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Release of putative exocytic transport vesicles from perforated MDCK cells

Mark K.Bennett, Angela Wandinger-Ness and Kai Simons

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, FRG

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Mechanically perforated MDCK cells were used to study membrane transport between the trans-Golgi network and the apical and basolateral plasma membrane domains *in vitro*. Three membrane transport markers—an apical protein (fowl plague virus haemagglutinin), a basolateral protein (vesicular stomatitis virus G protein), and a lipid marker destined for both domains (C6-NBD-sphingomyelin)—were each accumulated in the trans-Golgi by a 20°C block of transport and their behaviour monitored following cell perforation and incubation at 37°C. In the presence of ATP and in the absence of calcium ions a considerable fraction of the transport markers were released from the perforated cells in sealed membrane vesicles. Control experiments showed that the vesicles were not generated by non-specific vesiculation of the Golgi complex or the plasma membrane. The vesicles had well defined sedimentation properties and the orientation expected of transport vesicles derived from the trans-Golgi network.

Key words: influenza virus/intracellular transport/perforated cells/transport vesicles

Introduction

The polarized epithelial Madin–Darby canine kidney (MDCK) cell line has proven an excellent experimental model for studying the development and maintenance of cell surface polarity in simple epithelia (Rodriguez-Boulan, 1983; Simons and Fuller, 1985). MDCK cells grown in culture form an epithelial monolayer with intercellular tight junctions dividing the plasma membrane into apical and basolateral domains, each with a distinct protein and lipid composition (Richardson and Simmons, 1979; Louvard, 1980; van Meer and Simons, 1982). Studies on the development of cell surface polarity in MDCK cells have been greatly facilitated by the use of exogenous membrane markers, including the spike glycoproteins of enveloped viruses as well as fluorescent lipid probes (Rodriguez-Boulan and Pendergast, 1980; Roth *et al.*, 1983; Matlin and Simons, 1984; Rindler *et al.*, 1984; Pfeiffer *et al.*, 1985; van Meer *et al.*, 1987). Present evidence suggests that the components of the two plasma membrane domains follow a common intracellular transport pathway from their site of synthesis in the endoplasmic reticulum (ER) through the sequential compartments of the Golgi complex (Sabatini *et al.*, 1983; Simons and Fuller, 1985). Within the trans-most Golgi compartment, the trans-Golgi network (TGN), the two pathways are thought to diverge with the sorting of apical from basolateral membrane components followed by vesicular delivery directly to the appropriate plasma membrane domain (Griffiths and Simons, 1986; Hughson *et al.*, 1988).

Understanding the molecular machinery involved in the sorting and transport of plasma membrane components in MDCK cells requires the development of a system to study the process *in vitro*. *In vitro* reconstitution has been successful in the analysis of other steps in constitutive membrane transport. Cell-free systems have been used for the reconstitution of membrane transport between successive compartments of the Golgi complex (Balch *et al.*, 1984a; Rothman, 1987), from the ER to the Golgi (Balch *et al.*, 1987), within the endocytic pathway (Davey *et al.*, 1985; Gruenberg and Howell, 1986; Braell, 1987), and for the constitutive exocytic pathway (Woodman and Edwardson, 1986). In addition, transport from the ER to the Golgi complex has recently been reconstituted using cells permeabilized by mechanical perforation (Beckers *et al.*, 1987; Simons and Virta, 1987). In each of these cases, the reconstitution of transport was detected by the fusion of transport vesicles derived from a donor compartment with the membrane of an acceptor compartment. However, these *in vitro* systems do not readily allow discrimination between the factors required for vesicle formation and those required for fusion. Furthermore, the vesicular intermediates assumed to be involved in all of these transport steps have not yet been isolated.

We have developed a system in which the exit of a marker protein from the TGN and its delivery to the plasma membrane can be followed independently. For this purpose, MDCK cells were infected with fowl plague virus (FPV) and the proteolytic cleavage of haemagglutinin (HA), an apical protein, used to monitor transport from the TGN. In addition, the accessibility of antibodies directed against the exoplasmic domain of HA was used to measure plasma membrane appearance. The cleavage of FPV HA into the disulphide-linked HA1 and HA2 subunits is catalysed by an enzymatic activity similar to that described for the processing of prohormones (Garten *et al.*, 1981, 1982; Docherty and Steiner, 1982) and occurs late in transport. FPV HA has been shown to be predominantly uncleaved when accumulated in the TGN by a 20°C block of membrane transport (Matlin and Simons, 1983). Upon warming the cells to 37°C, cleavage of HA along with appearance of the cleaved HA on the apical cell surface occurred rapidly. Although the precise cellular location where processing occurs is not completely clear, it appears to be initiated either during transport vesicle formation or during vesicle transport (A.Wandinger-Ness and K.Simons, unpublished observation).

In this paper the cleavage of HA in mechanically perforated MDCK cells was used to establish conditions for

the *in vitro* reconstitution of transport from the TGN. The conditions which supported HA exit from the TGN (as measured by HA cleavage) did not support the delivery of HA to the apical plasma membrane. Instead, the putative transport vesicles derived from the TGN were released from the perforated cells in an ATP stimulated fashion. Two additional markers—the G protein of vesicular stomatitis virus (VSV), a basolateral protein, and *N*-6[7-nitro-2,1,3-benzoxadiazol-4-yl]aminocaproyl sphingosine (C6-NBD-ceramide), a fluorescent lipid marker whose metabolites are destined for both the apical and basolateral domains—were used to monitor transport from the TGN in the perforated cells. Like HA, both of these markers were released from the perforated cells in an ATP stimulated manner. We present evidence that the released markers are contained in transport vesicles.

Results

Cleavage of HA

The late proteolytic processing of FPV HA into the disulphide-linked products HA1 and HA2 was used as an assay to measure the *in vitro* reconstitution of transport from the TGN. MDCK cells, grown on polycarbonate filters, were infected with FPV for 3.5 h, pulse-labelled with [^{35}S]-methionine for 4 min, and the labelled HA accumulated in the TGN by incubation for 5 min at 37°C and for an additional 90 min at 20°C. The cells were then perforated using a modification of the procedure originally developed for MDCK cells grown on glass coverslips (see Materials and methods) and incubated at 37°C for 60 min under various conditions. Following the incubation, the HA was immunoprecipitated from cell lysates with an anti-FPV HA antibody, the immunoprecipitates were analysed on polyacrylamide gels (PAGE), and the amount of HA cleavage was quantitated. The HA2 cleavage product was used to quantitate the amount of cleavage in this and later experiments since it migrated as a well resolved band on PAGE whereas HA1 migrated as a broad band and was poorly resolved from the FPV nucleocapsid protein (Matlin and Simons, 1983). The results are expressed in terms of cleavage efficiency which is defined as the increase in cleavage that occurs in perforated cells divided by the increase in cleavage that occurs in intact control cells. Figure 1 illustrates the results obtained with two different incubation buffers which differed in their anion composition. One buffer was prepared with potassium acetate (KOAc) and the other with a mixture of the potassium salts of gluconate, glutamate and aspartate (GGA). Both buffers supported an ATP dependent increase in HA cleavage which was independent of calcium ions. The amount of HA cleavage observed in the absence of ATP was not significantly greater than the amount of cleavage at the start of the incubation as judged by a two-sided *t* test. Continued transport of HA in a small fraction of intact cells (<5% under the conditions used) may account for the slight increase in HA cleavage detected in the absence of ATP since the cleavage in intact cells was not inhibited by the ATP depleting system added to the incubation buffer. Buffers that have been used in other *in vitro* transport assays (Balch *et al.*, 1984a, 1987), having KCl as the primary salt, did not support an increase in cleavage (not shown). Because the anion composition of the GGA buffer is more physiological, it was used for the

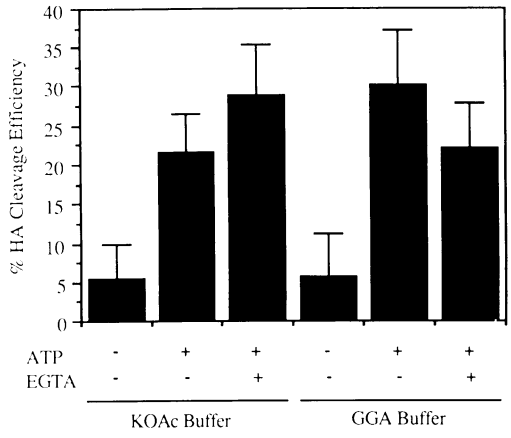

Fig. 1. HA cleavage efficiency. Filter cultures were infected with FPV, pulse-labelled with [^{35}S]methionine for 4 min, and the labelling stopped by the addition of medium containing excess unlabelled methionine as described in Materials and methods. Following a 90 min incubation at 20°C the cells were perforated and incubated under different buffer conditions at 37°C for 60 min. The amount of HA cleavage which occurred in intact control cells or perforated cells was quantitated. The amounts of HA cleavage (mean ± standard error of the mean from at least eight experiments) were as follows: no incubation, 37.9 ± 2.0%; intact cells incubated at 37°C for 60 min, 90.5 ± 0.7%; perforated cells incubated at 37°C for 60 min in KOAc buffer−ATP, 40.8 ± 1.2%; KOAc buffer+ATP, 49.2 ± 1.7%; KOAc buffer+ATP+EGTA, 53.1 ± 2.6%; GGA buffer−ATP, 40.9 ± 2.0%; GGA buffer+ATP, 53.8 ± 1.7%; GGA buffer+ATP+EGTA, 49.5 ± 2.1%. A two-sided *t*-test indicated that the amount of cleavage in the absence of ATP was not significantly greater than that at the start of the incubation (at the 90% confidence level), whereas the cleavage in the presence of ATP was significantly greater (at the 99.5% confidence level). The cleavage efficiency shown was calculated by dividing the increase in cleavage measured in perforated cells by the increase in cleavage measured in intact cells after incubation at 37°C for 60 min.

remainder of the experiments described in this paper. Under all conditions tested, the efficiency of cleavage in the perforated cells was relatively low, only 20−30% of that found in intact cells. This may be partly due to the fact that the 20°C block of transport not only causes an accumulation of transported protein in the TGN, but also slows down the transport from the ER to the Golgi (Matlin and Simons, 1983; de Curtis *et al.*, 1988). Under the conditions used in our *in vitro* experiments (Figure 1), transport from the ER to the TGN does not occur (see below). Therefore, the cleavage efficiency for HA which has already accumulated in the TGN before perforation is likely to be higher than that measured for the total HA, a portion of which remains in the ER during the incubation.

Release of membrane transport markers from perforated cells

Having established conditions that promote the cleavage of HA in perforated cells, we assayed for the appearance of the cleaved HA on the cell surface by surface immunoprecipitation. MDCK cells grown on filters were infected with FPV, pulse-labelled, and incubated at 20°C to accumulate the HA in the TGN as described above. Following perforation the cells were incubated at 37°C for 60 min under conditions which promoted HA cleavage (+ATP, ±EGTA) and subjected to surface immunoprecipitation with an antibody which recognized only the exoplasmic spike domain of HA. Ten percent of the labelled HA2 (a measure

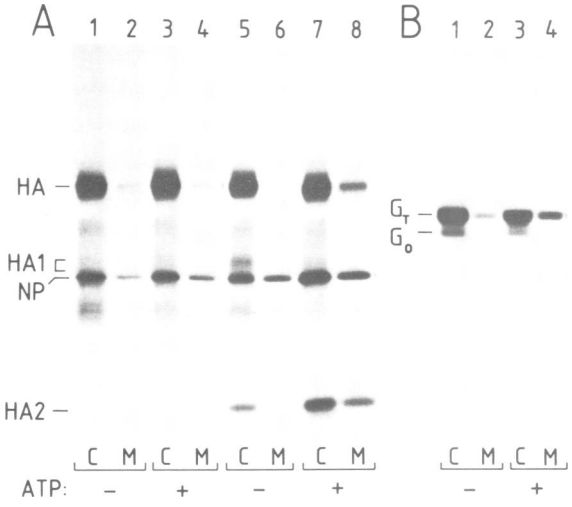

Fig. 2. Release of viral protein transport markers into the incubation medium. (**A**) FPV infected cells were perforated either (i) immediately after pulse-labelling with [^{35}S]methionine for 4 min (**lanes 1–4**) or (ii) after pulse-labelling for 4 min and incubating for 5 min at 37°C followed by an additional 90 min at 20°C in medium containing excess unlabelled methionine (**lanes 5–8**). The filter cultures were then incubated at 37°C for 60 min in GGA buffer containing 2 mM EGTA either in the presence or absence of ATP as indicated. The HA was immunoprecipitated from cell (C) and incubation medium (M) fractions and resolved by PAGE. The positions of the uncleaved HA, the cleavage products (HA1 and HA2) and the FPV nucleocapsid NP are indicated. (**B**) Filter cultures were infected with VSV, pulse-labelled with [^{35}S]methionine and incubated in medium containing excess unlabelled methionine as described in (A) above. The filter cultures were then perforated and incubated at 37°C for 60 min in GGA buffer containing 2 mM EGTA either in the presence or absence of ATP as indicated. The VSV G remaining with the cells (C) and released into the incubation medium (M) was immunoprecipitated, treated with endoglycosidase H, and resolved by PAGE. The positions of the terminally glycosylated (G_T) and core glycosylated (G_O) forms of VSV G are indicated. Each pair of lanes (C and M) represents the material from one-quarter of one 24 mm filter. The autoradiograms were exposed for 2–3 days.

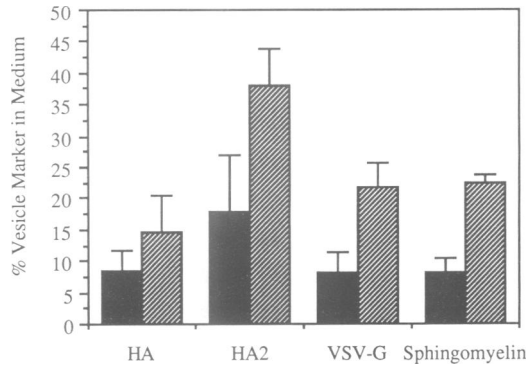

Fig. 3. Quantitation of membrane transport marker release. Filter cultures were infected with FPV or VSV and either (i) pulse-labelled with [^{35}S]methionine and subsequently incubated in medium containing excess unlabelled methionine, or (ii) labelled with C6-NBD-ceramide as described in Materials and methods. Following perforation the cultures were incubated at 37°C for 60 min in GGA buffer containing 2 mM EGTA either in the presence (striped bars) or absence (solid bars) of ATP. The distributions of the viral membrane proteins and of the fluorescent lipid C6-NBD-sphingomyelin between the cells and the incubation medium were quantitated as described in Materials and methods. The percentage of each marker released was calculated as the amount recovered in the incubation medium divided by the total amount present in the cells and the incubation medium. HA refers to uncleaved HA. The error bars represent the standard deviation of the mean from at least four experiments.

of cleaved HA) was precipitated by antibodies added to the cells prior to lysis (not shown). This value may be an overestimate of HA2 surface appearance since the antibody applied to the surface of perforated cells had access to the cytoplasmic compartment and was difficult to wash out efficiently prior to cell lysis. For comparison, intact cells were treated identically and the amount of surface HA2 measured by surface immunoprecipitation. In this case, 50% of the labelled HA2 was precipitated by anti-FPV HA antibodies added to the apical surface prior to lysis (not shown). These results suggested that while an early step in the process of transport from the TGN to the apical plasma membrane (HA cleavage) could be reconstituted in the perforated cells, the final step in the process entailing delivery of the transport marker to the plasma membrane did not occur under the conditions used.

This result raised the possibility that transport vesicles were formed from the TGN during the incubation but did not fuse with the apical plasma membrane. In order to determine whether these putative HA2 containing transport vesicles were released from the cells, the incubation medium and cell filter were analysed separately for the presence of HA2. As shown in Figure 2A, when the pulse-labelled HA was accumulated in the TGN at 20°C prior to perforation (lanes 5–8) there was an ATP dependent increase in both HA cleavage (compare lanes 5 and 7), as noted previously, and HA release into the medium (compare lanes 6 and 8) during the incubation at 37°C. Further characterization of the incubation medium (see below) indicated that all of the HA was within sealed membranous vesicles, consistent with the possibility that transport vesicles were released from the perforated cells.

Quantitation of the experiment shown in Figure 2A and similar experiments indicated that a greater proportion of HA2 as compared to uncleaved HA was recovered in the medium suggesting that markers present in later compartments along the transport pathway may be preferentially released. In order to test this possibility, the cells were perforated immediately following the pulse-labelling so that most of the labelled HA was in the ER at the start of the incubation. Under these conditions, very little HA was released from the cells either in the presence or absence of ATP (Figure 2A, lanes 1–4). This experiment also demonstrated the lack of transport from the ER to the TGN during the 37°C incubation as evidenced by the absence of HA cleavage (Figure 2A, lanes 1 and 3). The experiments shown in Figure 2A were carried out using GGA buffer containing 2 mM EGTA. When the EGTA was omitted or $CaCl_2$ was added to the incubation buffer, much less HA was released both in the presence and absence of ATP (not shown). Therefore, the remainder of the experiments described in this paper were carried out with GGA buffer containing EGTA to allow the further characterization of the material released from perforated cells.

It was possible that transport vesicles destined for the apical domain (the HA2 containing vesicles) were released from the cells because the apical membrane was disrupted by perforation to such an extent that vesicle fusion could no longer take place. Therefore, we studied the behaviour of two membrane transport markers that were destined for the basolateral membrane domain. First, the behaviour of the

well characterized basolateral protein VSV G was followed. The cells were infected with VSV for 3.5 h, pulse-labelled with [^{35}S]methionine for 4 min, and the VSV G accumulated in the TGN by incubating for 5 min at 37°C and for an additional 90 min at 20°C. Following perforation and incubation at 37°C for 60 min, the distribution of VSV G between the cells and incubation medium was determined by immunoprecipitation with an anti-VSV G antibody. As shown in Figure 2B, there was an ATP stimulated release of VSV G from the perforated cells (compare lanes 2 and 4). As with HA, preferentially the late transport forms were recovered in the medium. The core glycosylated form of VSV G was detected only in the cell samples and not in the medium (Figure 2B, lanes 2 and 4).

Quantitation of the results with the viral protein markers (summarized in Figure 3) indicated that there was a >2-fold stimulation by ATP of both FPV HA2 and VSV G release. A greater percentage of the apical marker (HA2) as compared to the basolateral marker (VSV G) was recovered in the medium both in the presence and absence of ATP. A significant amount of uncleaved HA was also released from the perforated cells. However, the release of uncleaved HA from the perforated cells was stimulated by ATP to a lesser extent than that of HA2 and VSV G.

The fluorescent lipid C6-NBD-ceramide was also used as a membrane transport marker (Lipsky and Pagano, 1983; van Meer *et al.*, 1987). At low temperatures C6-NBD-ceramide readily exchanges from liposomes into all cellular membranes and is converted into C6-NBD-sphingomyelin and -glucosylceramide in the Golgi complex. This conversion prevents the probe from flipping across the membrane, therefore trapping it in the luminal leaflet of the Golgi membrane. When the cells are warmed to 37°C, the C6-NBD-sphingomyelin is rapidly transported to both plasma membrane domains of MDCK cells. In order to follow transport of this marker in perforated cells, MDCK cells were incubated with C6-NBD-ceramide containing liposomes for 60 min at 20°C. The cells were then incubated for an additional 30 min at 20°C without liposomes and in the presence of 0.2% bovine serum albumin (BSA). The second incubation served two purposes. First, it allowed additional time for the metabolism and accumulation of the label in the Golgi complex. Second, the BSA efficiently extracted any C6-NBD-ceramide remaining on the cell surface and metabolites of C6-NBD-ceramide which had reached the surface. The cells were then perforated and incubated at 37°C for 60 min in GGA buffer containing 0.2% BSA. The medium and cell filter were separated, the lipids extracted and separated by 2-dimensional TLC, and the amount of C6-NBD-sphingomyelin quantitated. As with the viral protein markers, there was an ATP stimulated release of the fluorescent lipid marker (Figure 3). These results strongly suggested that a significant fraction of transport vesicles derived from the TGN, instead of fusing with the plasma membrane, were released from the perforated cells.

Topology of transport vesicle markers

Transport vesicles derived from the TGN should contain the viral spike proteins oriented such that the spike portion is sealed within the vesicle and the cytoplasmic tail is accessible on the vesicle surface. We tested the topology of the viral spike proteins by determining the accessibility of the spike protein domains to antibodies or trypsin. The results are

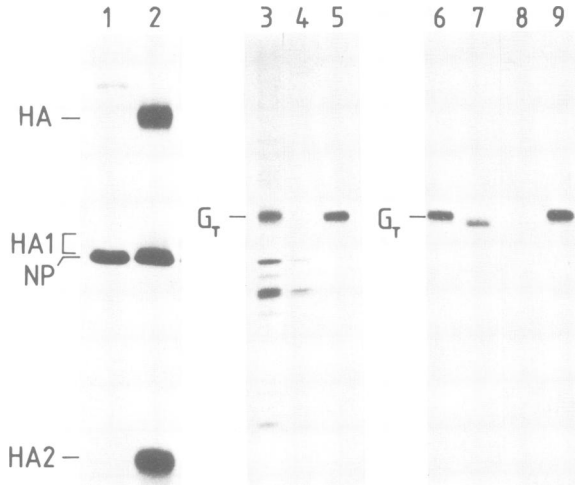

Fig. 4. Topology of viral protein tansport markers. Filter cultures were infected with FPV or VSV, and pulse-labelled for 4 min with [^{35}S]methionine. The labelling was stopped with the addition of excess unlabelled methionine as described in Materials and methods. Following a 90 min incubation period at 20°C the cells were perforated and incubated for 60 min at 37°C in GGA buffer containing EGTA and ATP. The incubation medium (500 μl) from perforated FPV infected cells was immunoprecipitated with an anti-FPV spike antibody in the absence (**lane 1**) or presence (**lane 2**) of 1% Triton X-100. The incubation medium (300 μl) from perforated VSV infected cells was immunoprecipitated with an antibody directed against the cytoplasmic tail of VSV G in the absence of detergent (**lane 3**), or with an anti-VSV G spike antibody in the absence (**lane 4**) or presence (**lane 5**) of 1% Triton X-100. In addition, the medium from perforated VSV infected cells (300 μl) was digested with trypsin as described in Materials and methods prior to immunoprecipitation with an anti-VSV G spike antibody. The sample were treated as follows: no trypsinization (**lane 6**); trypsinization in the absence of detergent (**lane 7**); trypsinization in the presence of 1% Triton X-100 (**lane 8**); trypsinization in the presence of 1% Triton X-100 and soybean trypsin inhibitor (**lane 9**). The additional bands present in lanes 3 and 4 are due to the absence of Triton X-100 during the initial steps of the immunoprecipitation and probably represent non-specific binding of proteins to the Pansorbin immunoabsorbant. The autoradiograms were exposed for 17 h (lanes 1 and 2) or 2 days (lanes 3–9).

shown in Figure 4. The topology of HA containing vesicles was tested by immunoprecipitating an incubation medium fraction with an antibody directed against the spike portion of HA either in the absence (lane 1) or presence (lane 2) of Triton X-100. Both HA2 and uncleaved HA were immunoprecipitated only in the presence of detergent indicating that the spike domain was protected within the lumen of the vesicles. The topology of VSV G containing vesicles was tested by immunoprecipitating an incubation medium fraction with a monoclonal antibody directed against the cytoplasmic tail of VSV G in the absence of Triton X-100 (lane 3) and with an antibody directed against the spike domain of VSV G either in the absence (lane 4) or presence (lane 5) of Triton X-100. In the absence of detergent, the cytoplasmic tail of VSV G, but not the exoplasmic spike domain, was accessible to antibodies. This result indicated that the spike domain of VSV G was also protected within the lumen of vesicles. The orientation of VSV G was further confirmed by subjecting the transport vesicles to trypsin digestion either in the presence or absence of detergent. In the absence of Triton X-100, trypsin digestion resulted in a slight increase in the mobility of VSV G (lane 7) relative

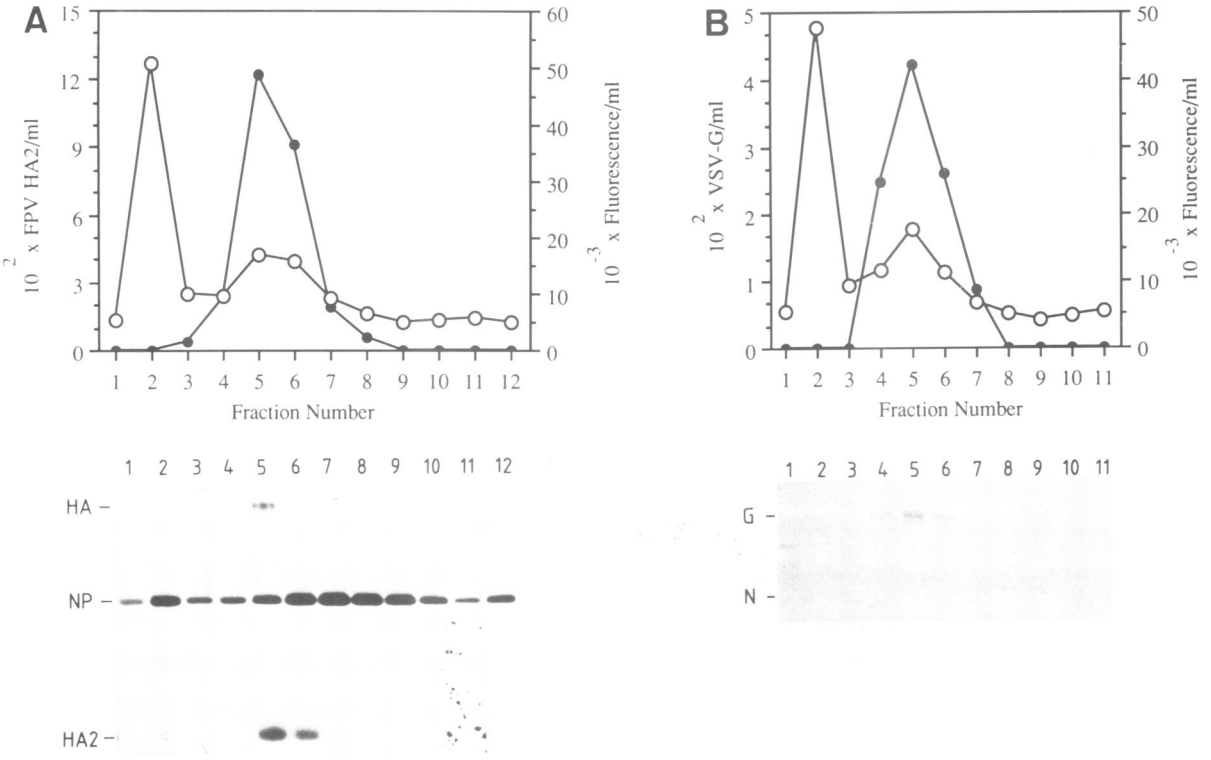

Fig. 5. Sedimentation of transport markers. Filter cultures were infected with FPV (**panel A**) or VSV (**panel B**), pulse-labelled, and then labelled with C6-NBD-ceramide at 20°C. Following perforation and incubation for 60 min at 37°C in GGA buffer containing 2 mM EGTA and ATP, the incubation medium (0.65 ml) was isolated and layered on top of an 11.5 ml linear 0.3–1.5 M sucrose gradient (in 10 mM Hepes pH 7.4, 2 mM EGTA, 1 mM DTT) prepared in an SW 40 polyallomer centrifuge tube. The gradient was subjected to centrifugation at 100 000 *g* for 14 h and then fractionated into 1 ml fractions. The amount of NBD-fluorescence in each fraction was quantitated (top, ○) and the viral proteins in each fraction were immunoprecipitated and resolved in PAGE (bottom). The bands labelled NP and N are the nucleocapsid proteins of FPV and VSV, respectively. The amount of HA2 and VSV G in each fraction was quantitated by densitometric scanning of the autoradiogram (top, ●). The units are arbitrary. The dense end (bottom) of the gradient is on the right side of the graph. The autoradiograms were exposed for 2 weeks.

to untreated VSV G (lane 6). This shift in mobility was due to the digestion of the cytoplasmic tail by trypsin (Katz *et al.*, 1977; Katz and Lodish, 1979). In the presence of Triton X-100, the VSV G was completely digested (lane 8) unless trypsin inhibitor was included in the reaction (lane 9). Quantitation of this result, correcting for the loss of one methionine present in the cytoplasmic tail (Rose and Gallione, 1981), indicated that at least 83% of the VSV G was within vesicles with an orientation expected of a transport vesicle.

Sedimentation behaviour of transport markers

The putative transport vesicles released from perforated MDCK cells were further characterized by determining their sedimentation behaviour. The cells were infected with either FPV or VSV and pulse-labelled as before. During accumulation of HA or VSV G in the TGN at 20°C the cells were also labelled with C6-NBD-ceramide. Following perforation and incubation at 37°C, the incubation medium was layered on top of a linear 0.3–1.5 M sucrose gradient. The gradient was subjected to centrifugation at 100 000 *g* for 14 h to allow the vesicles to reach their equilibrium density. The gradient was then fractionated and the amount of fluorescence and viral spike protein in each fraction quantitated. The results obtained when FPV infected cells or VSV infected cells were used are shown in Figure 5A and B, respectively. The fluorescent lipid marker (open circles) was present in two well resolved peaks. The first peak remained at the top of the gradient and was composed of unmetabolized C6-NBD-ceramide which was extracted by BSA during the incubation. The second peak sedimented half way through the gradient and contained predominantly C6-NBD-sphingomyelin. This peak corresponded to fluorescent lipid present in sealed membrane structures. The fact that the C6-NBD-sphingomyelin was not extracted by BSA during the incubation indicated that it was present in the luminal leaflet of the vesicles. Thus, like the viral protein markers, the fluorescent lipid marker was within vesicles with the topology expected of authentic transport vesicles. The sedimentation profiles (closed circles) of both HA2 (Figure 5A) and VSV G (Figure 5B) precisely overlapped with the C6-NBD-sphingomyelin peak. The equilibrium density reached by all three transport markers was 1.09–1.11 g/ml. There was no detectable difference in the density of HA2 and VSV G containing vesicles. Velocity sedimentation also resulted in co-migration of all three transport markers and indicated that the markers were contained in vesicles with a rather small size (not shown). The equilibrium sedimentation of uncleaved HA (Figure 5A) was similar to that of the HA2 and C6-NBD-sphingomyelin markers. However, on a velocity sedimentation gradient the uncleaved HA was resolved from the HA2 and C6-NBD-sphingomyelin (not shown). The viral nucleocapsid proteins (NP for FPV and N for VSV), which were contaminants of the immunoprecipitations, sedimented to a higher equilibrium density than the viral spike proteins (Figure 5A

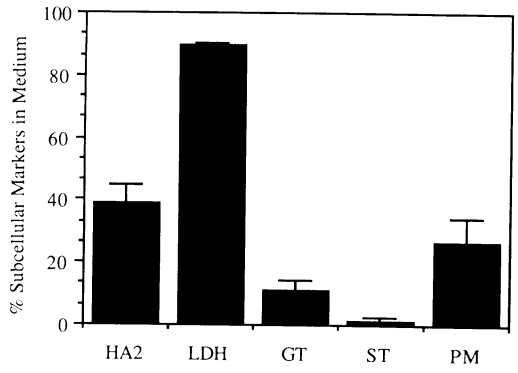

Fig. 6. Markers released from perforated cells. Filter cultures were infected with FPV for 3.5 h and incubated at 20°C for 90 min. The cells were perforated and incubated at 37°C for 60 min in GGA buffer containing 2 mM EGTA and ATP. The distributions of the cytosolic enzyme lactate dehydrogenase (LDH), the resident Golgi markers galactosyl-transferase (GT) and sialyl-transferase (ST), and ^{3}H-labelled plasma membrane protein (PM) between the cells and the incubation medium were quantitated. The percentage of each marker present in the medium is shown. For comparison, the percentage of HA2 released into the medium is also plotted. The error bars represent the standard deviation of the mean from at least three experiments.

and B) indicating that the capsid and the spike proteins had not formed an association. Furthermore, the C6-NBD-ceramide labelled vesicles were readily resolved from ^{35}S-labelled virus (both FPV and VSV) which reached an equilibrium density of 1.14−1.16 g/ml (not shown). These sedimentation results demonstrated that the three transport markers were contained in vesicles with very similar properties. In addition, they showed that the fluorescent metabolites of C6-NBD-ceramide could be used as a convenient marker to monitor the behaviour of the vesicles.

Release of other markers from perforated cells

The experiments presented thus far suggested that the viral proteins as well as the lipid markers were released from perforated cells in membrane structures with the properties expected of transport vesicles derived from the TGN. However, it was possible that the vesicles resulted from general Golgi vesiculation or were derived from inside-out plasma membrane fragments. To eliminate these possibilities, we determined the cell versus medium distribution of other cellular markers.

In these experiments the cells were infected with FPV for 3.5 h and then incubated at 20°C for 90 min to mimic the conditions used previously to accumulate the transport markers in the TGN. The cells were perforated and incubated at 37°C for 60 min in GGA buffer containing EGTA and ATP (conditions that promoted maximal release of the transport markers). The incubation medium and the cell filter were then separated and assayed for the distribution of different cellular markers. The results for the most relevant markers are summarized in Figure 6. The majority (90%) of the soluble cytoplasmic marker lactate dehydrogenase (LDH, M_r = 140 000) was found in the medium fraction after incubation, as expected for a soluble marker that is able to diffuse freely through the holes in the apical membrane. This result, along with the finding that 65% of the total cellular protein was released into the medium, left no doubt that the majority of cells were permeabilized. Galactosyl-transferase and sialyl-transferase, two resident trans-Golgi

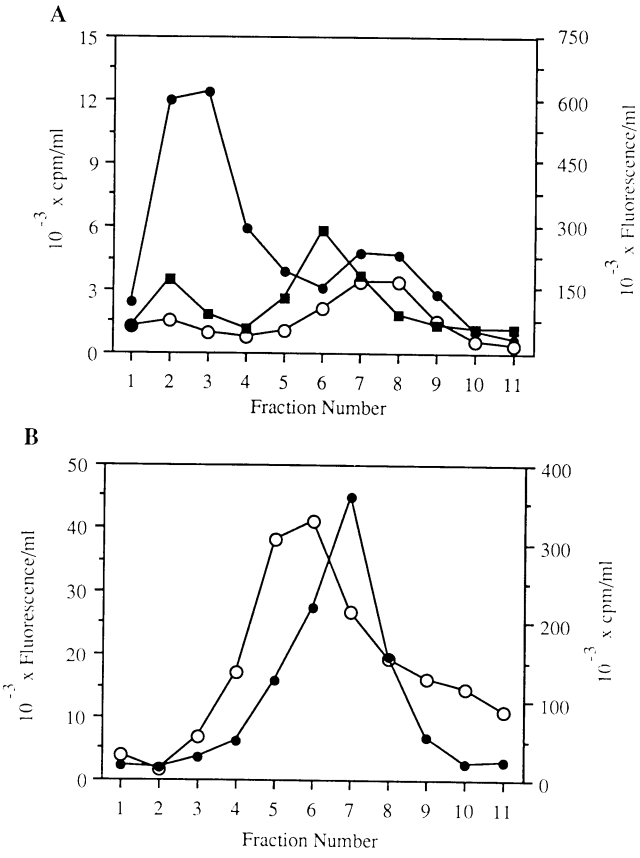

Fig. 7. Sedimentation of plasma membrane and membrane protein markers. (**A**) Filter cultures were labelled with C6-NBD-ceramide and then the apical surface was labelled with [^{3}H]borohydride as described in Materials and methods. Following perforation and incubation at 37°C for 60 min in GGA buffer containing 2 mM EGTA and ATP, the incubation medium (1.2 ml) was resolved on a linear 0.3−1.5 M sucrose gradient as described in the legend to Figure 5. The gradient was fractionated and the amount of NDB-fluorescence (■), acid insoluble radioactivity (●) and Triton X-114 extractable radioactivity (○) in each fraction quantitated. (**B**) Filter cultures were continuously labelled with [^{35}S]methionine for 14 h followed by labelling with C6-NBD-ceramide as described in Materials and methods. Following perforation and incubation at 37°C for 60 min in GGA buffer containing 2 mM EGTA and ATP, the particulate material from 600 μl of incubation medium was collected by centrifugation through a 300 μl cushion of 0.3 M sucrose (in 10 mM Hepes pH 7.4, 2 mM EGTA, 1 mM DTT) at 100 000 *g* for 60 min in a Ti 75 rotor. The particulate material was resuspended in 200 μl GGA buffer containing 2 mM EGTA and resolved on a linear 0.3−1.5 M sucrose gradient as described in the legend to Figure 5. The gradient was fractionated and the amount of NBD-fluorescence (○) and acid insoluble radioactivity (●) in each fraction determined.

or TGN markers (Roth and Berger, 1982; Roth *et al.*, 1985; Berger *et al.*, 1987), remained largely associated with the cells. The levels of galactosyl-transferase (11%) and sialyl-transferase (1.3%) recovered in the incubation medium were significantly lower than the level of the apical transport vesicle marker HA2 (39%). This suggested that the release of HA2 and the other transport markers was not solely the result of general vesiculation of late Golgi compartments. Less than 10% of the total internalized horseradish peroxidase (an endosomal marker) or β-*N*-acetylglucosaminidase (a lysosomal marker) were present in the medium fraction providing further evidence that most of the subcellular organelles remained associated with the perforated cells.

The distribution of the apical plasma membrane was

followed by labelling the apical cell surface of intact cells with [^{3}H]borohydride as described in Materials and methods. The cells were then perforated, incubated, and the distribution of acid insoluble ^{3}H-labelled material between the cell and medium fractions determined. As shown in Figure 6, a significant fraction of the plasma membrane label (27%) was present in the incubation medium. This raised the possibility that a portion of our transport markers was actually first delivered to the plasma membrane, and was subsequently released into the incubation medium as plasma membrane fragments or inside-out vesicles and therefore not in transport vesicles derived from the TGN. This problem was addressed by analysing the sedimentation behaviour of the plasma membrane marker.

Sedimentation behaviour of plasma membrane and membrane protein markers

Further analysis of the plasma membrane derived material in the incubation medium was performed using cells double-labelled with [^{3}H]borohydride and C6-NBD-ceramide. The incubation medium derived from these cells was resolved in an equilibrium sedimentation gradient (0.3−1.5 M sucrose as described above). Gradient fractions were analysed for acid insoluble radioactivity and fluorescence. As shown in Figure 7A, the ^{3}H-labelled plasma membrane protein was resolved into two peaks of acid insoluble radioactivity (closed circles). When the fractions were subjected to Triton X-114 phase separation, an assay for integral membrane proteins (Bordier, 1981; Pesonen *et al.*, 1984), only the second peak contained Triton X-114 extractable radioactivity (open circles). Therefore, this peak probably contained plasma membrane derived fragments or vesicles, while the majority of the ^{3}H-labelled protein was soluble. The reason for the release of apparently soluble protein from the apical surface is not clear, but it also occurred to the same extent when intact cells were incubated for 60 min at 37°C (not shown). This observation also suggested that the total amount of ^{3}H-labelled acid insoluble protein (Figure 6) present in the incubation medium was a poor measure of the actual amount of plasma membrane derived vesicles released from the perforated cells. The sedimentation of the fluorescent lipid marker (Figure 7A, closed squares) was clearly distinct from that of the plasma membrane derived vesicles. The plasma membrane marker sedimented further into the gradient than the fluorescent lipid transport marker ruling out the possibility that the transport markers were first delivered to the plasma membrane and then released as inside-out plasma membrane vesicles. The same conclusion was reached when pulse-labelled HA accumulated on the cell surface was used as a plasma membrane marker (not shown).

As shown in Figure 6, most of the soluble cellular protein appeared to leak out of the perforated cells, while the majority of the organelles remained cell associated. As part of the characterization of the incubation medium fraction we compared the sedimentation behaviour of total membrane protein in the medium fraction to that of a transport vesicle marker. The cells were first metabolically labelled with [^{35}S]methionine for 14 h to label all cellular proteins and then labelled with C6-NBD-ceramide which served as the transport vesicle marker. Following perforation and incubation, the incubation medium was subjected to centrifugation at 100 000 *g* for 60 min through a 0.3 M sucrose cushion to separate soluble from membrane associated particulate material. The particulate material was resuspended and subjected to equilibrium sedimentation on a linear 0.3−1.5 M sucrose gradient as previously described. The gradient was fractionated and analysed for fluorescence and acid insoluble radioactivity. The total membrane associated protein (Figure 7B, closed circles) clearly sedimented differently from the transport marker (Figure 7B, open circles) indicating that the transport vesicles could be resolved from other membranes recovered in the incubation medium. Figure 7B also demonstrated that the isolation of the fluorescent transport marker by high speed centrifugation did not alter its subsequent sedimentation properties (cf. Figure 5).

Discussion

Perforated MDCK cells and three different membrane transport markers (FPV, HA, VSV G and C6-NBD-sphingomyelin) were used to monitor membrane transport from the TGN *in vitro*. All three markers were released into the extracellular medium in an ATP stimulated manner. A number of observations suggested that the transport markers in the medium fraction were contained in transport vesicles derived from the TGN. The released transport markers were predominantly from late stages of the biosynthetic transport pathway. They were contained in sealed membranous vesicles exhibiting a well defined behaviour upon equilibrium sedimentation and the topology expected of transport vesicles. In experiments not shown here, we have determined the kinetics of HA cleavage and release into the medium during the 37°C incubation. Both the cleavage of HA (a late transport event), and the release of HA2 from the perforated cells occurred rapidly, reaching 50% of their maximum values within 5 min of incubation at 37°C. This rapid time course was at least as fast as that reported for transport from the TGN to the cell surface following release of the 20°C block of membrane transport (Matlin and Simons, 1984). The observation that the resident Golgi proteins galactosyl-transferase and sialyl-transferase remained largely associated with the perforated cells suggested that the trans-Golgi compartment or TGN did not vesiculate randomly. The vesicles containing the transport markers were readily resolved from both a plasma membrane marker and intact virus particles (both FPV and VSV) on equilibrium sedimentation gradients. Furthermore, the HA and VSV G recovered in the medium had an orientation opposite to that expected for intact virus. Therefore, other possible explanations for the recovery of the transport markers in the incubation medium (including general vesiculation of the TGN and/or plasma membrane, or even viral budding) were ruled out.

Using perforated MDCK cells grown on polycarbonate filters, we observed little or no transport of either VSV G from the ER to the Golgi (not shown) or of HA from the TGN to the apical plasma membrane. Transport from the Golgi to the basolateral plasma membrane was not quantitated, but the observed release of VSV G from the perforated cells suggested that this transport step may also be at least partially blocked. These results are in contrast to those recently reported for membrane transport in perforated MDCK cells grown on glass coverslips in which both the ATP dependent transport of VSV G from the ER to the Golgi (as judged by endoglycosidase H resistance) and

transport of C6-NBD-sphingomyelin from the Golgi complex to the basolateral plasma membrane were reconstituted (Simons and Virta, 1987). The main difference between the two systems was the substrate on which the cells were grown. The cells grown on glass coverslips were perforated shortly after reaching confluence and therefore, due to variability in the thickness of different regions of the cell, presented an irregular apical surface for the binding of the nitrocellulose filter. In contrast, the filter grown cells used in the present study form a monolayer of cuboidal cells of uniform thickness with a high density of apical microvilli. These morphological differences, along with the stronger adherence of the cells to the filter substrate, allowed perforation conditions to be used in this study that resulted in more extensive or widespread damage to the apical membrane. Scanning electron microscopic analysis of the filter grown cells before and after perforation showed dramatic morphological changes including the loss of microvilli over the entire apical surface (J.Kartenbeck, M. Bennett and K.Simons, unpublished observation). This may cause the filter grown cells to lose their cytosolic proteins more rapidly or to a greater extent than the cells grown on coverslips. Indeed, we found that the cells lost the majority of their cytosolic protein following perforation and estimated that the cytosol was diluted over 200-fold with incubation buffer. It is possible that a cytosolic protein required for membrane fusion was diluted below a critical concentration in our system so that all fusion events required for intracellular membrane transport were inefficient or blocked. *In vitro* reconstitution of membrane transport in other systems, all of which depend on successful delivery to detect transport, have required the addition of cytosolic protein (Balch *et al.*, 1984a, 1987; Davey *et al.*, 1985; Woodman and Edwardson, 1986). It may, therefore, be possible to reconstitute vesicle fusion in our system by the addition of a cytosolic fraction. We have taken advantage of the lack of fusion in the present study since it has allowed the identification and characterization of transport vesicles derived from the TGN.

Quantitation of the transport markers present in the incubation medium indicated that a larger percentage of the apical transport marker (HA2) as compared to the basolateral transport marker (VSV G) was released from the perforated cells, both in the presence and absence of ATP. One possibility for this difference is that the two markers are sorted into different vesicles during transport from the TGN, as is believed to occur during transport *in vivo*. The differences between the two markers could then reflect differences in the budding of the two types of vesicles or differences in the behaviour of the two types of vesicles within the cells (e.g. differential binding to the cytoskeleton or intracellular membranes, or fusion of some of the basolateral transport vesicles with the target membrane). The isolation and characterization of the putative apical and basolateral transport vesicles will be required to investigate these possibilities.

A substantial amount of uncleaved HA was also recovered in the incubation medium. The release of uncleaved HA was less stimulated by ATP than that of other transport markers. However, like the other transport markers, the uncleaved HA was contained within vesicles with the spike domain protected. The sedimentation behaviour of the uncleaved HA on process gradients was different from that of the other markers (not shown) suggesting that it was not contained in transport vesicles derived from the TGN. Since FPV HA never reaches a state of complete endoglycosidase H resistance (Kääriäinen and Pesonen, 1982) and the mobility shifts on PAGE caused by terminal glycosylation were not reproducibly detected, it could not be concluded whether the uncleaved HA in the medium fraction originated from the ER, from a Golgi compartment, or both.

Previous studies have shown that ATP is necessary for every step within the biosynthetic pathway requiring vesicular carriers, including: transport from the ER to the Golgi complex, between Golgi compartments (intra-Golgi), and from the Golgi complex to the plasma membrane (Balch *et al.*, 1986; Balch and Keller, 1986). The intra-Golgi transport step has been dissected further and been shown to involve at least three distinct ATP requiring steps; vesicle priming, vesicle processing, and vesicle fusion (Balch *et al.*, 1984b; Wattenberg *et al.*, 1986). Vesicular transport from the TGN to the plasma membrane in MDCK cells involves a number of sequential steps. These include the sorting of apical from basolateral membrane components, the formation, budding and targeting of transport vesicles, and the fusion of the transport vesicles with the appropriate plasma membrane domain. The ATP dependence of the two processes we measured using perforated cells, HA cleavage and release of transport markers, was different. The increase in HA cleavage observed in perforated cells was completely dependent on ATP. In contrast, 35–45% of the transport markers released in the presence of ATP were also released in its absence. This suggests that two separate steps in the transport pathway were reconstituted.

The cleavage of HA is thought to occur in transit between the TGN and the plasma membrane, although the intracellular compartment where processing occurs has not been identified. There is little cleavage of HA when it has been accumulated in the TGN at 20°C (Matlin and Simons, 1983). In the perforated cells, HA cleavage could be reconstituted even in the absence of cell surface delivery. Taken together these observations suggest that the cleavage is initiated in transport vesicles. By analogy, the proteolytic processing of prohormones to their mature forms occurs shortly after budding of the secretory granule from the TGN (Orci *et al.*, 1987; Tooze *et al.*, 1987), perhaps triggered by granule acidification. The proteolytic cleavage of HA is enzymatically similar to the processing of prohormones during regulated secretion (Garten *et al.*, 1981, 1982; Docherty and Steiner, 1982) and processing enzymes with similar specificities exist along the constitutive secretory pathway (Warren and Shields, 1984; Morrison *et al.*, 1985). Therefore, it seems likely that HA cleavage occurs during or shortly after apical vesicle formation. The ATP may be required for vesicle formation itself or for subsequent modification of the luminal environment of the vesicle.

It is not clear exactly which step in the transport of proteins from the TGN to the plasma membrane is reflected by the ATP-stimulated release of transport vesicles from the perforated cells. It may reflect the actual formation of vesicles. If so, then the transport vesicles recovered in the absence of ATP may represent vesicles which were 'primed' during the 20°C block of transport and were then released upon warming the perforated cells to 37°C. Coated buds have been observed on the TGN by electron microscopy in cells maintained at 20°C (Griffiths *et al.*, 1985) and may represent 'primed vesicles'. Stimulation of this process by

ATP may then reflect an ATP requirement for the formation of additional 'primed' vesicles. A similar ATP dependent 'priming' step has been observed for intra-Golgi transport *in vitro* (Balch *et al.*, 1984b). Another alternative is that the ATP-stimulated release of transport vesicle markers reflects an effect on pre-formed transport vesicles, for example, the disruption of vesicle−TGN, vesicle−cytoskeleton, or vesicle−target membrane interactions. The observation that $CaCl_2$ reduced the recovery of transport markers in the medium fraction indicates that other factors may also modulate the formation or behaviour of TGN derived transport vesicles. Using perforated MDCK cells and the assays we have developed, it should be possible to further define the sequence of events in membrane transport from the TGN to the plasma membrane, as well as to explore the ATP, cytosol and temperature dependence of these steps.

Transport vesicles within the constitutive biosynthetic transport pathway have proven very elusive, in part, because they are only transient intermediates under normal circumstances. An intermediate in the transport from the ER to the cis-Golgi has been identified, but not isolated (Lodish *et al.*, 1987). Whether this intermediate represents a transport vesicle or is derived from the ER transitional elements during homogenization has not been established. Electron microscopic studies of both tissue samples (Croze *et al.*, 1982) and isolated Golgi fractions (Balch *et al.*, 1984b; Melançon *et al.*, 1987) have identified coated vesicles which are believed to represent intra-Golgi transport vesicles. However, such vesicles have not been isolated and characterized. Recently constitutive secretory vesicles have been isolated and biochemically characterized from yeast secretory mutants (Holcomb *et al.*, 1987; Walworth and Novick, 1987). We are now in a position to isolate and further characterize the constitutive transport vesicles released from perforated MDCK cells. The isolation will be facilitated by the fact that the perforated cells provide a highly enriched starting material by preferentially releasing TGN derived transport vesicles while retaining the majority of other intracellular membranes. With the biochemical and functional characterization of these vesicles, we can begin to address the process of membrane sorting and transport from the TGN at a molecular level.

Materials and methods

Materials

Media and reagents for cell culture were purchased from Gibco Biocult and Seromed. Creatine phosphate and creatine kinase were obtained from Boehringer Mannheim, rhodamine-phalloidin and C6-NBD-ceramide were from Molecular Probes, and TPCK-treated trypsin was from Cooper Biomedical. Ovalbumin, asialofetuin, BSA, HRP, apyrase, ATP, protease inhibitors and unlabelled UDP-galactose were purchased from Sigma. Endoglycosidase H was from Seikagaku, Hoechst 33258 was from Serva, and Triton X-114 was from Fluka. [^{35}S]Methionine (800 Ci/mmol), UDP-D-[6-^{3}H]galactose (20 Ci/mmol), and CMP-*N*-acetyl-[^{14}C]neuraminic acid (250 mCi/mmol) were from Amersham. NaB[^{3}H]$_4$ (12.7 mCi/mmol) and unlabelled CMP-*N*-acetyl neuraminic acid were from New England Nuclear. Rabbit polyclonal antibodies generated against FPV HA and VSV G were prepared and affinity purified as described previously (Matlin *et al.*, 1981, 1982). The mouse monoclonal antibody directed against the cytoplasmic tail of VSV G was prepared as described by Kreis (1986).

Cell culture media

Growth medium consisted of Eagle's minimal essential medium with Earle's salts (E-MEM) supplemented with 10 mM Hepes pH 7.3, 10% (v/v) fetal calf serum, 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% (w/v) BSA, 100 U/ml penicillin and 100 μg/ml streptomycin. Pulse-labelling medium consisted of methionine-free E-MEM containing 10 mM Hepes pH 7.3, 0.2% BSA, 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 and 0.35 g/l sodium bicarbonate. Metabolic labelling medium consisted of E-MEM containing 10 mM Hepes pH 7.3, 5% (v/v) fetal calf serum and 1.5 mg/l unlabelled 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 resuspended in 9 ml of growth medium and 1.5 ml of the cell suspension was seeded on each of six 24 mm diameter, 0.4 μm pore size, premounted polycarbonate filters (a kind gift from Costar). The filter cultures were maintained at 37°C and 5% CO_2 for 2−3 days in a Petri dish containing 100 ml of growth medium.

Viral infection

Isolates of fowl plague virus (FPV/A/Rostock, Hav1 N1) were obtained from H.-D.Klenk, Justus Liebig University, Giessen, FRG, plaque purified on MDCK cells, grown in cells as described by Matlin and Simons (1983) and titred on MDCK cells as described by Matlin *et al.* (1981). A stock of phenotypically mixed VSV (Indiana strain) was prepared in Chinese hamster ovary C15.CF1 cells which express HA on their plasma membrane (Hearing *et al.*, 1985; Sambrook *et al.*, 1985) and titred on MDCK cells as described by Matlin *et al.* (1982). Infections were performed by rinsing the filter cultures with infection medium, placing the filter in a six-well culture dish, and applying 100 μl infection medium containing 20 p.f.u./cell of FPV or 50 p.f.u./cell of VSV to the apical side. After adsorption of the virus to the cells at 37°C for 1 h, the inoculum was removed and 1 ml infection medium was added to the apical side and 2.6 ml to the basal side of the filter. The infection was continued for an additional 2.5 h at 37°C, 5% CO_2.

Radioactive labelling

Pulse-labelling was performed by rinsing the filter cultures twice with 37°C phosphate-buffered saline containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS+), and placing the basal side of the filter on 100 μl of pulse-labelling medium containing 50 μCi [^{35}S]methionine layered on parafilm in a humid chamber at 37°C for 4 min. 100 μl pulse-labelling medium added to the apical surface prevented the cells from drying during the labelling. The labelling was stopped by transferring the filter cultures to a six-well culture dish and adding 1 ml water bath medium (supplemented with 0.2% BSA, 150 mg/l unlabelled methionine and 20 μg/ml cycloheximide) to the apical side and 2.6 ml to the basal side of each filter. The filter cultures were incubated at 37°C for 5 min, after which they were cooled rapidly by adding fresh medium equilibrated at 20°C. The incubation was continued at 20°C for an additional 90 min. Metabolic labelling was performed as follows. Two-day-old filter cultures were rinsed twice with 37°C PBS+ and placed in a six-well culture dish. One ml metabolic labelling medium was added to the apical side of the filter and 3 ml metabolic labelling medium containing 100 μCi [^{35}S]methionine was added to the basal side. The filters were incubated for 14 h at 37°C, 5% CO_2.

Perforation procedure

The perforation procedure for MDCK cells grown on glass coverslips (Simons and Virta, 1987) was modified for cells grown on polycarbonate filters. All the manipulations were performed at 4°C with cold buffers. The filter culture was rinsed twice with KOAc buffer (25 mM Hepes pH 7.4, 115 mM potassium acetate, 25 mM $MgCl_2$) and cut from the holder with a scalpel. Excess buffer was removed by blotting the edge on a Whatman No. 1 filter. The filter was placed in a 50 mm culture dish in a 20°C water bath (in the cold room). A nitrocellulose acetate filter (HATF, 0.45 μm pore, Millipore) was soaked in KOAc buffer, blotted between two pieces of Whatman filter for 1 min and placed directly on top of the filter culture. The nitrocellulose filter was covered with a Whatman filter and gently smoothed with a bent Pasteur pipette to remove any excess moisture and maximize the contact between the cells and the nitrocellullose filter. The Whatman filter was removed. The cells were allowed to bind to the nitrocellulose filter for 90 s. The nitrocellulose filter was then wetted by the addition of 200 μl KOAc buffer. The excess buffer was aspirated, and the nitrocellulose filter was separated from the filter culture. Staining of the filter cultures with Hoechst 33258 (a nuclear stain) and rhodamine-phalloidin (a filamentous actin stain) in the absence of detergent indicated that the majority of cells remained on the polycarbonate filter and were permeabilized by this procedure.

Incubation conditions

Following perforation, the filter culture was transferred to a 35 mm tissue culture dish containing 1 ml incubation buffer and incubated for 60 min in a 37°C water bath. The standard incubation buffers were KOAc buffer (see above) and GGA buffer (25 mM Hepes pH 7.4, 38 mM potassium gluconate, 38 mM potassium glutamase, 38 mM potassium aspartate and 2.5 mM $MgCl_2$) containing 1 mM dithiothreitol and in most cases 2 mM EGTA. In addition, +ATP samples contained 1 mM ATP and an ATP regenerating system consisting of 8 mM creatine phosphate and 50 μg/ml creatine kinase, and −ATP samples contained the ATP depleting enzyme apyrase (30 U/ml). In some cases the filter on which the cells were grown was cut into quarters following perforation. The filter quarters were incubated in separate wells of a 24-well dish containing 250 μl buffer. A moist Whatman filter was placed in the lid of the incubation chambers to minimize evaporation during the 37°C incubation.

C6-NBD-ceramide labelling and analysis

C6-NBD-ceramide containing liposomes were prepared as previously described (van Meer *et al.*, 1987). For labelling, the filter cultures were washed twice with PBS+, placed in a six-well culture dish, and 2.6 ml water bath medium was added to the basal side and 1 ml water bath medium containing C6-NBD-ceramide liposomes (26 nmol C6-NBD-ceramide/ml) was added to the apical side of the filter. The filters were incubated in a 20°C water bath for 60 min at which time the apical and basal medium was replaced with water bath medium containing 0.2% BSA and the incubation continued for 30 min at 20°C. Following perforation and incubation in GGA buffer containing 2 mM EGTA and 0.2% BSA, the lipids were extracted from both the perforated cells and the incubation medium (Blight and Dyer, 1959). The lipids were separated by two-dimensional high-performance TLC as described (van Meer and Simons, 1982) and the fluorescent spot corresponding to C6-NBD-sphingomyelin was scraped from the plate and extracted (van Meer *et al.*, 1987). Alternatively, the incubation medium was resolved by equilibrium sedimentation. The amounts of fluorescent lipids in the sucrose gradient fractions or in fractions extracted from TLC plates were measured using an SLM 8000 fluorometer (SLM Instruments, Inc.).

Immunoprecipitations

The total amount of HA or VSV G in the samples was quantitated as follows. First the samples were solubilized with lysis buffer [20 mM Tris pH 7.4, 2 mM EDTA, 2% (w/v) Triton X-100, 0.3 M NaCl, 10 μg/ml each of chymostatin, leupeptin, antipain and pepstatin]. Cell samples were lysed by shaking at room temperature for 10 min in 1 ml lysis buffer/filter and collecting the lysate. Incubation medium samples and gradient fractions were lysed by adding 1/4 volume of 5-fold concentrated lysis buffer. After lysis, 109 μl affinity purified anti-FPV HA (14 μg/ml) or affinity purified anti-VSV G (14 μg/ml) was added and the sample incubated at 4°C overnight. The antigen−antibody complex was precipitated with 10 μl of Pansorbin [10% (w/v) suspension of fixed *Staphylococcus* bacteria, Calbiochem], washed three times with 1 ml 20 mM Tris pH 8.6, 0.5 M NaCl, 1% (w/v) Triton X-100, 0.1% (w/v) SDS, 2 mM EDTA and once with 1 ml 10 mM Tris pH 7.4, 0.9% (w/v) NaCl. The immune complexes were released from the Pansorbin with SDS gel sample buffer. Surface immunoprecipitation of FPV HA was performed by adding 200 μl affinity purified anti-FPV HA (14 μg/ml) to the apical surface of intact or perforated cells and allowing binding to occur for 60 min at 4°C. Unbound antibody was removed by washing three times with cold PBS+. The cells were solubilized in lysis buffer and the antibody−antigen complex precipitated with Pansorbin as described above. Immunoprecipitation of the medium fraction without prior detergent solubilization was performed by adding 20 μl affinity purified antibody [anti-FPV HA (70 μg/ml), anti-VSV G (35 μg/ml), or anti-VSV G tail (150 μg/ml)] directly to 300 μl of the incubation medium. The samples were incubated for 60 min at 4°C, and the antigen−antibody complexes were precipitated with Pansorbin. The immunoprecipitates were then washed as described above.

Endoglycosidase H digestions

In some cases, the immunoprecipitated VSV G was digested with endoglycosidase H prior to gel electrophoresis in order to distinguish between core- and terminally-glycosylated forms. The antigen−antibody complexes were precipitated with Pansorbin as described above, washed twice with lysis buffer supplemented with 5 mg/ml BSA and 300 mM additional NaCl and once with lysis buffer supplemented only with an additional 300 mM NaCl. The samples were resuspended in 100 μl 200 mM sodium citrate pH 5.0 containing 10 μg/ml each of chymostatin, leupeptin, antipain and pepstatin. After addition of 5 μl endoglycosidase H (1 U/ml) per sample, they were incubated at 37°C overnight. The immune complexes were released from the Pansorbin by adding 1 μl 20% (w/v) SDS and heating at 95°C for 3 min. The protein was concentrated by precipitating with an equal volume of cold 40% (w/v) trichloroacetic acid (TCA) for 30 min. The precipitated protein was collected by centrifugation, washed with ice-cold ethanol and dissolved in SDS gel sample buffer.

Gel electrophoresis

Samples to be analysed by PAGE were dissolved in gel sample buffer [50 mM Tris pH 6.8, 2.5 mM EDTA, 2% (w/v) SDS, 5% (w/v) glycerol, 0.01% bromophenol blue and 2.5% (v/v) 2-mercaptoethanol]. VSV G samples were resolved on 10% polyacrylamide gels and FPV HA samples were resolved on 10% polyacrylamide gels containing 8 M urea. After electrophoresis, the gels were fixed in 45% methanol and 7% acetic acid and treated for fluorography using Entensify (Dupont). The amount of radioactivity in the viral protein was quantitated by cutting the appropriate region from the dried gel (using the autoradiogram as a template) and liquid scintillation counting in Rotiszint liquid scintillation cocktail (Roth). The amount of HA cleavage was calculated from the amount of radioactivity present in the uncleaved HA and HA2 bands. Since HA2 contains the same number of methionine residues as HA1 (Ward, 1981), the percent cleavage is calculated as (2 × HA2)/(uncleaved HA + 2 × HA2).

Plasma membrane labelling

Filter cultures which had been labelled with C6-NBD-ceramide were washed twice with cold PBS+ and excess liquid was aspirated. The basal side of the filter was placed on a 100 μl drop of PBS+ layered on parafilm. 400 μl 1 mM $NaIO_4$ in PBS+ was added to the apical side. The filters were incubated in a humid chamber on ice for 15 min. The $NaIO_4$ was removed, the filters were washed in two changes cold PBS+, and placed on a 100 μl drop of PBS+. A stock solution of $NaB[^3H]_4$ (0.1 mCi/μl) was prepared in 0.01 M NaOH and stored at −70°C. Just prior to use a small aliquot of the $NaB[^3H]_4$ was thawed and diluted to 5 μCi/μl in PBS+ and 200 μl of this solution was added to the apical side of the filter. The cells were labelled on ice in a humid chamber for 20 min with occasional agitation. The label was removed and the filters washed twice with cold PBS+. The cells were immediately perforated as described above. Approximately 80% of the acid insoluble radioactivity was removed with the nitrocellulose filter used to perforate the cells.

Biochemical assays

The susceptibility of the VSV G in the medium fraction to trypsin digestion was determined as follows. A 300 μl sample of the medium fraction was treated with 0.25 mg/ml trypsin (TPCK-treated) in the presence or absence of 1% Triton X-100 for 30 min at 0°C. The trypsin was inhibited by the addition of soybean trypsin inhibitor to a final concentration of 1.25 mg/ml and incubated for 15 min at 0°C. Finally, Triton X-100 (1% final concentration) was added to those samples treated in the absence of detergent.

Galactosyl-transferase activity was measured using ovalbumin and UDP-D-[6-^{3}H]galactose as substrates. The reaction mixtures contained the following in 125 μl: 50 mM Tris pH 7.5, 12.5 mM $MnCl_2$, 1.25 mg ovalbumin, 0.1 mM unlabelled UDP-galactose, 1.25 μCi UDP-D-[6-^{3}H]-galactose, and 100 μl cell or medium sample in 5 mM Hepes pH 7.4, 1% Triton X-100. The reaction was carried out at 37°C for 30 min and terminated by the addition of 0.9 ml cold 20% TCA. The precipitated protein was collected by centrifugation, washed twice with 20% TCA, dissolved in 0.1 M NaOH, 0.1% SDS, and counted in a liquid scintillation counter using Ready Safe scintillation cocktail (Beckman). Sialyl-transferase activity was measured using asialofetuin and CMP-*N*-acetyl-[^{14}C]neuraminic acid as substrates. The reaction mixtures contained the following in 125 μl: 50 mM sodium cacodylate pH 6.6, 0.63 μg asialofetuin, 40 μM unlabelled CMP-*N*-acetyl neuraminic acid, 0.31 μCi CMP-*N*-acetyl-[^{14}C]neuraminic acid and 100 μl cell or medium sample in 5 mM Hepes pH 7.4, 1% Triton X-100. The reaction was carried out at 37°C for 60 min, terminated by addition of 0.9 ml cold 20% TCA, and processed as described for the galactosyl-transferase assay.

Endosomes were labelled with horseradish peroxidase (HRP) by including 5 mg/ml HRP in the water bath medium added to both the apical and basolateral sides of the filter during incubations for 5 min at 37°C followed by 90 min at 20°C. The cells were perforated and incubated in GGA buffer containing EGTA and ATP for 60 min at 37°C. The distribution of HRP activity between the cells and the incubation medium was determined according to Gruenberg and Howell (1986).

Other markers were measured according to published procedures. β-*N*-acetyl-glucosaminidase activity was measured as described by Harms *et al.* (1980). Lactate dehydrogenase activity was measured using an assay kit obtained from Sigma. Total acid insoluble radioactivity was determined by TCA precipitation either on a Whatman 3MM filter (^{35}S-labelled

samples) or from solution (^{3}H-labelled samples) followed by liquid scintillation counting. Protein was determined by the method of Markwell *et al.* (1978) using BSA as a standard.

Triton X-114 extraction and phase separation was performed by a modification of the procedure described by Bordier (1981). One ml of a cold 1% (w/v) Triton X-114 solution in PBS was added to a 50 μl sample of each gradient fraction on ice. The samples were mixed and incubated at 37°C for 5 min to promote phase separation. The samples were subjected to centrifugation for 1 min in an Eppendorf microfuge and the aqueous supernatant discarded. The detergent phase was solubilized in scintillation cocktail for determination of radioactivity.

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