Distinct Transport Vesicles Mediate the Delivery of Plasma Membrane Proteins to the Apical and Basolateral Domains of MDCK Cells

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Abstract. Immunoisolation techniques have led to the purification of apical and basolateral transport vesicles that mediate the delivery of proteins from the *trans*-Golgi network to the two plasma membrane domains of MDCK cells. We showed previously that these transport vesicles can be formed and released in the presence of ATP from mechanically perforated cells (Bennett, M. K., A. Wandinger-Ness, and K. Simons, 1988. *EMBO (Euro. Mol. Biol. Organ.) J.* 7:4075–4085). Using virally infected cells, we have monitored the purification of the *trans*-Golgi derived vesicles by following influenza hemagglutinin or vesicular stomatitis virus (VSV) G protein as apical and basolateral markers, respectively. Equilibrium density gradient centrifugation revealed that hemagglutinin containing vesicles had a slightly lower density than those containing VSV-G protein, indicating that the two fractions were distinct. Antibodies directed against the cytoplasmically exposed domains of the viral spike glycoproteins permitted the resolution of apical and basolateral vesicle fractions. The immunoisolated vesicles contained a subset of the proteins present in the starting fraction. Many of the proteins were sialylated as expected for proteins existing the *trans*-Golgi network. The two populations of vesicles contained a number of proteins in common, as well as components which were enriched up to 38-fold in one fraction relative to the other. Among the unique components, a number of transmembrane proteins could be identified using Triton X-114 phase partitioning. This work provides evidence that two distinct classes of vesicles are responsible for apical and basolateral protein delivery. Common protein components are suggested to be involved in vesicle budding and fusion steps, while unique components may be required for specific recognition events such as those involved in protein sorting and vesicle targeting.

SEVERAL classes of proteins including lysosomal enzymes, plasma membrane proteins, and proteins of regulated secretion follow a common route from their site of synthesis in the ER through the cisternae of the Golgi complex. Upon arriving in the *trans*-Golgi network (TGN)[1] the proteins in transit must be sorted for delivery to their final destinations (for reviews see Griffiths and Simons, 1986; Klausner, 1989). In epithelial cells, the plasma membrane consists of two domains each with a distinct protein composition (for review see Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). This necessitates an additional sorting step for the segregation of apical from basolateral plasma membrane proteins.

MDCK cells form polarized monolayers in culture and have been used extensively as a model system to study protein sorting in epithelial cells (McRoberts et al., 1981). Newly synthesized plasma membrane proteins have been shown to be sorted intracellularly using MDCK cells infected with enveloped RNA viruses (Matlin and Simons, 1984; Misek et al., 1984; Rindler et al., 1985; Pfeiffer et al., 1985). The spike glycoproteins of such viruses follow the same intracellular transport pathway as endogenous plasma membrane proteins and are delivered differentially to the two domains without requiring additional virally encoded products (Rindler et al., 1984; Fuller et al., 1985; Jones et al., 1985; Caplan et al., 1986; Gottlieb et al., 1986*b*; Stephens et al., 1986). Influenza hemagglutin (HA) is transported to the apical domain, while vesicular stomatitis virus (VSV) G protein is delivered to the basolateral domain (Rodriguez-Boulan and Pendergast, 1980). The advantages afforded by using the viral glycoproteins as model apical and basolateral plasma membrane proteins are several fold. The proteins are highly expressed during viral infection and can be detected readily. Because host protein synthesis is inhibited by viral infection, the viral glycoproteins represent the sole proteins in transit. However, even in the absence of host protein synthesis, viral protein sorting continues for several hours after

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1. *Abbreviations used in this paper*: HA, hemagglutin; NSF, *N*-ethylmaleimide-sensitive factor; TGN, *trans*-Golgi network; VSV, vesicular stomatitis virus.

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infection (Rodriguez-Boulan and Pendergast, 1980; Fuller et al., 1984). This suggests that the proteins involved in sorting and proper delivery must be reused during multiple rounds of transport.

It is thought that apical and basolateral plasma membrane proteins are sorted as they emerge from the TGN by inclusion into separate vesicular carriers which are then targeted to the appropriate membrane domain. In vivo these transport vesicles are transient intermediates and not very abundant. Therefore, we have developed an in vitro system that allows the isolation of TGN-derived transport vesicles with the aim of identifying the molecular machinery involved in protein sorting and vesicle targeting (Bennett et al., 1988). MDCK cells were grown on permeable filter supports allowing them to attach tightly to the substrate and to form a fully polarized monolayer. The cells were then infected for short times with influenza or VSV and incubated at 20°C causing the transport markers to accumulate in the TGN (Matlin and Simons, 1983; Griffiths et al., 1985; Hughson et al., 1988). Subsequently, the cells were mechanically perforated with the aid of a nitrocellulose filter. This introduced holes in the plasma membranes but left the cells attached to the filter support with their subcellular organization intact (Bennett et al., 1989). Using this system, we demonstrated previously that HA and VSV-G protein, accumulated in the TGN before perforation, were released from the perforated cells in sealed membrane vesicles (Bennett et al., 1988). The vesicles had the expected topology for authentic transport vesicles and required ATP for their formation and release. The release of membranes from such perforated cells was quite specific: only low levels of resident Golgi markers, endocytic markers, or ER-derived vesicles were recovered in the incubation medium (Bennett et al., 1988). The TGN-derived transport vesicles have now been purified for further characterization and resolved into apical and basolateral fractions.

Materials and Methods

Materials

Media and reagents for cell culture were purchased from Gibco Biocult (Eggenheim, FRG) and Biochrom (Berlin, FRG). Creatine phosphate and creatine kinase were obtained from Boehringer Mannheim (FRG); immobilized papain from Pierce (Rodgau, FRG); and purified mouse IgG, ATP, BSA, gelatin, protease inhibitors, and Triton X-114 were from Sigma Chemical Co. (Deisenhofen, FRG). [^{35}S]methionine (800 Ci/mmol) was from Amersham Buchler GmbH (Braunschweig, FRG). Reagents for two-dimensional gel electrophoresis were obtained from the following vendors: agarose standard EEO (M_r, 0.16), Servalyt pH 5-7, and DTT from Serva (Heidelberg, FRG); Ampholines pH 5-7 and 3.5-10 from LKB Pharmacia (Freiburg, FRG); acrylamide and N,N'-methylenebisacrylamide for IEF, N,N,N',N'-tetramethylethylene diamine, ammonium persulfate and SDS from BioRad (Munich, FRG); acrylamide and N,N'-methylenebisacrylamide for SDS-PAGE from British Drug House (Poole, England); NP-40 from Fluka Neu-Ulm; urea (enzyme grade) from Bethesda Research Laboratories (Gibco BRL LTD, Paisley, Scotland); glycine, glycerol, ortho-phosphoric acid, and sodium hydroxide from Merck (Darmstadt, FRG). The mouse mAbs directed against the cytoplasmic domains of VSV-G protein and PR8 HA were prepared as described by Kreis (1986) and Hughson et al. (1988), respectively.

Cell Culture Media

Growth medium consisted of EME with Earle's salts (E-MEM) supplemented with 10 mM Hepes, pH 7.3, 10% (vol/vol) FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Infection medium was composed of E-MEM supplemented with 10 mM Hepes, pH 7.3, 0.2% (wt/vol) BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin. Pulse-labeling medium consisted of methionine-free E-MEM containing 10 mM Hepes, pH 7.3, 0.2% (wt/vol) BSA, and 0.35 g/l sodium bicarbonate (instead of the usual 2.2 g/l). Water bath medium was E-MEM supplemented with 10 mM Hepes, pH 7.3, 0.2% BSA, 0.35 g/l sodium bicarbonate, 20 μg/ml cycloheximide, and 150 mg/l unlabeled methionine (10-fold excess). Metabolic labeling medium consisted of E-MEM containing 10 mM Hepes, pH 7.3, 2.5% FCS, and 1.5 mg/l unlabeled methionine (1/10th the normal concentration).

Cell Culture

MDCK strain II cells were grown and passaged as described previously (Matlin et al., 1981). The cells from one confluent 75-cm^2 flask were seeded on six 24-mm diameter, 0.4-μm pore size, premounted Transwell polycarbonate filters as described (Bennett et al., 1988). Alternatively, for large scale isolation of vesicles the cells from one flask were resuspended in 20 ml of growth medium and seeded on a single 100-mm diameter, 0.4-μm pore size Transwell filter. The filters were transferred to special holders in a Petri dish containing 140 ml of growth medium. All filter cultures were maintained at 37°C and 5% CO_2 for 2–3 d. The Transwell filters were a kind gift from Hank Lane, Costar (Cambridge, MA).

Viral Infection

Fowl plague virus and VSV stocks were prepared as described (Bennett et al., 1988). Influenza PR8 and WSN ts61 stocks were prepared as described (Hughson et al., 1988). Infections were performed by rinsing the filters with infection medium and transferring small (24-mm) filters to six-well dishes and large (100-mm) filters to 10-cm dishes. The virus stocks were diluted in infection medium and added to the apical side of the filters, 100 μl per 24-mm filter and 1 ml per 100-mm filter. The concentration of fowl plague virus and PR8 used was 20 pfu/cell, of WSN ts61 4 pfu/cell, and of VSV 50 pfu/cell. The viruses were allowed to adsorb to the cells for 1 h at 37°C (31°C used for WSN ts61), the inoculum was removed and fresh infection medium was added to both sides of the filter. 1 ml per 24-mm and 10 ml per 100-mm filter was added to the apical side and 2.5 ml per 24-mm and 12 ml per 100-mm filter was added to the basolateral side. The infections were continued for an additional 2.5 h at 37°C (fowl plague virus, PR8, and VSV) or 3.5 h at 39°C (WSN ts61) in 5% CO_2. Subsequently, the medium was replaced with water bath medium and the infected cells were incubated for 2 h in a water bath at 20°C.

Radioactive Labeling

Pulse labeling of infected cells was done with 24-mm filters just before the 20°C incubation as described (Bennett et al., 1988). Metabolic labeling was done using 100-mm filters as follows. 2-d-old filter cultures were transferred to 10-cm dishes and washed twice with metabolic labeling medium at 37°C. 10 ml metabolic labeling medium was added to the apical side of each filter and 12 ml of the same medium containing 1 mCi [^{35}S]methionine was added to the basal side. The filters were incubated for 12 h at 37°C, 5% CO_2. For subsequent viral infection the apical medium was aspirated and the basolateral medium was collected, supplemented with 100 μCi fresh [^{35}S]methionine, and saved. After virus adsorption, fresh metabolic labeling medium was added to the apical side and the basolateral medium was readded for the duration of the infection.

Vesicle Isolation from Perforated Cells

Perforation of the 100-mm filters was done essentially as described for the 24-mm filters (Bennett et al., 1988) with a few minor changes. The filters were excised from the holders and transferred to 14-cm dishes containing ice-cold 25 ml KOAc buffer (25 mM Hepes, pH 7.4, 115 mM potassium acetate, 2.5 mM $MgCl_2$) to wash away cell culture medium. The filter culture was then placed in a 14-cm dish in a 20°C water bath (in the cold room) and overlaid with a prewetted nitrocellulose acetate filter (HATF 0.45-μm pore; Millipore Continental Water Systems, Bedford, MA). Excess moisture was removed by overlaying with a Whatman I filter (Whatman, Kent, England) and smoothing with a bent Pasteur pipette. After binding to the cells for 90 s the nitrocellulose filter was wetted with 2 ml KOAc buffer and the filters were carefully separated. After perforation, the filter culture was transferred to a 10-cm tissue culture dish containing 10 ml GGA buffer (25 mM Hepes, pH 7.4, 38 mM potassium gluconate, 38 mM potassium glutamate, 38 mM potassium aspartate, 1 mM DTT [added fresh], 2 mM EDTA, and 2.5 mM $MgCl_2$) and an ATP regenerating system consisting of 1 mM

ATP, 8 mM creatine phosphate, and 50 μg/ml creatine kinase. The perforated cells were incubated at 37°C for 1 h after which time the incubation medium was collected and clarified by centrifugation for 10 min at 1,000 g_{av}. The supernatant was overlaid onto a 2-ml 0.25 M sucrose cushion (in 10 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM DTT) in the bottom of an SW40 tube and the membranes were pelleted by centrifugation for 3 h at 200,000 g_{av} and 4°C. The supernatant was carefully aspirated with a drawn out Pasteur pipette and the wall of the tube was dried with a cotton swab. The membrane pellets were gently resuspended in 300 μl of 1.5 M sucrose (in 10 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM DTT) with a pipetteman in preparation for equilibrium density centrifugation.

Resolution of VSV-G Protein and HA Containing Vesicles on Linear Equilibrium Density Gradients

The membrane pellets were isolated from pulse-labeled VSV or fowl plague virus-infected cells (three 24-mm MDCK filter cultures used for each virus) after perforation. The pellets were resuspended and mixed in 300 μl of 1.7 M sucrose (in 10 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM DTT). The sample was overlaid with a 0.3–1.5 M linear sucrose gradient and after centrifugation for 13.5 h at 125,000 g_{av} in an SW40 rotor 1-ml fractions were collected. Affinity-purified polyclonal antibodies directed against VSV-G protein and fowl plague virus HA were mixed and used to immunoprecipitate the viral proteins from each gradient fraction as described by Bennett et al. (1988). The centrifuge tube was washed with 1 ml lysis buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 2% [wt/vol] Triton X-100, 0.3 M NaCl, and 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin) to solubilize pelleted material for immunoprecipitation.

Purification of Vesicles on Discontinuous Equilibrium Density Gradients

All sucrose solutions were prepared in 10 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM DTT. The membrane fractions obtained from up to three 100-mm filters were combined in a total volume of 300 μl 1.5 M sucrose. The sample was placed in the bottom of an SW60 tube and was overlaid with 1.9 ml 1.2 M sucrose followed by 1.8 ml 0.8 M sucrose. The vesicles were allowed to reach their equilibrium density by centrifugation of the gradients for 13 h at 125,000 g_{av} and 4°C in an SW60 rotor (Beckman Instruments, Inc., Palo Alto, CA) after which 0.3-ml fractions were collected. The peak fractions containing vesicles were recovered at the 0.8/1.2 M sucrose interface and were pooled.

Reagents for Immunoisolation

Mouse mAbs directed against the cytoplasmic domains of VSV-G protein (Kreis, 1986) and influenza PR8 (Hughson et al., 1988) were used in the form of concentrated hybridoma culture supernatants. The hybridomas were maintained in dense culture in RPMI medium containing 10% FCS for 2 d. The medium was harvested, concentrated 10-fold by ultrafiltration, and stored frozen in small aliquots for single use. Cellulose fibers were used as the solid support for immunoisolation (Luzio et al., 1976; for review see Richardson and Luzio, 1986). Microgranular cellulose powder was purchased from Whatman and activated for protein coupling (Hales and Woodhead, 1980). A polyclonal antibody directed against the Fc domain of mouse IgG was raised in sheep by immunizing with purified Fc fragments (prepared from mouse IgG digested with immobilized papain as described in the Pierce catalog). Sheep antimouse Fc was affinity purified on an Affi-gel 10 matrix (BioRad) with covalently bound mouse IgG and subsequently coupled to diazocellulose. 1 mg of protein added per mg of diazocellulose resulted in 200–300 μg coupled protein. The immunoadsorbent was stable for several months stored at 4°C as a slurry; 2-mg fibers/ml of PBS containing 5 mg/ml BSA and 0.02% (wt/vol) sodium azide. Before use the fibers were washed three times with PBS containing 0.1% gelatin to remove the BSA and azide.

Immunoisolation of Vesicles

For each immunoisolation experiment a gradient-purified vesicle fraction was divided into three equal aliquots. One aliquot was incubated with a control antibody and a second aliquot was incubated with the specific antibody, as follows. Each aliquot was diluted with 0.5 vol PBS containing 0.1% gelatin and a protease inhibitor cocktail (30 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin) in an Eppendorf tube (Eppendorf/Nethaler, Hamburg, FRG). To this mixture 10–20 μl concentrated hybridoma culture supernatant was added. The samples were incubated with rotation at 4°C for 3 h (VSV-G protein vesicles) or overnight (WSN HA vesicles). 1 mg immunoadsorbent (2 mg/ml in PBS containing 0.1% gelatin) was added and the samples were incubated with rotation for an additional 90 min at 4°C. The cellulose fibers were collected by centrifugation at 4°C and 2,500 rpm in a Microfuge 11 (Beckman Instruments, Inc.). The fibers were washed three times with 1 ml PBS containing 0.1% gelatin and once with PBS alone. After the last wash excess liquid was removed by brief centrifugation in an Eppendorf microfuge and the bound material was solubilized in 50 μl gel sample buffer. The third aliquot was diluted with ~3 vol 10 mM Hepes, pH 7.4, 2 mM EGTA (to give 0.2–0.25 M sucrose final) and the vesicles were pelleted by centrifugation for 3 h at 200,000 g_{av} and 4°C in a TL-100 ultracentrifuge (Beckman Instruments, Inc.) using a TLS-55 rotor. The membrane pellet was resuspended directly in 50 μl sample buffer and served as the starting material reference.

SDS-PAGE

SDS-PAGE was performed as described (Bennett et al., 1988). For SDS-PAGE immunoisolated samples were solubilized in twofold concentrated sample buffer (100 mM Tris, pH 6.8, 5 mM EDTA, 10% glycerol, 4% SDS, 0.02% bromo-phenol blue).

Two-dimensional Gel Electrophoresis

A combination of IEF and SDS-PAGE was used to resolve proteins in two dimensions essentially as described by Bravo (1984). For two-dimensional gel electrophoresis the samples were solubilized in 9.8 M urea, 4% (wt/vol) NP-40, 2% (vol/vol) ampholines pH 7–9 (LKB Instruments, Inc., Gaithersburg, MD), and 100 mM DTT. The tube gels used for the IEF dimension were 25 cm long and had an internal diameter of 2.5 mm. IEF gels were run at 1,200 V for 17 h. The pH gradient after electrophoresis ranged from 4.57 to 8.03 and was linear between pH 4.6–7.2. The second dimension resolving gels were 15% (wt/vol) acrylamide, 0.075% (wt/vol) N,N'-methylene-bisacrylamide and the stacking gels were 5% (wt/vol) acrylamide, 0.25% (wt/vol) N,N'-methylene-bisacrylamide. After electrophoresis the gels were fixed in 45% methanol and 7% acetic acid and treated for fluorography using Entensify (Dupont, NEN Research Products, Boston, MA).

Quantitation of Two-dimensional Gels

The autoradiographs of two-dimensional gels were digitized using an Osram CCD video camera and the Image (version 1.16) software program (developed by W. Rasband, National Institutes of Health, Bethesda, MD) designed for use with a Macintosh IIcx computer. The integrated densities of individual spots were calculated as follows: (N × mean density) − (N × background), where N was the number of pixels, mean density was the average density of the spot, and background was the average density of the area immediately surrounding the spot. The integrated densities were used to calculate the protein recovery after immunoisolation as follows: (SID − CID) ÷ (TID), where SID and CID refer to the integrated densities measured for samples immunoisolated with the specific and control antibody,

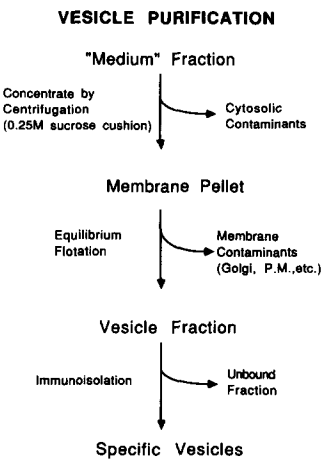

Figure 1. Schematic of vesicle purification.

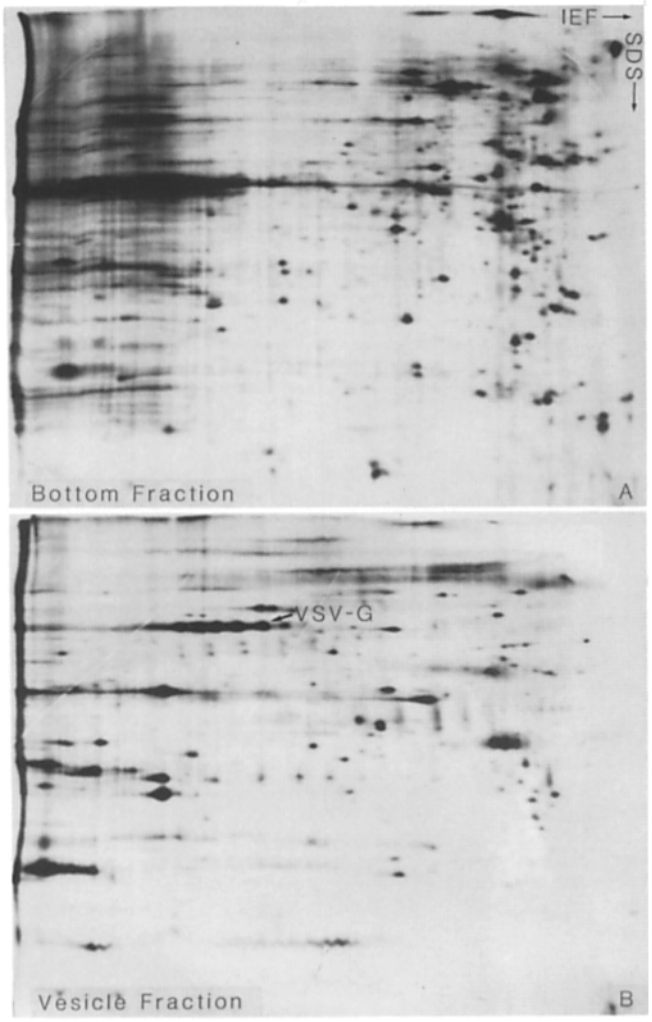

Figure 2. Gradient-purified vesicles have a characteristic protein composition. MDCK cells were metabolically labeled with [^{35}S]-methionine, infected with VSV, and perforated. A membrane pellet was prepared from the incubation medium and resolved on an equilibrium flotation step gradient. Aliquots from (*A*) the bottom-most fraction (representing material which did not enter the gradient) and from (*B*) the peak vesicle fraction were analyzed on two-dimensional gels.

respectively, and TID was the integrated density of the starting material sample. The recoveries were normalized to the recovery of the respective viral protein, VSV-G protein, or influenza HA and used to calculate the apical to basolateral ratios shown in Table II.

Triton X-114 Extraction and Phase Separation

Triton X-114 extraction and phase separation were performed by a modification of the procedure described by Bordier (1981). The vesicles released from perforated cells were purified on equilibrium step flotation gradients as described above. The peak fractions were pooled and the vesicles were pelleted by centrifugation in a TL-100 (Beckman Instruments, Inc.) as described under Immunoisolation of Vesicles. The vesicle pellet (obtained from the cells on a single 100-mm filter) was resuspended in 50 μl 1% Triton X-114 (wt/vol) in PBS and transferred to an Eppendorf tube. The sample was incubated on ice for 15 min, transferred to 37°C for 5 min to promote phase separation, and then subjected to centrifugation for 1 min in an Eppendorf microfuge. The aqueous phase was carefully transferred to a new tube with a drawn out Pasteur pipette and the proteins were precipitated with −20°C acetone and 20 μg BSA added as carrier protein. The precipitate was collected by centrifugation and dissolved in 50 μl two-dimensional gel sample buffer. The detergent phase was washed with 50 μl cold PBS, the phases were separated as above, and the aqueous phase was discarded. The detergent phase was dissolved in 50 μl two-dimensional gel sample buffer.

EM

For the morphological characterization of the gradient-purified vesicle fraction, samples were embedded in Epon as follows. A gradient-purified vesicle fraction was fixed in suspension with 2% glutaraldehyde in 120 mM cacodylate buffer, pH 7.4, for 30 min at room temperature. The fixed vesicles were collected by centrifugation in an airfuge (Beckman Instruments, Inc.) for eight min at 10 psi (60,000 *g*). The pellet was postfixed for 30 min in a solution of 1.5% osmium tetroxide in cacodylate buffer. After several washes with 50 mM veronal-acetate buffer at pH 7.5, the pellet was stained for 40 min in the dark using a solution of 1% magnesium uranyl acetate in 50 mM veronal-acetate buffer, pH 5.2. The sample was dehydrated and embedded in epon. Ultrathin sections were stained and observed on a Philips EM301 at 80 kV. Serial sections were analyzed and confirmed that the structures shown in Fig. 3 *a* were vesicles and not cross sections of tubules.

Immunocytochemistry was performed on ultrathin frozen sections to identify specific transport vesicles in the gradient-purified vesicle fraction. The sucrose gradient fraction containing the vesicles was diluted to 0.25 M sucrose and the membranes were collected by centrifugation for 3 h at 200,000 g_{av} and 4°C in a TL-100 ultracentrifuge (Beckman Instruments, Inc.) using a TLS-55 rotor. The pellets were fixed with 4% paraformaldehyde in 200 mM Hepes buffer for 5 min followed by fixation with 8% paraformaldehyde in the same buffer for 1 h. The pellets were infiltrated with 2.1 M sucrose in PBS for 20 min and mounted on copper stubs for sectioning at −110°C using a glass knife and an ultramicrotome (Model OMW 4; Reichert, Vienna, Austria) with a cryoattachment.

Immunolabeling was performed as described previously (Griffiths et al., 1984). The antibody used to label VSV-G protein containing vesicles was a mouse mAb directed against the cytoplasmic domain of VSV-G protein (Kreis, 1986). The antibody used to label HA containing vesicles was a mouse mAb specific for the cytoplasmic domain of fowl plague virus HA (data not shown). For immunolabeling hybridoma culture supernatants were diluted 1:1 with 10% FCS. The bound antibodies were visualized with a linker rabbit antimouse antibody followed by protein-A conjugated to 9 nm gold. 25 vesicles of each were analyzed for the diameter measurements.

Results

Isolation of Transport Vesicles from Perforated Cells

MDCK cells grown on permeable filter supports were mechanically perforated by overlaying the monolayer with a nitrocellulose filter, allowing a short drying time and then gently peeling the filters apart (Bennett et al., 1988, 1989). The perforated cells were incubated at 37°C in the presence of an ATP regenerating system. After 1 h the incubation medium was collected and used to purify the transport vesicles for further study. A summary of the purification procedure is shown in Fig. 1.

In the first step, transport vesicles and any other membranes released during the incubation were concentrated by centrifugation through a 0.25 M sucrose cushion. This step also served to remove most of the cytosolic proteins which were released from the perforated cells and constituted the bulk of the protein present in the incubation medium. Next, the membrane pellet was carefully resuspended in a dense sucrose solution, overlaid with a discontinuous 0.8:1.2 M sucrose gradient and subjected to equilibrium flotation. It was our experience that flotation of the transport vesicles to their equilibrium density removed residual cytosolic components and membrane contaminants more effectively than sedimentation. The purification achieved by this step is most clearly

demonstrated by comparing the two-dimensional protein pattern of the peak vesicle fraction (recovered at the 0.8:1.2 M sucrose interface) with that of the bottom most fraction (representing material which did not enter the gradient). The samples shown in Fig. 2 were derived from cells metabolically labeled with [^{35}S]methionine and infected with VSV. Essentially all of the VSV-G protein, which served as our transport marker, was recovered in the peak vesicle fractions, while the cytosolic viral nucleocapsid proteins N and M remained largely in the bottom fraction (see Fig. 4, lanes *11* and *P*). In addition, the overall protein composition of the vesicle fraction was highly simplified relative to the complex protein pattern comprising the bottom fraction. Interestingly, many of the proteins in the vesicle fraction appeared to be sialoglycoproteins as judged by their heterogeneous charge distribution in the isoelectric focusing dimension (see also Fig. 7, *A* and *D*). Such proteins constitute only a very minor fraction of the total cellular protein and are generally not seen when whole MDCK cell lysates are resolved on two-dimensional gels (Kondor-Koch et al., 1985).

Analysis of the interface fraction by EM showed it to contain primarily vesicles with an average diameter of 100 nm (Fig. 3 *a*). The exocytic vesicles were identified by immunogold labeling vesicle fractions isolated from infected cells. Antibodies directed against the cytoplasmic domains of VSV-G protein or influenza HA together with protein-A gold were used to visualize the vesicles containing these transport markers on cryosections. A large number of cryosections prepared from independent samples were analyzed. The gold particles were consistently found clustered on membranes with the expected morphology of carrier vesicles (representative pictures are shown in Fig. 3, *b* and *c*). No such labeling was observed when the first antibody was omitted (not shown). The average diameter of VSV-G protein containing vesicles determined from such cryosections, was found to be 84 (±11) nm. The average diameter of influenza HA vesicles was 78 (±15) nm, not significantly different from that of the VSV-G protein containing vesicles. Thus, in only two steps, we were able to isolate a crude vesicle fraction from perforated MDCK cells. This fraction contained TGN-derived exocytic transport vesicles and had a distinctive protein composition.

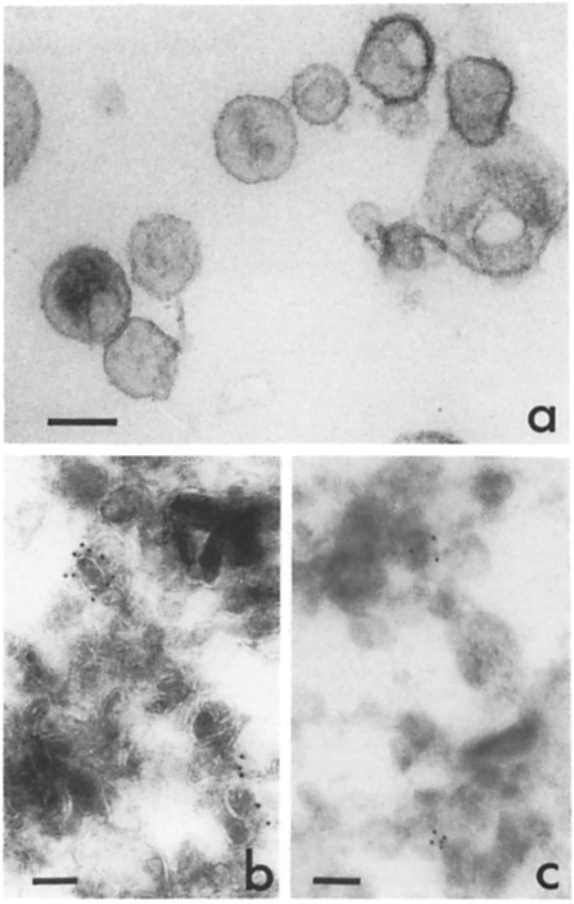

Figure 3. Morphology and immunogold labeling of gradient-purified transport vesicles. *a* shows an ultrathin section of an epon-embedded vesicle fraction. Vesicle fractions isolated from virally infected cells and purified on discontinuous equilibrium flotation gradients were concentrated by centrifugation. Immunocytochemistry was used to label cryosections of the membrane pellets. The vesicle membranes containing the viral transport markers were labeled with antibodies directed against the cytoplasmic domains of the viral proteins and visualized with protein-A conjugated to 9 nm gold. *b* shows VSV-G protein containing vesicles and *c* shows influenza HA containing vesicles.

Resolution of Apical and Basolateral Vesicles by Equilibrium Flotation

Previous work using MDCK cells had shown that newly synthesized apical and basolateral proteins were delivered directly to the appropriate plasma membrane domain after exit from the TGN (Matlin and Simons, 1984; Misek et al., 1984; Rindler et al., 1985; Pfeiffer et al., 1985; Caplan et al., 1986). This predicted the existence of two vesicular carriers with the necessary information to target proteins to the respective domain. Therefore, it was of interest to specifically isolate putative apical or basolateral vesicles from the crude vesicle fraction for comparison. The spike glycoproteins of enveloped RNA viruses were used as transport markers to tag specific vesicle fractions. Influenza HA served as the apical transport marker and VSV-G protein as the basolateral marker.

Previously, we failed to detect any differences in the densities of HA and VSV-G protein containing vesicles (Bennett et al., 1988). However, we have repeated the experiment using a mixed vesicle fraction. This was prepared by pooling the vesicles isolated from cells separately infected with VSV or with influenza virus. Here we have used fowl plague influenza virus, because the HA is cleaved during exit from the TGN into the disulphide-linked HA1 and HA2 subunits (Matlin and Simons, 1983; Bennett et al., 1988; Wandinger-Ness and Simons, unpublished observations). This has the advantage that HA cleavage can be used as a marker to distinguish the protein remaining in the Golgi (uncleaved) from that present in transport vesicles (cleaved). After perforation, the membrane fractions released from infected cells were pooled, concentrated, and overlaid with a linear 0.3–1.5 M sucrose gradient. Allowing the vesicles to reach their equilibrium density by flotation rather than by sedimentation allowed us to detect a slight but reproducible difference in the densities of apical and basolateral vesicles, marked by the HA2 and VSV-G proteins, respectively (Fig. 4, *A* and *B*). The peak of HA2 (fraction *5*) corresponded to a density of 1.099 g/ml and that of VSV-G protein (fraction *6*) to a density

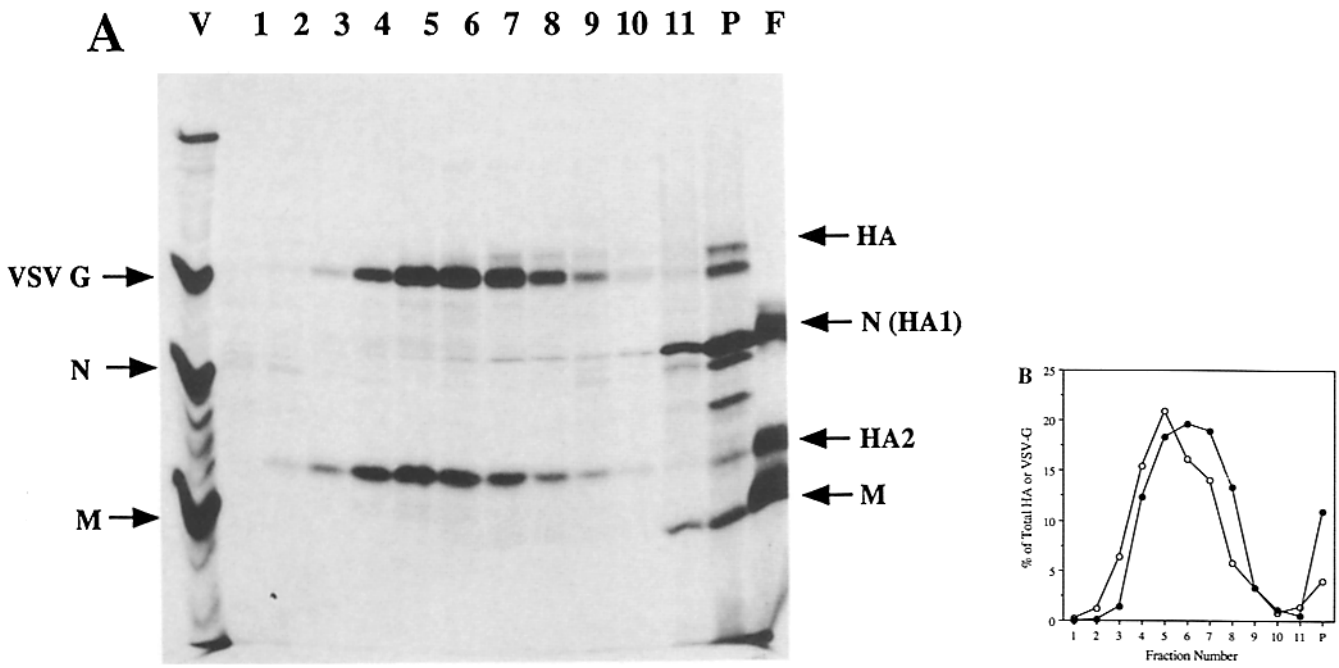

Figure 4. VSV-G protein and HA containing vesicles have different equilibrium densities. The membrane pellets isolated from pulse-labeled VSV or fowl plague virus–infected cells were resuspended together and overlaid with a 0.3–1.5 M linear sucrose gradient. After centrifugation to equilibrium 1-ml fractions were collected and the viral proteins immunoprecipitated from each fraction as well as from the resuspended pellet. The immunoprecipitates were resolved on a 10% SDS-polyacrylamide gel in the presence of 8 M urea (Bennett et al., 1988) and the corresponding autoradiogram is shown in *A*. The lane numbers correspond to the fraction numbers, where one is the top of the gradient and *P* represents the pellet fraction. The lanes marked *F* and *V* correspond to ^{35}S-labeled fowl plague virus and VSV viral standards, respectively. The viral proteins are indicated. HA1 migrates as a diffuse band and is poorly resolved from N protein. Therefore, HA2 is used to follow the cleaved form of HA. The autoradiogram shown in *A* was quantitated by densitometric scanning. *B* shows the amounts of HA2 (*open circles*) and VSV-G protein (*filled circles*) in each fraction expressed as a percentage of the total protein recovered on the gradient. 90% of the total HA was present in the cleaved form.

of 1.113 g/ml. As measured by densitometric scanning, 90% of the total HA recovered on the gradient was of the cleaved form and was primarily distributed in fractions *4–7* with very little HA2 seen at the bottom of the gradient (Fig. 4 *B*). The small amount of uncleaved HA remained largely in the pellet.

These results provided the first evidence that apical and basolateral membrane proteins might be transported in distinct carrier vesicles. However, since their densities were too similar to allow purification on this basis, we took advantage of the availability of mAbs directed against the cytoplasmic domains of VSV-G protein and influenza HA to selectively immunoisolate specific vesicle fractions (Kreis, 1986; Hughson et al., 1988). An mAb directed against the cytoplasmic domain of HA derived from influenza strain PR8 has been well characterized (Hughson et al., 1988). This antibody also recognizes the HA of WSN ts61, a temperature-sensitive variant with a cytoplasmic domain identical to that of PR8 (Ueda and Kilbourne, 1976; Winter et al., 1981; Nakajima et al., 1986). Therefore, these viruses were used in the following experiments.

Purification of Transport Vesicles by Immunoisolation

The strategy we used to immunoisolate specific vesicle fractions is shown in Fig. 5 (see also Materials and Methods). Briefly, a vesicle fraction, purified on a discontinuous flotation gradient, was first incubated with the specific or control antibody in solution. Subsequently, cellulose fibers with covalently bound sheep antimouse Fc antibodies were added as the solid support. After binding, the fibers were recovered by centrifugation and the bound material was solubilized in sample buffer for analysis by gel electrophoresis. In each immunoisolation experiment, the vesicle fraction was divided into three equal samples. One sample was incubated with the specific antibody. A second sample was incubated with an

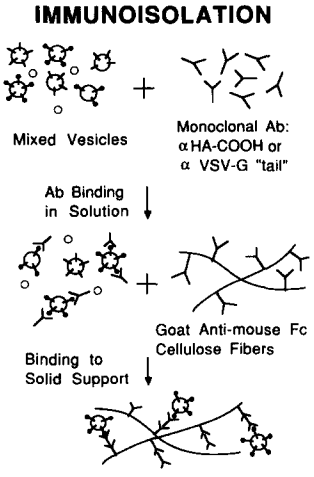

Figure 5. Schematic of vesicle immunoisolation.

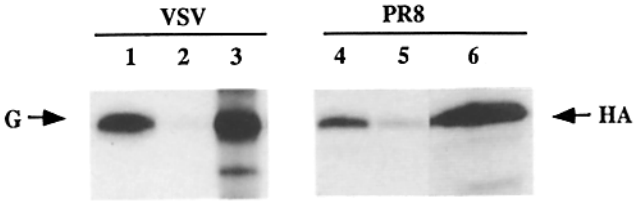

Figure 6. VSV-G protein and HA containing vesicles are recovered by immunoisolation with antitail antibodies. A gradient-purified vesicle fraction was prepared from pulse-labeled cells infected with VSV or influenza PR8 HA. Vesicles containing VSV-G protein (lanes *1–3*) or HA (lanes *4–6*) were immunoisolated with antibodies directed against the cytoplasmic domains of the respective viral proteins. Samples containing VSV-G protein were incubated with the primary antibodies for 3 h at 4°C and those containing HA were incubated overnight at 4°C. (lane *1*) VSV-G protein recovered on cellulose fibers using the anti-VSV-G protein tail antibody. (lane *2*) Background level of VSV-G protein recovered in cellulose fibers using the antibody specific for PR8 HA as a control. (lane *3*) Total VSV-G protein present in the starting material used for immunoisolation. (lane *4*) PR8 HA protein recovered on cellulose fibers using the anti-HA tail antibody. (lane *5*) Background level of HA recovered on fibers using the antibody specific for VSV-G protein as a control. (lane *6*) Total HA present in the starting material used for immunoisolation.

irrelevant antibody as a control for nonspecific binding. A third sample was left untreated and served as the starting material reference.

Using this procedure, 78 (±24)% of the total VSV-G protein and 40 (±3)% of the total PR8 HA were specifically recovered on the cellulose fibers under optimal conditions (Fig. 6 and Table I). The amount of nonspecific binding to the fibers was ~10% and was similar for both HA and VSV-G protein containing vesicles. Comparing several different solid supports for immunoisolation, cellulose fibers were found to give good yields with the lowest nonspecific binding. The lower recovery of PR8 HA as compared to VSV-G protein may be due to differences in antibody affinity. We have varied both the temperature and time of incubation with the specific antibody in order to improve the recovery of HA containing vesicles. Overnight incubation at 4°C gave the best results (Table I). Reduced antibody accessibility due to the relatively small cytoplasmic domain of HA (only 10 amino acids) may also account for the lower recovery. For this reason, vesicles were also isolated from cells infected with WSN ts61. In this strain, transport of the HA from the ER to the Golgi is temperature sensitive and HA can be reversibly accumulated in the ER at nonpermissive temperatures (Ueda and Sugiura, 1984; Rodriguez-Boulan et al., 1984; Rindler et al., 1985). Upon shifting the cells to permissive temperatures the HA is transported synchronously to the Golgi and its exit from the TGN is blocked by incubating at 20°C (Rodriguez-Boulan et al., 1984; Hughson et al., 1988). The increased antigen concentration in the TGN and in the budding transport vesicles may account for the higher recoveries (61 ± 16%) of HA vesicles obtained with the temperature-sensitive influenza strain (Table I).

Protein Composition of Apical and Basolateral Vesicle Fractions

One expectation if apical and basolateral membrane proteins

Table I. Viral Proteins Are Efficiently Recovered by Immunoisolation

	% Recovery (3 h binding)*		% Recovery (overnight binding)	
Viral protein	Specific	Control	Specific	Control
VSV-G (n = 5)‡	78 ± 24	9.7 ± 9.6	ND	ND
PR8 HA (n = 2)	13 ± 2.2	6.8 ± 2.8	40 ± 3.0	17 ± 5.2
WSN HA (n = 3)	ND	ND	61 ± 16	9.3 ± 4.2

* Both densitometric scanning of autoradiographs (Materials and Methods) and liquid scintillation counting (Bennett et al., 1988) were used to quantitate the proteins on gels. Percent recoveries in specific and control samples are relative to the amount of protein in the starting material sample.
‡ n represents the number of data sets.

are indeed contained in separate vesicles is that the protein composition of the vesicles should be distinct. Therefore, we analyzed the protein patterns of the immunoisolated vesicle fractions on two-dimensional gels. For this purpose, MDCK cells were metabolically labeled overnight with [^{35}S]methionine. The cells were then infected with WSN ts61 or VSV and labeling was continued during infection. The cells were perforated, and transport vesicle fractions were isolated by differential centrifugation and immunoisolation.

The typical patterns obtained for VSV-G protein and HA (WSN ts61) containing vesicles are shown in Fig. 7. The top panels (*A* and *D*) show the protein patterns of the crude vesicle fraction used for immunoisolation. The middle panels (*B* and *E*) show the proteins specifically immunoisolated together with the viral proteins. The bottom panels (*C* and *F*) show the protein patterns resulting from nonspecific binding to the cellulose fibers. The viral proteins are indicated for reference in the top panels. VSV-G protein and a soluble form, G_s (Garreis-Wabnitz and Kruppa, 1984; Graeve et al., 1986), are sialylated heterogeneously (Fuller et al., 1985) and hence are seen as multiple spots (Fig. 7 *A*). The charge heterogeneity observed for WSN HA (Fig. 7 *D*) can not be attributed to differential sialylation due to the action of the viral neuraminidase (see below) and is most likely due to sulfation (Pinter and Compans, 1975). Its migration as a doublet was indicative of the fact that WSN ts61 HA was not uniformly glycosylated and some remained endoglycosidase H sensitive even though both forms were detected at the cell surface (data not shown). The ratio of the two forms varied in cells infected with different virus stocks, but had no apparent effect on the immunoisolation results. Heterogeneous glycosylation of WSN ts61 HA has been observed previously (Palese, 1977; Rodriguez-Boulan et al., 1984) and may be attributed to an alteration of the tertiary protein structure by the mutation causing the temperature-sensitive phenotype (Nakajima et al., 1986).

The protein patterns of the crude vesicle fractions obtained from VSV as compared to influenza-infected cells were very similar (Fig. 7, *A* and *D*). The most obvious difference was the apparent shift in the migration of several glycoproteins toward more basic regions of the isoelectric focusing gel when the samples were isolated from influenza-infected cells (proteins indicated with arrowheads). This was most likely caused by the action of the viral neuraminidase

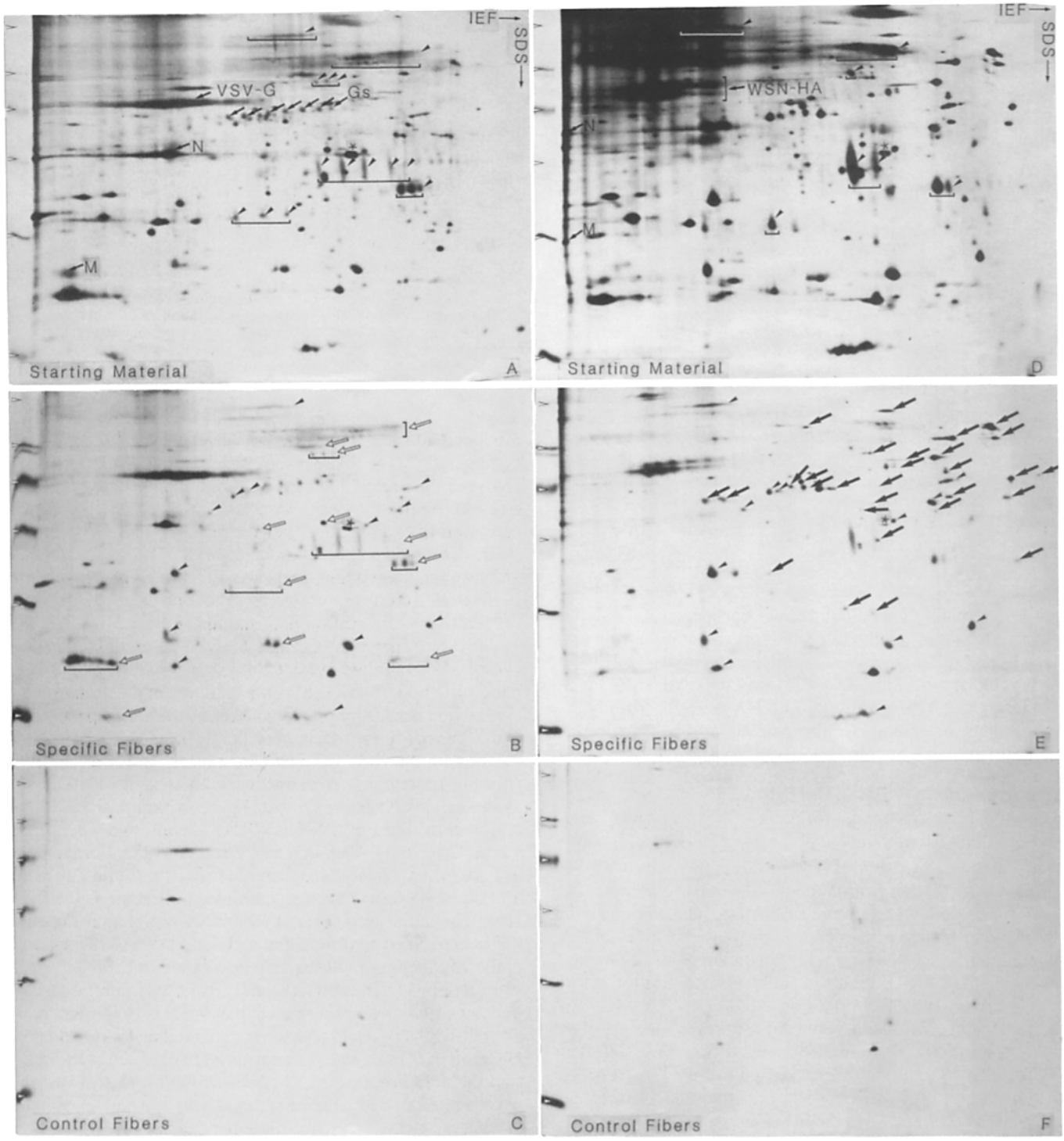

Figure 7. Protein composition of immunoisolated apical and basolateral vesicle fractions. A gradient-purified vesicle fraction was isolated from VSV (*A–C*) or WSN ts61-infected cells (*D–F*) and divided into three equal aliquots. The vesicles in one aliquot were concentrated by centrifugation and served as the starting material reference (*A* and *D*). A second aliquot was incubated with the antibody directed against the cytoplasmic domain of VSV-G protein. This antibody was used to specifically immunoisolate VSV-G protein containing vesicles (*B*) or as a control for nonspecific binding of WSN HA containing vesicles (*F*). A third aliquot was incubated with the antibody directed against the cytoplasmic domain of WSN HA. This antibody was used to specifically immunoisolate WSN HA containing vesicles (*E*) or as a control for nonspecific binding of VSV-G protein containing vesicles (*C*). In *A* and *D*, small arrows designate the viral proteins, while arrowheads indicate sialoglycoproteins whose mobility is altered in the presence of influenza neuraminidase. In *B* and *E*, large arrows indicate proteins associated preferentially with VSV-G protein (*open arrows*) or HA containing vesicles (*solid arrows*), respectively. Arrowheads indicate proteins associated with both VSV-G protein and HA containing vesicles. The directions of IEF and SDS electrophoresis are as indicated. An asterisk marks the position of actin. The position of ^{14}C-labeled molecular weight markers included in the second dimension are indicated by (>) on the left hand side of all the gels. The markers from top to bottom are: myosin (200 kD), phosphorylase b (92.5 kD), BSA (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), and lysozyme (14.3 kD).

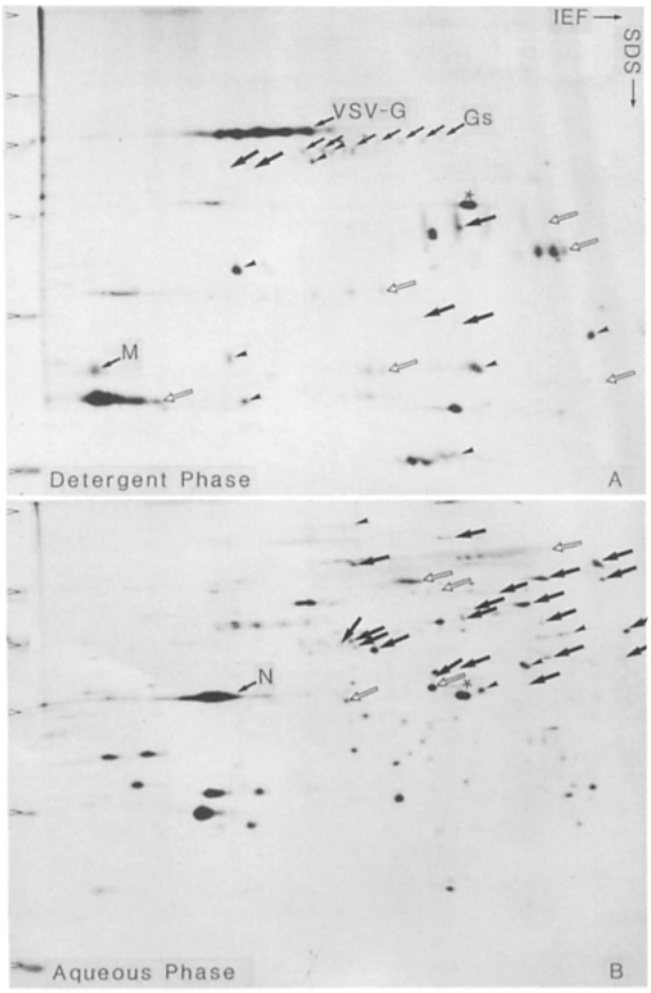

Figure 8. Membrane and peripheral vesicle proteins identified by Triton X-114 phase separation. Two 100-mm filter cultures of MDCK cells were metabolically labeled and infected with VSV. The cells were perforated and a gradient-purified vesicle fraction was prepared. One-half of the vesicle fraction was subjected to Triton X-114 extraction and phase separation. The proteins in the resulting detergent (*A*) and aqueous (*B*) phases were resolved on two-dimensional gels. The viral proteins are labeled and indicated by small arrows. Arrowheads designate proteins present in both apical and basolateral vesicles, solid arrows mark apical specific proteins, and open arrows indicate basolateral specific proteins. Note: several proteins shown in Figs. 7 and 9 were only seen on a longer exposure, including: A15 (aqueous), A17 (aqueous), A24 (in both phases), A25 (detergent), B13 (detergent), and C7 (aqueous). An asterisk marks the position of actin and the directions of IEF and SDS electrophoresis are as indicated. The molecular weight markers designated by (>) are as in Fig. 7.

Table II. Quantitation of Vesicle Proteins Identified on Two-dimensional Gels

Protein	Apical/Basolateral ratio*	Mol wt‡	Localization§
A3	14.3	108,000	peripheral
A4	6.0	92,000	peripheral
A5	10.8	91,000	peripheral
A14	15.3	65,000	peripheral
A16	10.1	69,000	peripheral
A23	38.0	46,000	membrane
A26	20.0	29,000	membrane
B4	0.48	54,000	peripheral
B5	0.11	52,000	peripheral
B8	0.42	35,000	membrane
B9	0.42	24,000	membrane
B12	0.14	23,000	membrane
C3	1.9	68,000	peripheral
C4	1.3	66,000	membrane
C8	2.2	54,000	peripheral
C9	1.2	39,000	membrane
C10	2.6	27,000	membrane
C11	3.1	24,000	membrane
C13	2.4	20,000	membrane

* Densitometric scanning was used to quantitate two data sets obtained with each virus and recoveries were calculated as described in Materials and Methods. The apical to basolateral ratio was calculated as: (Recovery relative to WSN) ÷ (Recovery relative to VSV).
‡ Average value determined from three separate gels.
§ Based on Triton X-114 phase partitioning.

on sialoglycoproteins. The viral neuraminidase is synthesized and transported together with HA to the TGN where it can catalyze the selective removal of sialic acid residues. Since such sialoglycoproteins are usually seen as multiple spots representing different degrees of sialylation the sequential removal of sialic acid residues by neuraminidase results in a reduction of the net negative charge on the proteins. Hence their migration in isoelectric focusing gels is altered and fewer spots are observed. This effect has been clearly documented for VSV-G protein in cells coinfected with influenza virus (Fuller et al., 1985).

Comparing the immunoisolated samples (Fig. 7, *B* and *E*) with the starting fractions, a subset of proteins which are clearly coisolated with the viral proteins can be seen. Proteins that were also not detected to any significant degree in the control samples (Fig. 7, *C* and *F*) and were, therefore, considered to be specifically isolated are labeled (*arrows* and *arrowheads*). As expected, residual cytosolic proteins, e.g., actin (marked by an *asterisk*) and the viral nucleocapsid proteins, still present in trace amounts in the vesicle fraction were not specifically isolated.

It was of primary interest to compare the protein patterns of HA and VSV-G protein containing vesicles (Fig. 7, *B* and *E*). After a careful comparison of several data sets, it became apparent that while there were many similarities there were also differences in the two patterns. A number of proteins were present in both fractions (Fig. 7, *B* and *E*, *arrowheads*). Several proteins were selectively recovered with HA containing vesicles, regardless of which influenza strain was used, PR8 or WSN ts61 (Fig. 7 *E*, *solid arrows*). Another group of proteins was specifically associated with the VSV-G vesicle fraction (Fig. 7 *B*, *open arrows*). Interestingly, some of the proteins characterisitic of apical vesicles (Fig. 7 *E*) were less prevalent in the gradient-purified vesicle fractions isolated from VSV-infected cells (Fig. 7 *A*). One possible explanation for this is that fewer apical vesicles are formed in the absence of cargo molecules.

Further Characterization of Vesicle Proteins

Additional information about the proteins present in the vesi-

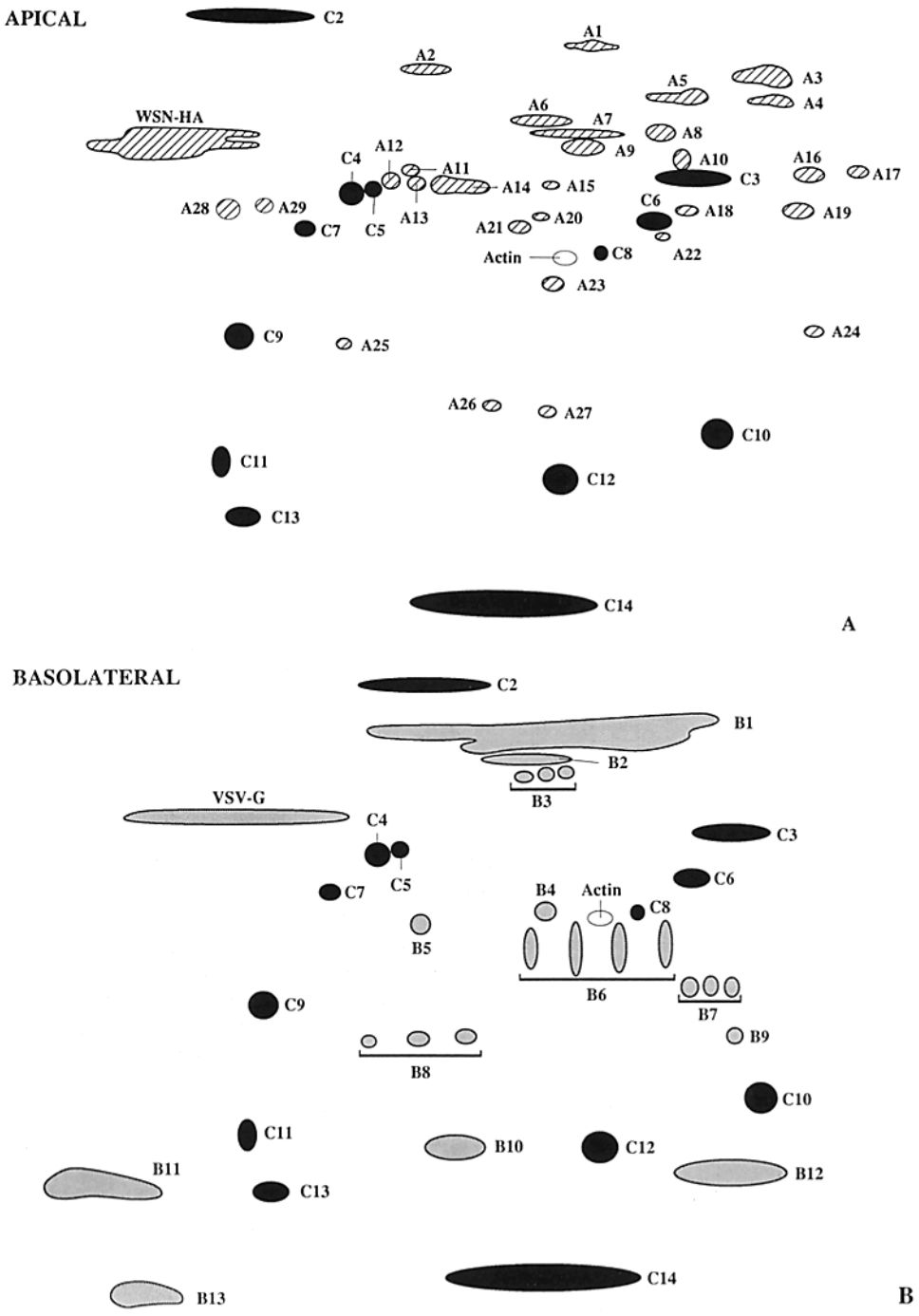

Figure 9. Schematic of apical and basolateral specific vesicle proteins. *A* shows the proteins preferentially associated with apical vesicles as shaded forms with numbers preceded by *A* (apical). *B* shows the proteins preferentially associated with basolateral vesicles as shaded forms with numbers preceded by *B* (basolateral). Filled forms represent proteins associated with both apical and basolateral vesicles and are designated by numbers prefaced with *C* (common).

cle fraction was obtained from Triton X-114 phase partitioning. This method has been used extensively to distinguish membrane and peripheral proteins (Bordier, 1981). Membrane proteins, with some exceptions, partition into the detergent phase, whereas peripheral proteins are typically recovered in the aqueous phase. For this experiment, cells were metabolically labeled with [^{35}S]methionine and infected with VSV. A crude vesicle fraction was prepared and divided into two equal parts. One sample was left untreated as a starting material reference. The second sample was subjected to Triton X-114 phase partitioning and separated into an aqueous and a detergent phase. All three samples were analyzed by two-dimensional gel electrophoresis.

The recovery of all the proteins after phase separation was close to 100% and only the samples representing the detergent (Fig. 8 *A*) and aqueous (Fig. 8 *B*) phases are shown. In general, the proteins partitioned into one of the two phases. One notable exception was actin which was distributed equally in both phases (marked by an *asterisk* in Fig. 8, *A* and *B*). Since actin is a soluble cytosolic protein, its presence in the detergent phase may be best explained by some association with membrane proteins. The fact that actin was not significantly recovered in the immunoisolated samples (Fig. 7, *B* and *E*) suggests that it is not associated with the transport vesicle membranes.

The partitioning of proteins into the aqueous or detergent

phases was used to tentatively classify their localization as peripheral or membrane, respectively (Table II). As expected the transmembrane VSV-G protein was recovered exclusively in the detergent phase. Although VSV G_s lacks the transmembrane domain it was also recovered in the detergent phase (Fig. 8 *A*). This is explained by the fact that it forms trimers with the transmembrane form of VSV-G protein (Garreis-Wabnitz and Kruppa, 1984). Based on the results of our immunoisolation experiments, the vesicle proteins were divided into three groups. One group of proteins comprised those present in both apical and basolateral vesicles (Fig. 8, *arrowheads*). A second group consisted of those found primarily in apical vesicles (Fig. 8, *solid arrows*) and a third group of those present mostly in basolateral vesicles (Fig. 8, *open arrows*). Both membrane and peripheral proteins could be identified among all three groups (Fig. 8 and Table II).

We have schematically illustrated the vesicle proteins comprising each of the three groups in Fig. 9. This summary is based on the visual comparison of four independent data sets obtained with each virus. *A* and *B* show the apical and basolateral proteins, respectively. The proteins have been assigned numbers according to their group. The numbers identifying apical proteins are prefaced with *A*, those identifying basolateral proteins with *B* and those representing proteins in both vesicles with *C* (for common proteins). This classification was further verified by densitometric scanning of the gels.

Two data sets obtained for each virus were quantified and the recovery of individual proteins relative to HA or VSV-G protein following immunoisolation was determined as described in Materials and Methods. An apical to basolateral ratio was calculated by dividing the recovery of a protein together with HA by its recovery with VSV-G protein. Values for representative members of each class are tabulated in Table II. Proteins considered to be apical or basolateral specific were differentially isolated with the two viral proteins. It is clear from the data in Table II that the proteins judged specific for the basolateral vesicles are not enriched to the same extent as the apical-specific proteins. This might be explained by previous findings showing that apical proteins are "missorted" in the basolateral direction while basolateral proteins are strictly excluded from the apical side (see Wandinger-Ness and Simons, 1990). Moreover, some of the proteins characteristic of apical vesicles were less prevalent in the vesicle fraction isolated from VSV-infected cells (see above). The proteins identified visually as being in both fractions were isolated to a similar extent with both viral proteins. These proteins marked C constitute interesting candidates for machinery common to both vesicle types.

Discussion

Perforated MDCK cells were used to isolate and purify transport vesicles derived from the TGN and responsible for plasma membrane protein delivery to the cell surface. Influenza HA or VSV-G protein were used as transport markers to identify the vesicles during purification. The procedures used were chosen to permit the specific isolation of TGN-derived transport vesicles. By incubation at 20°C in the presence of cycloheximide the viral proteins were accumulated in the TGN (Matlin and Simons, 1983; Griffiths et al., 1985; Hughson et al., 1988). The perforated cells were then incubated under conditions shown previously to preferentially cause the budding and release of TGN vesicles (Bennett et al., 1988). Analysis of a gradient-purified vesicle fraction by EM showed it to contain an abundance of noncoated vesicles and the average diameter of the cryosectioned vesicles containing the viral markers was ~80 nm. This is within the size range reported for other transport vesicles involved in exocytic membrane traffic (Griffiths et al., 1985; Orci et al., 1986, 1989; de Curtis and Simons, 1989). Further purification of these vesicles was achieved by immunoisolation techniques, using antibodies specific for the cytoplasmic domains of the viral proteins. The immunoisolated vesicle fractions had a relatively simple characteristic protein composition when analyzed by two-dimensional gel electrophoresis. Abundant cytosolic proteins such as tubulin (by comparison with published migration on two-dimensional gels, Kondor-Koch et al., 1985) and actin were not present in the fraction. A number of the vesicle proteins were identified as sialoglycoproteins due to the influence of viral neuraminidase activity on their mobility in isoelectric focusing gels. Carbohydrate side chains bearing sialic acid residues are characteristic of glycoproteins that have reached the *trans*-Golgi (Roth et al., 1985), providing strong evidence that the immunoisolated vesicles are derived from the TGN. Additional evidence is provided by the observation that 90% of the HA present in the vesicle fraction was cleaved in experiments done with fowl plague virus. This cleavage is a late event in transport and first occurs during or immediately after exit of the HA from the TGN (Matlin and Simons, 1983; Bennett et al., 1988; A. Wandinger-Ness and K. Simons, unpublished observations).

Transport vesicles mediating membrane traffic along the constitutive pathway have proven difficult to isolate due to their transient nature under normal conditions. Only recently has it been possible to isolate and characterize Golgi-derived vesicles in two other mammalian systems which can be compared with our results (Malhotra et al., 1989; de Curtis and Simons, 1989). Malhotra and colleagues purified a nonclathrin-coated vesicle fraction from rabbit liver Golgi after GTPS treatment. A number of vesicle-specific proteins were identified by SDS-PAGE and silver staining. Most of the proteins were thought to represent components of the vesicle coat, and only one protein of 42 kD was suggested to be derived from the Golgi membranes. The failure to detect other vesicle membrane proteins may be attributed to the lack of sensitivity afforded by silver staining. Since the vesicles isolated from perforated cells do not have any obvious coat by EM, it is unlikely that any of the proteins identified in this work correspond to such putative coat proteins. There are at least three proteins in the 42 kD molecular mass range and further work is required to determine whether any of them are the same as the 42 kD protein seen in the coated Golgi vesicles. De Curtis and Simons (1989) described the isolation of exocytic carrier vesicles derived from the TGN and BHK cells. Because the BHK vesicle proteins were analyzed by NEPHGE a direct comparison with the present results was not possible. However, when an immunoisolated vesicle fraction was prepared from BHK cells according to the published procedure and resolved on IEF gels the two-dimensional gel electrophoresis patterns had features similar to those of the MDCK vesicles (data not shown). The

methods used to isolate the TGN-derived carrier vesicles from the two cell lines are very different. Therefore, the similarity of the protein compositions is striking and taken as further confirmation that these are indeed vesicle-specific proteins with potentially important functions in membrane traffic.

In MDCK cells both apical and basolateral plasma membrane proteins follow the same biosynthetic route from the ER to the TGN where they are subsequently sorted for delivery to the appropriate membrane domains (Rindler et al., 1984; Fuller et al., 1985). Using influenza HA and VSV-G protein as model apical and basolateral proteins, respectively, we have for the first time been able to isolate two classes of exocytic vesicles which mediate the delivery to the appropriate membrane domains. The vesicles were distinguishable on the basis of two different criteria. Apical vesicles were slightly less dense than their basolateral counterparts when resolved on equilibrium density gradients. In addition, each vesicle fraction contained a set of unique protein components which were shown to be enriched 2–38-fold in the respective fraction. As expected, some of these proteins could also be detected on the plasma membrane following selective surface biotinylation (A. Brändli and K. Simons, unpublished results). In each case, the domain-specific plasma membrane localization of a protein corroborated its classification as an apical or basolateral vesicle protein. Unfortunately, it is technically very difficult to analyze the vesicle fractions immunoisolated on cellulose fibers by electron microscopy. Preliminary findings have shown that the vesicle morphology can best be observed by cryo-electron microscopy. Work is underway to develop immunolabeling techniques for analyzing the vesicles by this method.

In addition to having unique protein components, apical and basolateral vesicles were observed to share a number of common components. The exact functions of all these proteins still remains to be determined. Some of the proteins may prove to be plasma membrane proteins which are endocytosed and recycle via the TGN (Snider and Rogers, 1985; Duncan and Kornfeld, 1988; Reichner et al., 1988; Jin et al., 1989). Thus far only a very selected subset of plasma membrane proteins are known to take this route in MDCK cells (Brändli and Simons, 1989). The fact that host protein synthesis is inhibited during viral infection, while proper sorting of viral glycoproteins still continues, suggests that at least some of these proteins may play a key role in mediating vesicular membrane traffic. The shared components could be part of a molecular complex generally required either for the formation of a vesicle or for its fusion with the target membrane. Precedence for this suggestion is given by the recent finding that a single cytosolic protein, *N*-ethylmaleimide-sensitive factor (NSF) (Balch et al., 1984; Glick and Rothman, 1987; Malhotra et al., 1988; Block et al., 1988), is involved in several intracellular membrane fusion events (Diaz et al., 1989; Beckers et al., 1989; Wilson et al., 1989). NSF together with three other proteins is thought to form a "fusion machine" which is assembled when a vesicle reaches the proper target membrane (Malhotra et al., 1988; Weidman et al., 1989). None of the common vesicle proteins appeared to correspond to NSF based on a comparison of their molecular weights. This may be a consequence of the fact that NSF dissociates from membranes incubated in the presence of magnesium and ATP (Glick and Rothman, 1987). As yet, the integral membrane receptor for NSF has not been isolated so it remains to be seen whether it is among the vesicle membrane proteins we have identified. Both apical and basolateral vesicles have been shown to associate with microtubules in vitro (van der Sluijs et al., 1990). Therefore, it is also possible that some of the proteins common to both vesicle fractions function in microtubule binding.

The proteins unique to apical or basolateral vesicle fractions are good candidates for components involved in protein sorting or vesicle targeting. Thus far, the only known proteins with a potential role in exocytic vesicle targeting are the small ras-related GTP-binding proteins, Yptlp and Sec4p, identified in yeast (Gallwitz et al., 1983; Salminen and Novick, 1987; Goud et al., 1988; Segev et al., 1988; Walworth et al., 1989; Baker et al., 1990). Mutations in these two proteins have been shown to block secretion at different stages of the pathway with the concomitant accumulation of vesicles (Salminen and Novick, 1987; Segev et al., 1988). It has been proposed that this family of low molecular weight GTP-binding proteins functions to ensure the unidirectional delivery of vesicles to the correct target membrane (Bourne, 1988; Walworth et al., 1989). Several low molecular weight GTP-binding proteins have been identified in the immunoisolated vesicle fractions using GTP overlay blots (M. K. Bennett, A. Wandinger-Ness, and K. Simons, unpublished results). Probably due to their low abundance and slow turnover these GTP-binding proteins could not be correlated with any of the ^{35}S-labeled proteins. The cDNAs encoding ras-like GTP-binding proteins are currently being cloned from MDCK cells in order to identify putative apical or basolateral specific GTP-binding proteins.

Based on their partitioning after Triton X-114 phase separation the vesicle proteins have been tentatively classified as membrane or peripheral. Both types of proteins are found among the components unique to apical or basolateral vesicle fractions. The sorting of proteins into endocytic vesicles has been suggested to be mediated by a scaffold of peripheral and coat proteins which interact directly with the cargo molecules, thus, obviating the need for a specific integral membrane sorting protein (Pearse, 1985, 1988; Brodsky, 1988). Several observations make it seem unlikely that this is the case for exocytic sorting. First, soluble proteins are secreted from MDCK cells in a polarized fashion (Kondor-Koch et al., 1985; Gottlieb et al., 1986*a*; Urban et al., 1987; Caplan et al., 1987). Such polarized secretion must be mediated by a membrane receptor (for review see Wandinger-Ness and Simons, 1990). Second, the lumenal domains of membrane proteins have been shown to contain important information for proper sorting in epithelial cells (Jones et al., 1985; McQueen et al., 1986, 1987; Roman and Garoff, 1986; Kilpatrick et al., 1987; Roth et al., 1987). Taken together, these observations predict the existence of one or more integral membrane sorting receptors. Having identified several membrane proteins unique to apical or basolateral vesicles, it will be of interest to test whether these proteins have a direct role in protein sorting.

It is now possible to study the individual protein components of purified TGN-derived transport vesicles further either by molecular cloning or with antibody probes. Identifying their functions in vesicular membrane traffic will be facilitated by available in vitro assays for microtubule binding (van der Sluijs et al., 1990), vesicle budding (Bennett et

al., 1988), as well as for vesicle fusion (Woodman and Edwardson, 1986). Ultimately, this will lead to an understanding of the mechanisms underlying protein sorting and vesicle targeting.

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