al., 1993). In contrast, basolaterally directed proteins such as the VSV G protein are expected to be excluded from complexes formed by glycosphingolipids during transit through the Golgi (Brown & Rose, 1992; Kurzchalia et al., 1992). Basolateral sorting signals have recently been localized to the cytoplasmic domain of several receptors, some of which are similar to the signals previously identified for rapidly endocytosing proteins (Matlin, 1992; Mostov et al., 1992). These signals may interact with accessory proteins such as cytosolic adaptor molecules (Hopkins, 1991) and lead to exclusion from the apical vesicles (Hunziker et al., 1991).

In this work, we used detergent insolubility as a measurement of the association of proteins into rafts. The Triton-insoluble residues from MDCK cells are membraneous in appearance and are rich in lipid, consisting of sphingolipid, glycerolipid, and cholesterol in about equal amounts (Brown & Rose, 1992). Interestingly, artificial liposomes of the same lipid composition that contain no protein are also insoluble in TX-100 (Brown, 1992). Thus, the lipids themselves constitute the vehicle for Triton insolubility. One interpretation is that Triton leaves the putative glycolipid domains in cellular bilayers insoluble. In contrast, the CHAPS-insoluble pellet from MDCK cells contains much less lipid (~40% w/w) than the Triton-insoluble membrane fraction (>95% w/w), but the lipid that does remain is of a similar composition, with the notable exception of the Forssman antigen and lactosylceramide which are depleted from the CHAPS-insoluble pellet. Therefore, the CHAPS complex seems to be dominated more by protein–protein interactions and the lipid left may be bound to protein, considering that the complex was isolated by centrifugation through a CHAPS-containing 0.9 M sucrose cushion (Helenius & Simons, 1975). In addition, whereas practically all of the GPI-anchored proteins are insoluble in TX-114, these proteins are partially extracted in soluble form by CHAPS. Since GPI-anchored proteins and raft lipids behave similarly during detergent extraction, they may associate directly with each other. Previous studies using various detergents to solubilize GPI-linked proteins and sphingolipids lend credence to their similarity in behavior (Banerjee et al., 1990); Hooper & Turner, 1988).

The total protein compositions of the two detergent-insoluble complexes were analyzed by 2-D gel electrophoresis. Several proteins previously identified in post-TGN carrier vesicles from MDCK cells were present (Figure 3). The protein composition is very simple. The CHAPS- and Triton-insoluble complexes are qualitatively similar in composition, but the abundance of individual components varies significantly. C14 and VIP21 are the major proteins of the CHAPS complex, C9, C11, and C13 dominate after TX-114 extraction, and C10 and A26 are minor components in both detergent-insoluble complexes (Figure 4). A26 is the only one of these proteins that is enriched in apical transport vesicles; all of the other components are common to both apical and basolateral vesicle classes. No basolaterally enriched protein was identified.

Although all detergent-insoluble proteins are potential candidates for (sorting and targeting) functions in post-Golgi transport, it should be pointed out that detergent insolubility as a means for defining protein association has obvious limitations. Associating proteins may, of course, be removed from the complexes during detergent extraction. The question of whether other apical (non-GPI) passenger proteins behave like influenza HA during intracellular transport remains to be determined. We have found that ~20% of the apical protein human aminopeptidase N was CHAPS-insoluble in

overexpressing MDCK cells (K. Fiedler, O. Norén, & K. Simons, unpublished results).

Another surprising finding was that the treatment of both exocytic carrier vesicles and mechanically scraped MDCK cells with iodonaphthyl azide (Figure 7), a cross-linking agent previously thought to label hydrophobic domains of transmembrane proteins rather nonselectively, resulted in a remarkably simple pattern in which VIP21 and C9 were selectively labeled; recall that VIP21 and C9 were also found in the glycolipid-enriched, detergent-insoluble complexes. Together these findings suggest that iodonaphthyl azide may preferentially partition into glycolipid microdomains and might be useful for identifying proteins associated with glycolipid rafts.

On the basis of the behavior of VIP21 in CHAPS, we were previously able to purify this protein for microsequencing (Kurzchalia et al., 1992). Antibodies produced against VIP21 peptide epitopes have been used to identify its localization in the Golgi complex, intracellular vesicles, and the cell surface, as expected for a protein present in TGN-derived vesicles (Dupree et al., 1993). Over 90% of the VIP21 on the cell surface is concentrated in plasma membrane invaginations celled caveolae, structures known to contain GPI-anchored proteins (Anderson et al., 1992) and possibly enriched in glycosphingolipids (Montesano et al., 1982; Parton et al., 1988). Caveolae are found in all cell types (Severs, 1988) and on both the apical and the basolateral cell surfaces in MDCK cells. Taking VIP21 and GPI-anchored proteins as markers for the rafts [for a further discussion, see Dupree et al. (1993)], we therefore have to assume that, in addition to membranes of the secretory pathway, surface caveolae are also contributing to the composition of the Triton- and CHAPS-insoluble complexes.

While we consider the glycosphingolipid rafts within the membrane as platforms for lateral segregation of lipids and proteins in the bilayer, such rafts may be involved in other activities as well. For example, a complex of GPI-anchored proteins and protein tyrosine kinases involved in the T-cell activation cascade contains glycolipids (Stefanova et al., 1991; Thomas & Samelson, 1992; Cinek & Horejsi, 1992). Thus, rafts might locally concentrate and cluster factors involved in signal transduction processes into spatially segregated subdomains on the cell surface (Brown, 1992; Cinek & Horejsi, 1992). Further, the myelin membrane formed by oligodendrocytes and Schwann cells (Martenson, 1992) is rich in glycolipids and could involve rafts associating in the myelin membrane (Pfeiffer et al., 1993). Although clustering of glycosphingolipids has been demonstrated in several systems (Thompson & Tillack, 1985; Curatolo, 1987; Boggs, 1987; Masserini et al., 1988), more work is necessary to define the specificity of the interactions leading to clustering among lipids and among lipids and proteins. Nevertheless, it should now be possible to begin to connect the physical chemistry of glycosphingolipids with their biological functions.

ACKNOWLEDGMENT

We thank Vesa Olkkonen and Sanjay Pimplikar for helpful discussions, Paul Dupree, Steve Pfeiffer, and Sara Courtneidge for reading the manuscript critically, and Hank Lane for kindly providing the Transwell filters. We also gratefully acknowledge Kirsten Mundt for excellent help and Hilkka Virta for expert technical assistance.

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