

VIP21, A 21-kD Membrane Protein is an Integral Component of *Trans*-Golgi-Network-Derived Transport Vesicles

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Abstract. In simple epithelial cells, apical and basolateral proteins are sorted into separate vesicular carriers before delivery to the appropriate plasma membrane domains. To dissect the putative sorting machinery, we have solubilized Golgi-derived transport vesicles with the detergent CHAPS and shown that an apical marker, influenza haemagglutinin (HA), formed a large complex together with several integral membrane proteins. Remarkably, a similar set of CHAPS-insoluble proteins

was found after solubilization of a total cellular membrane fraction. This allowed the cloning of a cDNA encoding one protein of this complex, VIP21 (Vesicular Integral-membrane Protein of 21 kD). The transiently expressed protein appeared on the Golgi-apparatus, the plasma membrane and vesicular structures. We propose that VIP21 is a component of the molecular machinery of vesicular transport.

EPITHELIAL cells, which form a boundary between an organism and the surrounding environment, are polarized, consisting of apical and basolateral surfaces. These surfaces are physiologically and biochemically distinguishable and reflect the existence of two plasma membrane domains (for review see Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989; Simons and Wandinger-Ness, 1990). An excellent model for studying the development and maintenance of this cell-surface polarity of epithelial cells is the MDCK cell line (Rodriguez-Boulan, 1983; Simons and Fuller, 1985). Studies on cell polarity in this system have been greatly facilitated by the use of the spike glycoproteins of enveloped RNA viruses that bud from different surfaces of the MDCK cell (Rodriguez-Boulan and Sabatini, 1978; Rodriguez-Boulan and Pendergast, 1980; Matlin and Simons, 1984; Misek et al., 1984; Pfeiffer et al., 1985). Influenza haemagglutinin (HA)¹ is delivered exclusively to the apical domain, while vesicular stomatitis virus (VSV) G protein is delivered to the basolateral domain (Rodriguez-Boulan and Pendergast, 1980). An understanding of the sorting and delivery of these proteins is basic to an understanding of the polarity of epithelial cells.

Apical and basolateral plasma membrane proteins in MDCK cells are sorted in the *trans*-Golgi network (TGN) by inclusion into separate vesicular carriers that transport the proteins to the correct destination (Griffiths and Simons,

1986; Hughson et al., 1988). The molecular mechanisms of this process remain unclear. However, the elucidation of the general machinery involved in vesicle budding, transport and membrane fusion has recently gained pace. Yeast genetics (Novick et al., 1980) and an in vitro assay of protein transport (Balch et al., 1984; Rothman and Orci, 1992) have been two successful strategies. Thus, *N*-ethyl maleimide-sensitive factor (NSF) (Wilson et al., 1989), soluble NSF attachment proteins (SNAPs) (Clary et al., 1990), the rab-related small GTP-binding proteins YPT1 and SEC4 (Salminen and Novick, 1987; Segev et al., 1988), the Arf (Stearns et al., 1990) family, and many other yeast proteins (Kaiser and Schekman, 1990; Nakano and Muramatsu, 1989; Newman et al., 1990) have been identified or further characterized.

An alternative to these approaches for dissecting the machinery is to isolate transport vesicles and then to characterize the components. Isolation of clathrin-coated vesicles has allowed the identification of the coat proteins (including clathrin and the adaptin family) and an understanding of the physical structure of the coat (for reviews see Pearse and Robinson, 1990; Morris et al., 1989). Additionally, the cotamer proteins (COPs) were shown to be components of the Golgi-derived non-clathrin-coated vesicles (Malhotra et al., 1989; Serafini et al., 1991; Duden et al., 1991). Purification of synaptic vesicles in large quantities has yielded information about their composition (Südhof and Jahn, 1991). Other types of vesicles such as those mediating transcytosis (Sztul et al., 1991) and the yeast constitutive secretory pathway have also been purified (Holcomb and Schekman, 1987; Walworth and Novick, 1987), and their characterization is starting to reveal novel proteins.

To identify molecular machinery involved in the epithelial

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1. *Abbreviations used in this paper:* GPI, glycosylphosphatidyl inositol; HA, influenza haemagglutinin; 1-D, one dimensional; PNS, postnuclear supernatant; TGN, *trans*-Golgi network; 2-D, two dimensional; VSV, vesicular stomatitis virus.

sorting process, an in vitro system was developed which allowed the isolation of TGN-derived, exocytic vesicles from MDCK cells (Bennett et al., 1988). This system was based on the ability of MDCK cells to form a fully polarized monolayer when grown on a permeable support. After infection with influenza or VSV viruses and accumulation of the glycoproteins in the TGN by incubation at 20°C (Matlin and Simons, 1983), the cells were mechanically perforated using a nitrocellulose filter. Sealed vesicles containing HA or the VSV G protein could be collected from the incubation medium. It was further possible to separate apical and basolateral vesicles by immunoisolation with antibodies against the cytoplasmically exposed domains of the glycoproteins (Wandinger-Ness et al., 1990). The protein patterns of the two populations of vesicles contained a number of proteins in common and also proteins that were specifically enriched in one or the other of the populations. Since there is no synthesis of cellular proteins in the conditions of viral infection, the non-viral proteins in these vesicles are probably essential, recycled, components of the machinery involved in sorting, vesicle budding, movement or fusion, and therefore interesting candidates for further analysis.

In this study we have asked the question whether the apical marker HA forms complexes with the constituents of the transport vesicles. Our hypothesis was that proteins of such complexes could be components of the putative sorting machinery. This speculation on complex formation is supported by the data obtained by Skibbens et al. (1989), which showed that the solubility of HA in Triton-X-100 dramatically changes as it is transported to the plasma membrane of MDCK cells. The authors detected insolubility of the HA in a late phase of the transport pathway after addition of complex sugars in the Golgi complex but before insertion of the protein in the plasma membrane. Furthermore, Brown and Rose (1992) have shown that proteins with a glycosylphosphatidyl inositol (GPI) anchor become associated with detergent-insoluble material enriched in glycosphingolipids during transport to the apical surface. As it has been shown that GPI anchorage is sufficient to direct apical targeting in MDCK cells (Lisanti and Rodriguez-Boulan, 1990), this association into a detergent-insoluble complex may be involved in the sorting process.

To find proteins involved in sorting, we extracted exocytic vesicles bearing the HA with several detergents under different experimental conditions and analyzed the sedimentation behavior of HA on sucrose gradients. After solubilization with the detergent CHAPS, the HA was found almost exclusively in a high molecular weight complex. The complex contained a few prominent small integral-membrane proteins. Furthermore, a similar protein pattern in the low molecular weight range was detected if a total cellular membrane fraction was solubilized with CHAPS, strongly suggesting that the CHAPS insolubility was an unusual characteristic of these membrane proteins. This property allowed us to isolate the complex in a sufficient amount for microsequencing. A cDNA encoding one of these proteins, VIP21 (Vesicular Integral-membrane Protein of 21 kD), was cloned and sequenced. The protein was localized in BHK cells by transient expression of the protein tagged with a *c-myc* epitope at the NH₂ terminus, and using anti-*c-myc* antibodies for indirect immunofluorescence and immunoelectron microscopic studies. The VIP21 was localized to membranes

of the Golgi-apparatus, the plasma membrane and vesicular structures. We propose that VIP21, as a constituent of the transport vesicles, is part of the machinery involved in vesicular transport of proteins between the TGN and the plasma membrane.

Materials and Methods

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 Sigma Chemical Co. (St. Louis, MO). The transfection reagent DOTAP was a product of Boehringer Mannheim GmbH (Mannheim, Germany). The mouse cell line producing mAbs 9E10 against the human *c-myc* epitope EQKLISEED (Evan et al., 1985) was purchased from The European Collection of Animal Cell Cultures (Nr 85102202).

Cell Culture

Cell culture media were as described previously (Bennett et al., 1988; Wandinger-Ness et al., 1990). MDCK strain II cells were grown and passaged as described previously (Matlin et al., 1981). For isolation of transport vesicles, cells from one confluent 75-cm² flask were seeded on a 10-cm diam, 0.4- μ m pore size Transwell filter in 20 ml of medium. The filters were mounted in a special holder in a Petri dish containing 140 ml of growth medium. The Transwell filters were a kind gift from Hank Lane (Costar, Cambridge, MA). For large scale preparation of total cellular membrane fractions, cells were grown on cell culture plates (Nunc, Roskilde, Denmark) (24.5 \times 24.5 cm). Again, cells from one 75-cm² flask were seeded on one plate containing 100 ml of the medium. Cultures were maintained at 37°C and 5% CO₂ for 2–3 d, when vesicles were prepared or for 2.5 d when the total cellular membrane fraction was prepared.

Viral Infection, Radioactive Labeling, and Isolation of TGN-derived Vesicles from Mechanically Perforated Cells

VSV and influenza WSN ts61 stocks were prepared according to Bennett et al. (1988) and Hughson et al. (1988), respectively. The filters were rinsed with infection medium and returned to the original 10-cm dishes. The virus stock in infection medium (0.5 ml) was added to the apical side of the filter at a multiplicity of 5 pfu/cell (WSN ts61) or 50 pfu/cell in the case of VSV. After adsorption to the cells for 1 h at 31°C for WSN ts61 or 37°C for VSV, the viral inoculates were aspirated and fresh infection medium was added to the apical side (10 ml) and to the basolateral side (12 ml). The infections were continued for an additional 3.5 h at 39°C (WSN ts61) or 2.5 h at 37°C (VSV). Subsequently the medium was replaced with water bath medium and cells were transferred to a water bath at 20°C for 1.5 h.

Metabolic or pulse labeling of the cells grown on filters was carried out according to previously described procedures (Bennett et al., 1988; Wandinger-Ness, 1990), adjusted when necessary for usage of 10-cm dishes. Cells grown on Nunc cell culture plates (24.5 \times 24.5 cm) were metabolically labeled as follows. A 2-d-old culture (\sim 80% confluent) was washed three times with PBS containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂ (PBS[+]) at 37°C. Subsequently, 100 ml of metabolic labeling medium containing 1 mCi of [³⁵S]methionine was added, and the plate incubated for 12 h at 37°C, 5% CO₂.

The TGN-derived vesicles from the cells grown on 10-cm filters were prepared according to Wandinger-Ness et al. (1990). The final purification of the vesicles was carried out on discontinuous equilibrium density gradients. The membrane fractions obtained from three filters were combined in a total volume of 0.3 ml, and made 1.5 M in sucrose. The sample was overlaid in an SW-60 tube with 1.9 ml 1.2 M sucrose followed by 1.8 ml 0.8 M sucrose. All sucrose solutions were prepared in 10 mM Hepes (pH 7.4), 2 mM EGTA, and 1 mM DTT (added fresh). The vesicles were centrifuged for 13 h at 38,000 rpm (125,000 *g*_{av}) at 4°C. Fractions of 0.3 ml were collected and the radioactivity of the aliquots was counted. The vesicles were recovered in the peak fractions at the 0.8/1.2 M sucrose interface.

Solubilization of TGN-derived Vesicles with CHAPS

Solubilization of TGN-derived vesicles was carried out with 20 mM

CHAPS prepared in 50 mM Hepes (pH 7.5) (CHAPS buffer). Vesicles (200 μ l) after the equilibrium flotation gradient step were mixed with an equal volume of 2 \times CHAPS buffer. After incubation for 30 min on ice, the mixture was diluted with 1 \times CHAPS buffer (200 μ l) and applied to a 10–30% (wt/vol) sucrose gradient (2 \times 1.8 ml). The sucrose solutions contained 10 mM CHAPS and 50 mM Hepes (pH 7.4). The centrifugation was carried out in a SW-60 rotor for 2.5 h at 38,000 rpm. After fractionation of the gradients, sodium deoxycholate to a final concentration of 0.3% and 2 μ g BSA were added to each 0.2-ml fraction and proteins were precipitated by equal volume of 20% TCA. After incubation for 1 h on ice, the precipitates were collected, washed twice with cold acetone, dried at 37°C, and solubilized in SDS-PAGE sample buffer. The pellet fraction was solubilized directly with sample buffer.

The CHAPS pellet was solubilized in two-dimensional (2-D) sample buffer at 30°C for 2-D gel analysis. Unsolubilized vesicles were pelleted in a TL-100 centrifuge (Beckman Instruments, Palo Alto, CA), using TLS-55 rotor at 54,000 rpm for 3 h.

SDS-PAGE and 2-D Gel Electrophoresis

SDS-PAGE on 10% gels was performed as described (Bennett et al., 1988). Resolution of proteins in two dimensions by IEF and SDS-PAGE, based on the method of Bravo (1984), was performed according to Wanger-Ness et al. (1990). After electrophoresis the gels were fixed in 45% methanol and 7% acetic acid and treated for fluorography using Entensify (Dupont, New England Nuclear Research Products, Boston, MA). For quantification of one-dimensional (1-D) gels, the fluorographs were digitized using a Sony CCD video camera (Cologne, Germany) and the Image (version 1.16) software program designed for use with a Macintosh IIcx computer.

Isolation of CHAPS Complex from the Total Cellular Membrane Fraction

A procedure for isolation of a total cellular membrane fraction and its subsequent treatment with CHAPS is described for six Nunc cell culture plates. For analytical purposes, the procedure was scaled down to one plate, which was metabolically labeled with [³⁵S]methionine.

The 2.5-d-old cells from each plate were washed twice with PBS(+), scraped by a rubber policeman in 12 ml PBS(+) and transferred to a centrifuge tube. The plates were washed with additional 3 ml of PBS(+) and the pooled suspensions were centrifuged for 5 min at 1,500 rpm. To change the buffer, 12 ml of 250 mM sucrose, 3 mM imidazole, pH 7.4 (Gorvel et al., 1991) were added to the pellet and centrifuged for 10 min at 3,000 rpm. Each of six pellets was resuspended in 2 ml of the imidazole buffer and homogenized by passing 10 times through a blue pipette tip and 15 times through a 22 G needle. Microscopic analysis of the homogenate showed that the cells had been disrupted to ~90% whereas nuclei were still intact. The homogenate was transferred to an Eppendorf tube and centrifuged for 10 min at 3,000 rpm. The supernatant is called the postnuclear supernatant (PNS).

Sucrose (7.2 g) was added to the pooled PNS fractions (~12 ml) and dissolved by warming to 30°C. Subsequently the volume was adjusted to 15 ml (1.5 M sucrose) and DTT to final concentration of 1 mM was added. The suspension was transferred to two SW-27 tubes. The solutions were overlaid with 15 ml of 1.2 M sucrose followed by 10 ml of 0.8 M sucrose. Both sucrose solutions were prepared in the imidazole buffer with 1 mM DTT. After centrifugation in SW-27 rotor at 27,000 rpm for 20 h, fractions of 2 ml were collected from the top of the gradient. Fractions 4–6, corresponding to the interface between 0.8 and 1.2 M sucrose were pooled. We called these fractions the cellular membrane fraction.

The cellular membrane fraction was extracted with CHAPS as follows. CHAPS (8 ml of 100 mM) and 2 ml of 1 M Hepes (pH 7.4) were added to 12 ml of the membrane fraction, the volume adjusted to 40 ml, and the mixture incubated for 30 min at 4°C. Fractions (10 ml) were overlaid onto a 10–30% sucrose gradient (2 \times 12.5 ml) in 10 mM CHAPS and 50 mM Hepes (pH 7.4). The centrifugation was performed in SW-27 rotor for 4 h at 27,000 rpm. The supernatant was carefully aspirated with a drawn out pipette and the tube wall was dried with a swab.

Pellets were extracted with 0.5 ml of 50 mM Tris (pH 7.4), 1 mM DTT, and 1% Triton-X-114 (wt/vol). The suspensions were kept on ice for 30 min with periodical vortexing, pooled, and centrifuged in an Eppendorf centrifuge for 20 min at 12,000 rpm. The supernatant was incubated at 37°C for 15 min and subsequently centrifuged for 5 min at room temperature to separate the detergent and aqueous phases. Proteins in the both phases were either analyzed by 2-D gel electrophoresis (analytical experiment) or precipitated by addition of cold TCA at final concentration of 10% (prepara-

tive isolation). Before the precipitation step, the detergent phase was diluted to 0.9 ml with distilled water. The precipitates formed after 1 h on ice were collected by centrifugation and washed three times with ice-cold acetone. The pellet was dried at 37°C and subjected to SDS-PAGE on a 15% gel (1.5 mm).

Amino Acid Sequence Analysis

Protein bands from 1-D gels were excised after Coomassie blue staining. From six Nunc plates we obtained ~1 μ g of VIP21. After washing with water the gel pieces were lyophilized and rehydrated in 100 μ l of 100 mM NH₄HCO₃, 0.5 mM CaCl₂ containing 1 μ g trypsin. After digestion (37°C for 24 h) peptide fragments were extracted from the gel slice with 2 \times 100 μ l of 1% trifluoroacetic acid/0.01% Tween 20, then 70% trifluoroacetic acid/0.01% Tween 20, and 50% trifluoroacetic acid/acetonitrile/0.01% Tween 20, (modified from Eckerskorn and Lottspeich, 1989). The combined fractions were concentrated and subjected to RP-HPLC. Automated Edman degradation of peptides was performed using a sequencer (model 477A; Applied Biosystems Inc., Foster City, CA) connected to an on-line PTH analyzer (model 120, Applied Biosystems).

Molecular Cloning of VIP21

Total RNA was prepared from confluent MDCK cells using the guanidine hydrochloride procedure (Chirgwin et al., 1979). MDCK cDNAs were synthesized with the first-strand synthesis kit of Stratagene with oligo dT as the primer. G-tailing of cDNAs and PCR was carried out according to Chavrier et al. (1992). The gene-specific oligonucleotide was 5' (T/C)TTTACIAC(A/G)TC(A/G)TT(A/G)TTT(A/G)(A/G)TG 3'. For screening of the lambda ZAPII MDCK cDNA library (Chavrier et al., 1990), duplicate nitrocellulose filters were prehybridized for 2 h at 42°C in 5 \times SSC, 50% formamide, 5 \times Denhardt's solution, 1% SDS (Sambrook et al., 1990). Hybridization was carried out in the same solution, supplemented with ³²P-labeled probe overnight at 55°C.

In vitro protein synthesis was carried out according to the manufacturer (Promega Biotec, Madison, WI). Nucleotide sequences were determined using the T7 sequencing kit (Pharmacia, Uppsala, Sweden).

A construct encoding VIP21 with the *c-myc* epitope 9E10 (Evan et al., 1985) at the NH₂ terminus was created by the polymerase chain reaction with the VIP21 cDNA in pBluescript as the template. A 70b oligonucleotide encoding the amino acids MEQKLISEEDLGG and the first six amino acids of VIP21 and a 17b oligonucleotide that anneals to the T7 promoter were the primers. The amplified product was cloned in pGEM1 under the T7 promoter and the sequence verified to eliminate the possibility of amplification errors.

Sequence analysis was carried out with the University of Wisconsin GCG programs (Madison, WI) (Devereux et al., 1984). EMBL release 30, GenBank release 70 and Swissprot release 20 databases were searched for homology to VIP21.

Transfection

BHK 21 cells were grown in Glasgow's modified Eagle's medium supplemented with 5% FCS, 2 mM glutamine, 100 U/ml penicillin, 10 μ g/ml streptomycin. The cells on the day of transfection were ~70% confluent. The cells, after washing twice with serum-free medium, were infected with T7 RNA polymerase-recombinant vaccinia virus (Fuerst et al., 1986), using 3–5 pfu/cell. The infection was carried out at room temperature for 30 min. Then the cells were washed with serum-free medium and transfected using DOTAP reagent (Boehringer Mannheim GmbH) according to the manufacturers instructions.

Immunofluorescence Microscopy

Cells grown on 10-mm round coverslips after transfection for 3 h and subsequent incubation at 37°C were washed with PBS and permeabilized with 0.5% saponin in 80 mM Pipes (pH 6.8), 5 mM EGTA, 1 mM MgCl₂ for 5 min. The cells were fixed with 3% formaldehyde in PBS for 15 min. After fixation the cells were washed three times with PBS, aldehyde groups quenched with 50 mM NH₄Cl in PBS (10 min) and then again washed with PBS. The ascites solution of monoclonal antibodies 9E10 against the human *c-myc* was added in 1:5 dilution. After rinsing the cells with PBS, primary antibody was visualized with goat anti-mouse rhodamine-conjugated antibodies diluted in PBS. The coverslips after washing were mounted on glass slides in Moviol and viewed with an Axiophot Photomicroscope (Carl Zeiss, Oberkochen, Germany).

Immunoelectron Microscopy

Transfected cells after 4 h were removed from the culture dishes according to Green et al. (1981). They were then fixed with 8% paraformaldehyde in 250 mM Hepes (pH 7.35), prepared for cryosections, and labeled with antibodies and protein A gold as described previously (Griffiths et al., 1984, 1985).

Results

An Apical Marker Influenza HA Is Found in a Large, Detergent-Insoluble Complex within the Transport Vesicles

Our experiments were aimed at identifying components of the machinery of vesicle traffic from the TGN in epithelial MDCK cells. Candidates for the step of protein sorting might be proteins forming a tight complex with apically delivered proteins, for instance with the influenza virus HA. To address the question whether transport vesicles bearing HA contain such complexes, we used a mild detergent solubilization approach. Transport vesicles (hereafter referred to as TGN-derived vesicles) were obtained from mechanically perforated MDCK cells which were grown on a permeable support and infected with influenza virus (Bennett et al., 1988; Wandinger-Ness et al., 1990).

TGN-derived vesicles obtained from cells pulse labeled with [³⁵S]methionine were extracted with various detergents (for example Triton-X-100, Na-deoxycholate, octylglucoside, CHAPS) to find one which on one hand solubilized most of the vesicular proteins but on the other hand left the HA in a high molecular weight complex. The sedimentation properties of the HA on a sucrose gradient containing the same detergent were followed after extraction. The quantity of HA in each fraction was determined by densitometric analysis of fluorographs after SDS-PAGE.

Using the zwitterionic detergent CHAPS, ~90% of the HA was found in the pellet of the gradient (Fig. 1 A). Some other detergents, for example octylglucoside, solubilized the vesicular HA almost entirely and it sedimented as a trimer in fractions 4 to 6 (data not shown). In a series of experiments we tried to measure the sedimentation coefficient of the complex. It appears to be heterogeneous in size, giving a smear on the gradient, and the calculated S value is over 500 (data not shown). The finding of CHAPS insolubility is in general agreement with previous reports of detergent insolubility of apically sorted proteins (Skibbens et al., 1989; Brown and Rose, 1992).

The solubilization properties of the apical HA upon CHAPS treatment were different from those of the basolateral VSV-G protein. After treatment of TGN-derived vesicles from the VSV infected cells in the same way as the HA-bearing vesicles, the distribution of the G protein on the gradient dramatically differed from the HA profile (Fig. 1 B). Approximately 85% of the G protein remained on the top of the gradient and only about 15% was recovered in the pellet. Therefore the sedimentation of the apical HA into the pellet was not due to the lack of the solubilization of the vesicles by CHAPS. The apical and basolateral virus marker proteins in the vesicles interact differently with the vesicular proteins, and this could reflect the differences in their association with the vesicular machinery.

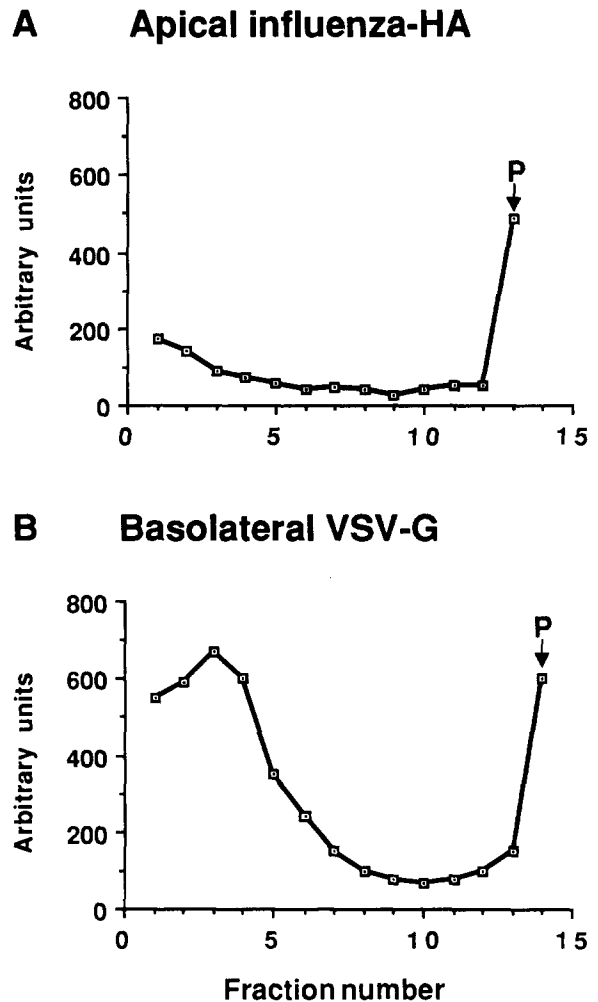


Figure 1. Apical membrane protein influenza HA is found in a large, CHAPS-insoluble complex within the transport vesicles. (A) Distribution of the influenza HA on a sucrose gradient after solubilization of TGN-derived vesicles obtained from influenza infected MDCK cells with the detergent CHAPS. (B) Distribution of a basolateral marker, VSV G-protein, after analogous treatment. TGN-derived vesicles were isolated from pulse-labeled cells. The sucrose gradients (10–30%) were fractionated from the top. Proteins from the fractions (0.4 ml) and the pellet (P) were subjected to SDS-PAGE. The amount of HA or G-protein were quantified by densitometric scanning of fluorographs.

2D Gel Analysis of the CHAPS Complex

The protein content of the pellet obtained after the CHAPS treatment was analyzed by using the 2D gel system described previously (Wandinger-Ness et al., 1990). Fig. 2 shows that there are differences between the protein pattern of the CHAPS-pellet (Fig. 2 B) and the starting vesicular material (Fig. 2 A). First, CHAPS solubilizes the low molecular weight form of the HA while leaving only the high molecular weight form in the pellet. Since it is known that the high molecular weight form is the post-Golgi modified form, only this TGN form is a part of the CHAPS complex. Second, it is clear that the protein composition of the CHAPS pellet is much more simple than that of the vesicles. Some proteins were almost entirely solubilized whereas others were detergent insoluble.

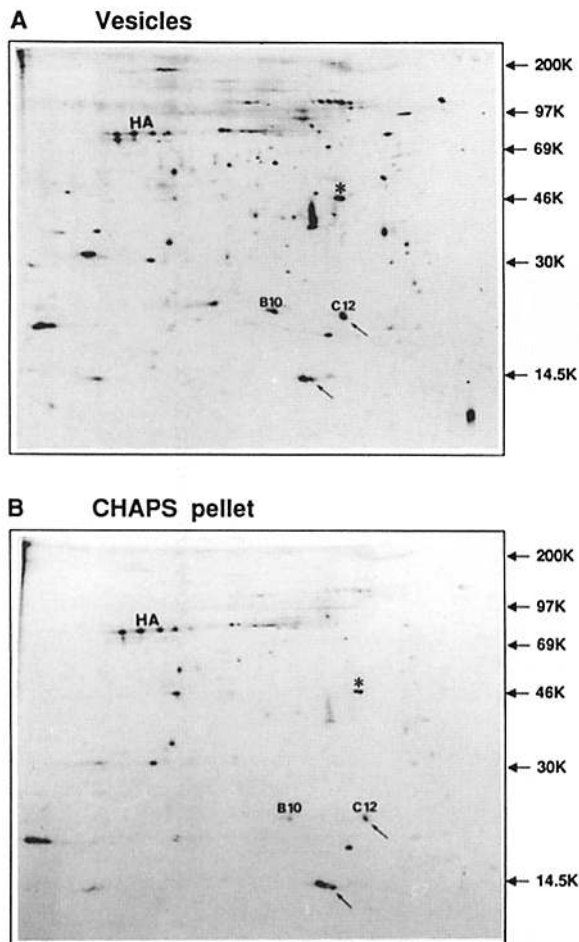


Figure 2. Protein composition of the CHAPS-insoluble complex from TGN-derived vesicles. *B* shows the 2D gel protein pattern of the pellet, obtained by the same procedures of detergent solubilization and sucrose gradient centrifugation as in Fig. 1. (*A*) Separation of an equivalent amount of the starting vesicles. (HA) Influenza hemagglutinin, (notice the existence of two molecular weight forms). The asterisk (here and on other 2D gels) designates actin. Similarly, arrows indicate proteins C12 and C14 (lower spot).

An immunoisolation technique has been previously used to separate apical and basolateral vesicles (Wandinger-Ness et al., 1990). 2D gel analysis allowed the characterization of vesicular proteins as specifically apical, specifically basolateral or common to both vesicle types (designated in Fig. 7 and 9 therein as *A*, *B*, and *C*, respectively). The protein pattern of the CHAPS pellet shows no preferential enrichment of the proteins from a definite class, although some small proteins of the common class (C12, C14) are quite dominant. Significantly, these and many of the other proteins of the CHAPS pellet are integral membrane proteins as demonstrated by Triton-X-114 extraction (Wandinger-Ness et al., 1990) or labeling with a hydrophobic crosslinker iodonaphthylazide (data not shown). The protein pattern of the CHAPS pellet obtained from TGN-derived vesicles of non-infected MDCK cells was practically identical to the pattern seen in Fig. 2 except that the influenza HA and neuraminidase proteins were missing (data not shown). In all likelihood the newly synthesized endogenous apical proteins in non-infected cells are too numerous to be seen as more than weak spots.

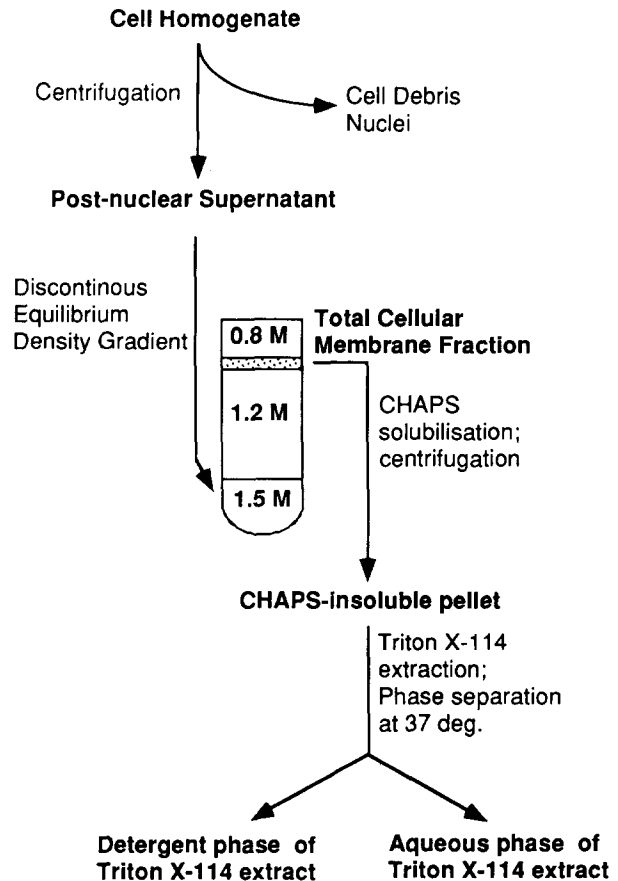


Figure 3. Scheme for the purification of the CHAPS-insoluble complex from cell homogenate and its subsequent biochemical analysis.

Isolation of the CHAPS-insoluble Complex from a Crude Total Membrane Fraction of Cellular Extracts

How many proteins are found in a high molecular weight complex when a crude membrane fraction is treated with CHAPS? If CHAPS insolubility is a specific property of the vesicular proteins, material could be obtained in higher quantities and this would facilitate biochemical characterization of the vesicular complex and its components.

Fig. 3 shows our experimental procedure for isolation of the CHAPS complex from a total membrane preparation. MDCK cells were grown on large (24 cm × 24 cm) cell culture dishes, labeled metabolically with [³⁵S]methionine, and after homogenization through a syringe, a PNS was prepared. The PNS was centrifuged on a discontinuous equilibrium density gradient. The floated membrane fraction was collected from the interface between 1.2 and 0.8 M sucrose. Because this purification step is very similar to the last step of the purification of TGN-derived vesicles (see Materials and Methods), this fraction should contain TGN-derived vesicles among other light membrane components. The equilibrium density centrifugation step simplified the protein pattern of the PNS considerably and enriched several proteins including some of those seen in the vesicles (Fig. 4, cf. *A* and *B*). Among these the small proteins C12 and C14 (arrows) were particularly prominent, suggesting that the TGN-derived vesicles could be present in the total membrane fraction.

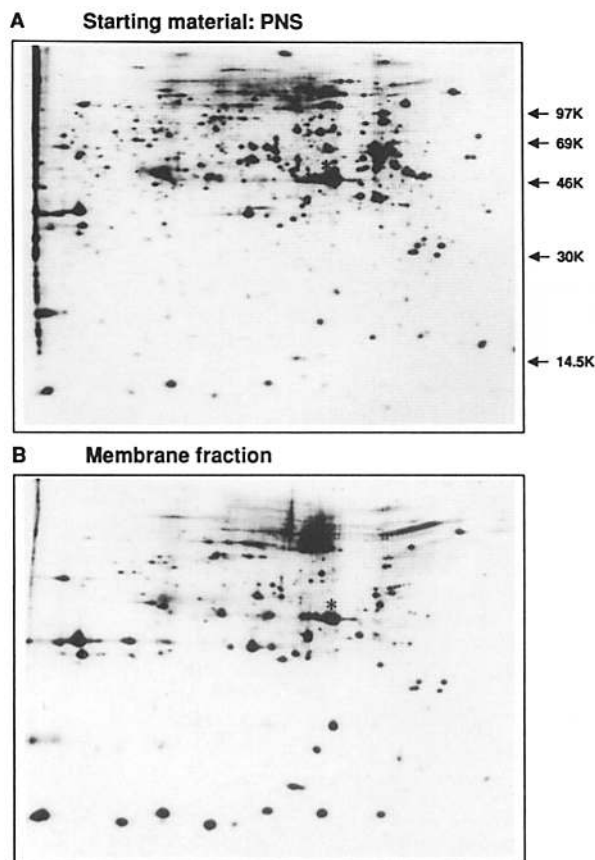


Figure 4. 2D gel protein patterns of the post-nuclear supernatant (A) and the total cellular membrane fraction (B). The quantities of the analyzed material were equivalent.

These membranes were treated with CHAPS and centrifuged on a sucrose gradient in a similar way to the TGN-derived vesicles. Very different radioactivity profiles of sucrose gradients were obtained when TGN-derived vesicles or the membrane fraction were treated with CHAPS (Fig. 5, A and B, respectively). Whereas, ~10% of the vesicular radioactivity was recovered in the pellet, the CHAPS-insoluble material of the membrane fraction did not exceed 0.5%, suggesting that the insolubility is a property of a small subset of proteins. The protein composition of the CHAPS-insoluble pellet is much simpler than the floated membrane fraction (Fig. 6). Again C12 and C14 are among the dominant proteins, demonstrating the remarkable specificity of the extraction.

To purify these proteins for further analysis we extracted the CHAPS pellet with Triton X-114 and separated the extract into detergent and the aqueous phases. The C12 and C14 proteins were enriched almost 50 times in the detergent phase (Fig. 6 B) leaving them the only major proteins in this size range.

We demonstrated that the proteins purified according to this procedure are identical to the proteins C12 and C14 of the TGN-derived vesicles. The detergent phase of the extract was mixed with the vesicular preparation from the influenza virus-infected cells and 2D gel analysis performed. As seen in Fig. 7, the spots of the C12 and C14 fully coincide with the protein spots of the detergent phase. Thus, these

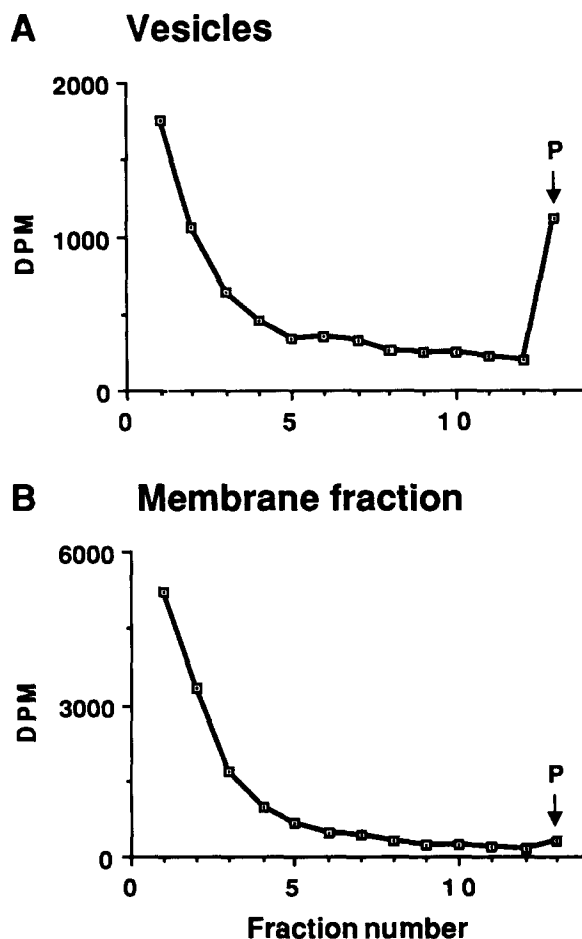


Figure 5. The relative amount of CHAPS-insoluble material in TGN-derived vesicles is significantly higher than that of the total membrane fraction. Vesicles from metabolically labeled cells (A) or the total membrane fraction (B) were treated with CHAPS and subjected to gradient centrifugation similarly to described in Fig. 1. The radioactivity of aliquots from the fractions and solubilized pellets (P) was counted.

CHAPS-insoluble proteins from the crude membrane fraction are identical to those in the vesicles.

Cloning of cDNAs That Encode a Protein of the CHAPS Insoluble Complex

We used the procedure for enrichment of the membrane proteins in preparative quantities. A preparation from cells grown on six large (24 cm × 24 cm) plates was separated by 1D SDS-PAGE and the band of 21 kD (C12), the only major protein of this size, was cut out. In situ digestion with trypsin and subsequent peptide microsequencing were performed as described in the Materials and Methods section. Initially, the sequence R/K I/E F/I S/C N/L I/V N C D P K from a mixture of two peptides was obtained. The six COOH-terminal amino acids I/V N C D P K were used to design a degenerate oligonucleotide (see Materials and Methods). A 5' RACE polymerase chain reaction was carried out using this oligonucleotide as the gene-specific primer, and a general second primer that can anneal to the 5' end of all the cDNAs present (Frohman et al., 1988). Using MDCK cDNA as a template, a single fragment of 260

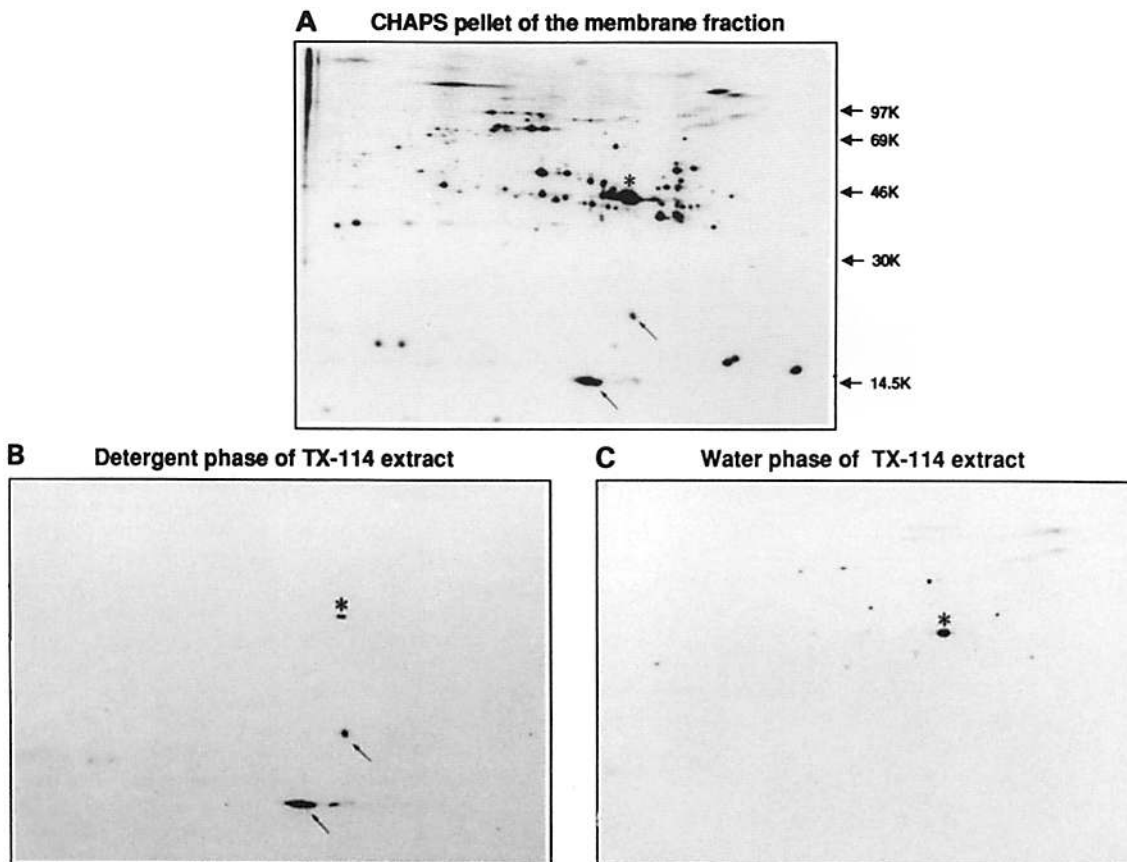


Figure 6. Protein composition of the CHAPS-insoluble complex from the total cellular membrane (**A**) and its extract with TX-114. **B** and **C** show the detergent and water phases of the extract, respectively.

bp was amplified. The sequence of this fragment encoded a short open reading frame that included the amino acid sequence K E I D L V N C D P K at the COOH terminus. A peptide with this amino acid sequence could have been present in the original mixture. It was therefore probable that the PCR-generated fragment was derived from the 5' end of a cDNA encoding C12.

To obtain a full-length cDNA clone, the PCR-generated fragment was used to screen 300,000 plaques of a lambda ZAPII MDCK cDNA library. Approximately 100 clones hybridized strongly with the fragment. The nucleotide sequence of two populations of clones with inserts of 1.0 and 2.0 kbp was determined and differed only in the 3' non-translated region and the polyA addition site. The nucleotide sequence of the shorter cDNA clone is shown in Fig. 8. The cDNAs encode a protein of 178 amino acids with a predicted molecular mass of 20,606 D, in good agreement with the molecular weight estimated of C12 from PAGE. The predicted protein also contains sequences identical to those obtained from subsequent microsequencing of four further tryptic peptides of C12. These peptides are boxed in Fig. 8. There is no signal sequence at the NH₂ terminus, but there is a single long hydrophobic region between amino acids 102 and 134, underlined in Fig. 8, which according to the prediction method of von Heijne (1987) could serve as a signal anchor sequence. The hydrophobic region could form a transmembrane span as expected for the C12 membrane protein.

To confirm that the cDNA encodes the C12, labeled pro-

tein was prepared by in vitro transcription and translation of the clone and analyzed by 2D PAGE. The protein appears to be posttranslationally modified by the reticulocyte lysate, since there are three forms of the protein on this gel. The nature of the modification, which changes the pI but does not greatly affect the mobility in SDS-PAGE, is not clear. The sample was mixed with the TX-114 detergent extract of the total membrane fraction. The most anodic form of the protein was found to have identical mobility in the 2D gel system as C12 (Fig. 9). Thus the cDNA encodes this protein. On inspection of the 2D gels of vesicles, it appeared that the major form of the protein was also present and had been named B10 (Fig. 2 A) (Wandinger-Ness et al., 1990) indicating that the modification occurs in vivo. The B10 was assigned as basolateral in the previous 2D gel analysis of the immunisolated apical and basolateral vesicles. However, the apical to basolateral ratio was similar for both, only 0.42 for B10 compared to 0.6 for C12, so that both forms of the protein must now be considered common components. The pI of the predicted protein sequence is 5.95, similar to that measured of B10, suggesting that C12 is the modified form of B10. We named the protein VIP21 (Vesicular Integral membrane Protein of 21 kD).

The Cellular Localization of NH₂-terminally Tagged VIP21

Clear indication that the VIP21 was indeed a constituent of the transport pathway from the Golgi to the plasma mem-

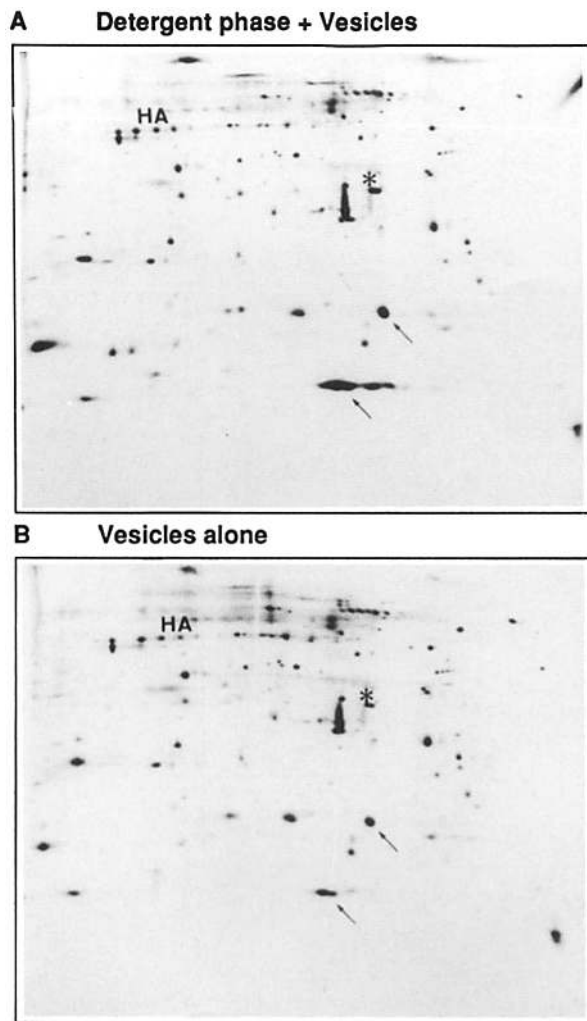


Figure 7. Small molecular weight proteins of the CHAPS-insoluble complex isolated from the total cellular membrane fraction are identical to transport vesicle proteins C12 (VIP21) and C14. (A) Mixture of TX-114 detergent phase (see Fig. 6 B) and the vesicles from influenza infected cells. (B) Vesicles alone.

brane was provided by immunofluorescence and immunoelectron microscopy. A construct encoding the human *c-myc* epitope fused to the NH₂ terminus of the VIP21 was transiently expressed in BHK cells using the vaccinia T7 expression system.

Immunofluorescence of the cells expressing the construct after 3 h shows a clear juxtannuclear and also diffuse punctate staining (Fig. 10). Immunoelectron microscopy confirmed that the expressed protein is found in post-Golgi locations. Three structures were predominantly labeled: invaginations on the plasma membrane (Fig. 11, A and B), possible vesicular profiles (B and inset on C), and at this time of transfection Golgi (Fig. 11 C). The invaginations on the plasma membrane were not clathrin coated (Fig. 11 B, arrow indicates a clathrin-coated pit for comparison). Membranes in the vicinity of the Golgi apparatus were more strongly labeled than the stacks themselves. The results are consistent with a post-Golgi location of VIP21.

Discussion

According to our working hypothesis, segregation of apical and basolateral membrane proteins within the TGN occurs

	CGGTTCCTTGAATAACAGCCAGGGAAACCTCGGAGCCTTACCCAGCCACCGGCCAGC	GGCACGAG	8
			68
	ATGTCGGGGCAATAACGTAGACTCCGAGGGACCTCTACACCGTTCCCATCCGGGAG		128
1	M S G G K Y V D S E G H L Y T V P I R E		
	CAGGGCAACATCTACAAGCCCAACAACAGCCATGGCGGAGGATGAGCGAGAAGCAG		188
21	Q G N I Y K F N N K A M A E E M S E K Q		
	GTGTAGACGGCCACCAAGGAATCGACCTGGTCAACCGGACCCCAAGCATCTCAAC		248
41	V Y D A H T K <u>E I D L V N R D P K</u> <u>R L N</u>		
	GACGACGTGGTCAAGATTGATTTGAAGATGTGATTGCAGACCAGAAGGAACACACAGT		308
61	<u>D D V V K</u> <u>I D F E D V I A E</u> P E G T H S		
	TTTGATGGCATCTGGAAGGCCAGCTTCAACACCTTCACTGTGACAAAATACTGGTTTTAC		368
81	F D G I W K A S F T T F T V T K Y W F Y		
	CGTTGTCTGTCGCCCTCTTTGGCATCCCAATGGCACTCATATGGGCCATTTACTTTGCC		428
101	R <u>L L S A L F G I P M A L I W G I Y F A</u>		
	ATTCCTTTCTTCTGCACATCTGGGCGTGTGCGCGCATTAAAGATTTCTCTGATTGAG		488
121	I L S F L H I W A V V P C I K S F L I E		
	ATTCAGTGCATCAGCCGTGTCTATTCATCTACGTCCACACCTTCTGTGACCCGTTCTTT		548
141	I Q C I S R V Y S I Y V H T F C D P F F F		
	GAGGCTGTGGCAAAATATTCAGCAATATCCGCATCAACATGCAGAAAGAAACATAAATG		608
161	E A V G K <u>I F S N I R</u> <u>I N M Q K</u> E T *		
	ACATTTAAGGATAGCAGTTAACCTGATTCITTTTT		644

Figure 8. Nucleotide sequence of VIP21 cDNA and amino acid sequence of the encoded protein. Boxed regions indicate microsequenced peptides obtained by digestion of VIP21. The putative membrane spanning segment of the protein is underlined. The asterisk denotes the stop codon. These sequence data are available from EMBL/GenBank/DBS under accession number Z12161.

in a signal-dependent way. We have previously suggested that the formation of an apical carrier vesicle would require both the signal-mediated inclusion of specific proteins and the simultaneous exclusion of others (Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990). We speculated that this process could be accomplished by the cooperative sorting of apical proteins with sphingolipids. Apical proteins associated with putative apical sorting receptors and vesicular machinery could interact with clusters of glycosphingolipids within the TGN membrane. The GPI-linked proteins almost exclusively found on the apical surface would be included in these clusters as well (Lisanti and Rodriguez-Boulant, 1990). The ability of sphingolipids to form microdomains has previously been suggested (Thompson and Tilkack, 1985). In this model the proteins and lipids, assembled together in "scaffolds", would bud off as a vesicle containing the necessary machinery to ensure specific delivery to the apical membrane. Conversely, basolateral proteins should lack the property to participate in the cooperative assembly of the scaffolds and thus would be excluded from the budding apical vesicles. The basolateral sorting signals recently identified in some proteins localize to the cytosolic domains and some are similar in structure to the signals previously identified for rapidly endocytosing proteins (Mostov et al., 1992). These signals could either be specifically recognized (Hopkins, 1991) or function as signals for exclusion from the apical vesicles, perhaps by the binding of cytosolic adaptor proteins (Hunziker et al., 1991) that cannot be fitted into the apical scaffolds.

A prediction of our model is that the behavior of apical and basolateral passenger proteins in the transport vesicles should be quite different: apical proteins are expected to be found in protein-glycolipid complexes, whereas basolateral proteins not. Based on this, we have developed a strategy to detect and to isolate putative complexes bearing an apical protein. Consistent with this, after the TGN-derived vesicles were treated with the detergent CHAPS, the apical marker influenza HA was found in an insoluble complex of over 500S which contained other integral membrane proteins. The complex is too large to be derived from a single vesicle.

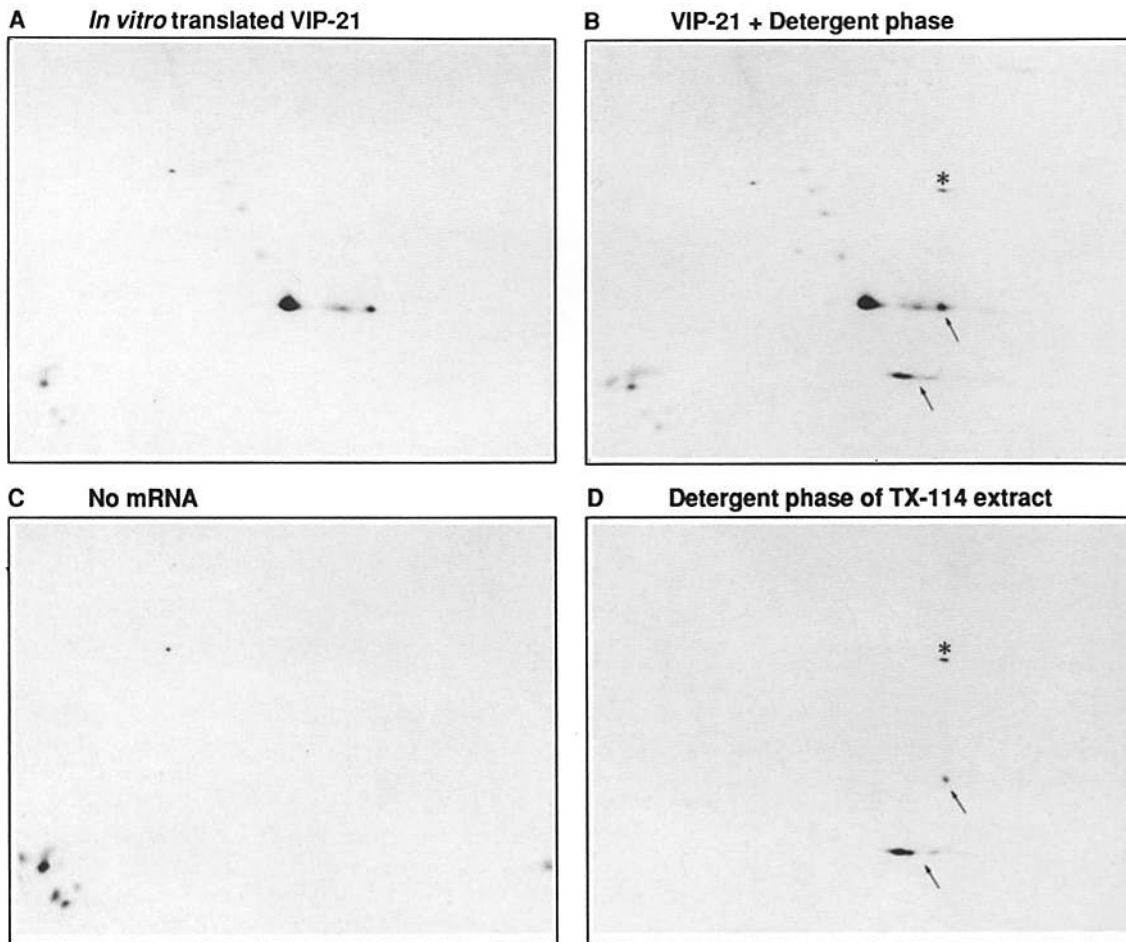


Figure 9. Comparison of in vitro translated VIP21 with the biochemically isolated protein. In vitro-translated VIP21 (A) and the detergent phase of the TX-114 extract of CHAPS-insoluble complex (D) were mixed and subjected to co-electrophoresis (B) on a 2D gel. (C) In vitro translation without addition of exogenous mRNA.

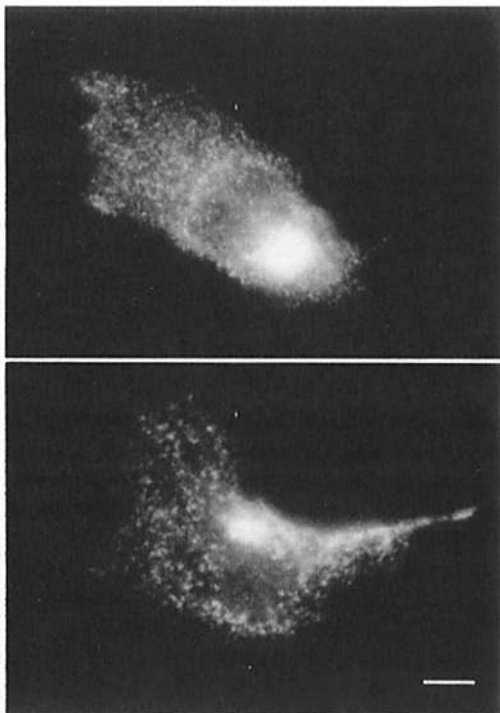


Figure 10. Immunofluorescence of BHK cells transfected with a construct encoding VIP21 with a c-myc epitope fused to the NH₂ terminus. Bar, 5 μ m.

It is likely that CHAPS treatment leads to the building of large structures comprising many scaffolds, and perhaps also containing lipids. Under similar solubilization conditions, the basolateral marker VSV G protein behaved quite differently, being almost entirely solubilized. We interpret these different detergent solubilities of the vesicle marker proteins as a reflection of the sorting process occurring in the TGN and the association of apical proteins with lipid domains and the putative sorting machinery.

How unusual is the CHAPS insolubility found in these vesicles? Treatment with CHAPS of a light-membrane fraction prepared from an MDCK PNS yielded a pellet showing considerably simplified 2D gel patterns compared to the starting membranes. Astoundingly, in the low molecular weight region the protein pattern was almost identical to that found after apical vesicle solubilization. The dominant small proteins are C14 and VIP21 (C12), also dominant components of the vesicular CHAPS-insoluble material. This demonstrates that CHAPS insolubility is a highly unusual feature of these proteins, which we speculate is dependent on special protein-protein or protein-lipid interactions, or both. Surprisingly among several membrane proteins only two were found in large quantities in the TX-114 extract of this sample.

The existence of the CHAPS-insoluble complex allowed us to develop a strategy for isolation of these proteins in quantities sufficient for peptide microsequencing. Purifica-

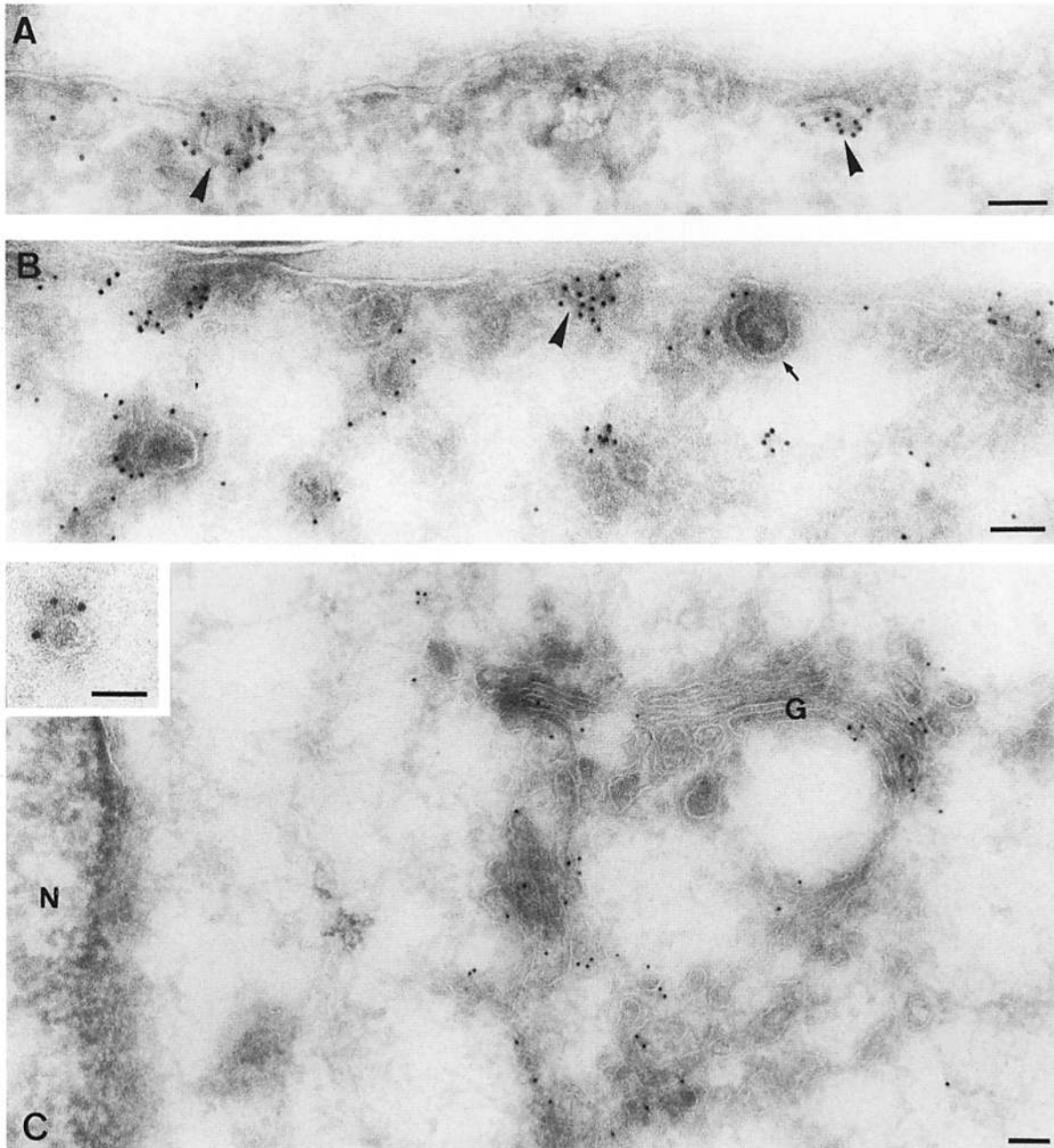


Figure 11. Immunoelectron micrographs of BHK cells transfected with a construct encoding VIP21 with a *c-myc* epitope fused to the NH₂ terminus. Expressed protein was detected with an anti-*myc* antibody followed by 9 nm protein A gold. Labeling is evident in clusters associated with non-(clathrin)-coated invaginations (*arrowheads*) of the plasma membrane (*PM*) (*A* and *B*), vesicular profiles (*inset*), and the Golgi (*G*). No labeling is evident on the ER surrounding the nucleus, *N*. An arrow indicates a clathrin-coated pit. Bars: (*A-C*) 100 nm; (*inset*) 50 nm.

tion via this detergent solubilization can be compared in specificity to an affinity chromatography procedure, although a priori the detergent CHAPS might not be expected to act as such a specific agent. The VIP21 protein encoded by the cDNA shows no homology to other proteins in the Swissprot or PIR databases. As expected it has a predicted hydrophobic region capable of spanning the membrane. This could serve as an internal signal sequence according to the prediction rules of von Heijne (1987), but would not be cleaved. It is likely that the long NH₂-terminal sequence preceding the signal (100 amino acids) remains cytosolic. In

further agreement with the membrane protein orientation rule of Hartmann et al. (1989), we predict that the COOH terminus is luminal (type II). It cannot be excluded however, that the hydrophobic region forms a loop in the membrane leaving the COOH terminus in the cytosol.

The protein has no predicted glycosylation, sulfation, phosphorylation or other modification sites described in the Prosite database (Bairoch, 1991). However, 2D gel analysis demonstrated that the *in vitro*-translated protein is modified by the reticulocyte lysate, changing the pI by one unit. Interestingly, two forms of VIP21 are present in the TGN-

derived vesicles. The nature of the modification is under investigation.

The cellular localization of VIP21 confirmed the validity of our assumption that CHAPS-insoluble proteins belong to post-Golgi compartments. In immunofluorescence studies and immuno-electron microscopy of the transiently expressed cDNA, VIP21 was found in the Golgi apparatus, vesicular structures, and to some extent on the plasma membrane. Although further studies will be required to determine the steady state distribution of VIP21, we can conclude that the steady-state localization lies beyond the Golgi apparatus, as the protein is not retained either in the ER or in the Golgi apparatus. A vesicular membrane protein when recycled might be present in the TGN, vesicles, plasma membrane, and endosomes, consistent with these observations. Interestingly, the expressed protein appeared to be associated with non-clathrin coated plasma membrane invaginations. Recent studies with the antibodies against the COOH-terminal peptide (residues 161-178) confirm that the localization of the endogenous VIP21 in BHK and MDCK cells is the same as that observed in this study with the epitope-tagged protein (Dupree, P., R. G. Parton, T. V. Kurzchalia, and K. Simons, unpublished observations).

VIP21 is one of the few vesicular integral-membrane proteins identified so far (see Südhof and Jahn, 1991). This protein is common to both apical and basolateral vesicles, based on the densitometric analysis of their 2D gel patterns (Wandinger-Ness et al., 1990). There are two possibilities to explain this distribution. VIP21 could have a function common to both vesicle types and would not be involved in specifying apical or basolateral delivery. However, the alternative view that the protein confers specificity to the apical vesicular machinery cannot yet be excluded. VIP21 could be moving with the basolateral vesicles as a passenger for delivery to the site where apical transcytosis takes place. Newly synthesized apical proteins are sorted directly from the TGN to the apical membrane in MDCK cells whereas in hepatocytes and to some extent also in intestinal CaCO₂ cells sorting occurs first after basolateral delivery (see Mostov et al., 1992). The apical proteins are endocytosed and routed by transcytosis from the basolateral early endosomes to the apical surface domain. Also this process requires machinery that could partially be the same as that used for apical delivery from the TGN. Only further work on the function of VIP21 will clarify its roles and itinerary in the cell. Nevertheless, the success of the CHAPS extraction makes us confident that this protein is an important component of the apical vesicle scaffold.

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