The EMBO Journal vol.12 no.4 pp.1597-1605, 1993

Caveolae and sorting in the *trans*-Golgi network of epithelial cells

Paul Dupree, Robert G.Parton, Graça Raposo[1], Teymuras V.Kurzchalia[2] and Kai Simons[3]

Cell Biology Programme, European Molecular Biology Laboratory, Postfach 102209, 6900 Heidelberg, Germany and [1]Centre d'Immunologie, INSERM, CNRS, Marseille, France
[2]Present address: Max-Delbrück Centre for Molecular Medicine, Robert-Rössle Strasse 10, D-1115 Berlin-O, Germany
[3]Corresponding author

VIP21 is a 21 kDa membrane protein present in TGN-derived transport vesicles isolated from the epithelial MDCK cell line. The membrane topology and subcellular localization of VIP21 were studied using antibodies against the N- and C-terminal domains. The protein was found to have a structure with little or no exposure to the exoplasmic side of the membrane. VIP21 was localized to the TGN, consistent with its presence in TGN-derived transport vesicles. Unexpectedly, it was also very abundant in the non-clathrin-coated plasma membrane invaginations called caveolae. We have previously proposed that VIP21 is associated with glycosphingolipid-enriched membrane domains in the TGN which may be involved in the sorting of proteins into vesicles directed to the apical plasma membrane. Caveolae are specialized lipid structures with similarities to the glycolipid microdomains in the TGN. The presence of VIP21 in both locations suggests that the mechanisms governing inclusion of proteins into caveolar plasma membrane domains are related to the processes of protein and lipid sorting at the TGN. This connection is confirmed by the recent finding that the amino acid sequence of VIP21 is almost identical to that of caveolin, a protein previously localized to caveolae.

Key words: β-adrenergic receptor/caveolae/endocytosis/glycolipid/VIP21

Introduction

In simple epithelial cells, the protein composition of the apical plasma membrane differs from that of the basolateral plasma membrane, reflecting their different vectorial transport and barrier functions (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). Furthermore, the lipid composition of the plasma membrane domains is polarized (Simons and van Meer, 1988). The apical membrane has an unusually high glycosphingolipid content, which may protect the cell against a harsh external environment. This observation led to the proposal that glycolipids and apical proteins are co-sorted in the *trans*-Golgi network (TGN) into the vesicular carriers that transport membrane and proteins to the plasma membrane (Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990). According to this hypothesis, glycolipid self-association into microdomains or 'rafts', floating in an otherwise more fluid lipid bilayer, form the basis for the budding of an apical vesicle. These rafts could form the scaffold onto which apically destined proteins attach, either by direct interaction with the lipids or indirectly via interaction with sorting machinery or other apically sorted proteins. Proteins destined for the basolateral membrane would be excluded from these structures.

The Madin-Darby Canine Kidney (MDCK) cell line is an excellent model system for studying the sorting process, forming polarized cell sheets when grown on permeable filter supports (Rodriguez-Boulan, 1983). The spike glycoproteins of enveloped viruses have been successfully used as model plasma membrane proteins in these cells (Matlin and Simons, 1984; Wandinger-Ness *et al.*, 1990). Influenza haemagglutinin (HA) is transported along the apical pathway, whereas the vesicular stomatitis virus G (VSV-G) protein follows the route of basolateral plasma membrane proteins (Rodriguez-Boulan and Pendergast, 1980). Using antibodies against the viral spike glycoproteins, the TGN-derived vesicular carriers have been isolated and their protein composition analysed by two-dimensional gel electrophoresis (Bennett *et al.*, 1988; Wandinger-Ness *et al.*, 1990). It was shown that the apical and basolateral vesicles have similar but distinct protein compositions. Under the conditions of viral infection, synthesis of endogenous proteins is almost completely abolished (Wandinger-Ness *et al.*, 1990), yet transport from the TGN to the plasma membrane continues for many hours. It is therefore probable that vesicle components are recycled to allow multiple rounds of transport. The components of the vesicles isolated under these conditions are thus good candidates for proteins involved in vesicular traffic in the cycle between the TGN, plasma membrane and the pathway back to the TGN.

One prediction of the glycolipid-based sorting hypothesis is that there should be specific interactions between glycolipids and some apically-directed proteins or vesiculation machinery (Simons and van Meer, 1988). This led us to investigate the detergent solubility properties of influenza HA and the other apical vesicle proteins (Kurzchalia *et al.*, 1992). Indeed, influenza HA, together with a subset of these proteins, was found not to be solubilized under conditions that solubilized the basolateral marker protein, VSV-G. This insolubility was remarkably specific, allowing purification of one protein of the vesicles, in just two detergent solubilization steps from crude cell membranes, in sufficient quantities for microsequencing and molecular cloning. The protein was named VIP21 (*v*esicular *i*ntegral membrane *p*rotein of 21 kDa).

As a protein potentially cycling between the TGN and the plasma membrane, the subcellular distribution of the endogenous VIP21 protein could give information about its role in intracellular traffic. Using antibodies raised against the N- and C-terminal domains of the protein, we found that neither was extracellular. As expected of a component of TGN-derived transport vesicles, VIP21 was localized to the Golgi. However, VIP21 was also abundant in the plasma membrane domains termed caveolae. Caveolae show several

interesting similarities with the glycolipid rafts of apical vesicles, and the presence of VIP21 in both structures strongly suggests a functional relationship between them. We therefore propose that related but distinct processes govern inclusion of lipids and proteins into both caveolae and apical carrier vesicles.

Results

Specificity of anti-VIP21 polyclonal antibodies

To determine the subcellular distribution and topology of VIP21, polyclonal antisera were raised in rabbits against synthetic peptides derived from the extreme N- and C-termini of the protein. These peptides were chosen because it was thought that these epitopes would be more accessible than those closer to the hydrophobic, membrane-spanning domain. Furthermore, these antibodies would be useful in determination of the protein topology. After affinity purification of the peptides used for immunization, the antisera were tested for monospecificity by Western blot analysis of MDCK membrane proteins (Figure 1A). The C-terminal antiserum (VIP21-C) reacted with two polypeptides of apparent molecular weight 21 and 20 kDa, corresponding to the two forms of VIP21 present *in vivo*, differing in pI by ~0.7 units (Kurzchalia *et al.*, 1992). Both these forms can be synthesized *in vitro* from a single cDNA clone, suggesting that they differ by a post-translational modification (Kurzchalia *et al.*, 1992). By comparison with the two-dimensional gels, it was clear that the 21 and 20 kDa bands correspond to the VIP21 polypeptides of higher and lower pI respectively. The N-terminal antiserum (VIP21-N) was also specific for VIP21, but recognized only the 21 kDa band. This suggests that the modification is within the N-terminal 20 amino acids of the higher mobility form and prevents interaction of the VIP21-N antibody with the protein. The modification is unlikely to be proteolysis at the N-terminus, since according to the amino acid sequence this would not cause such a large change in pI.

To confirm that both antibodies recognized the same protein, the VIP21-N-reactive polypeptide was immunoprecipitated from solubilized MDCK membranes and the precipitated protein was probed by Western analysis with the VIP21-C antiserum (Figure 1B). This confirmed that the antisera indeed recognized the same protein. However, both modified and unmodified VIP21 could be immunoprecipitated, suggesting that either VIP21 is part of a heterogenous oligomer that is resistant to detergent solubilization or that such a structure forms during immunoprecipitation. These data together show that both the sera recognize VIP21 alone.

Subcellular distribution of VIP21

MDCK cells were examined by immunofluorescence to determine the subcellular localization of the endogenous protein. The VIP21-C antiserum labelled a tubulo-reticular structure near the nucleus (Figure 2A), as well as showing some more diffuse punctate staining. The staining was specific, since it could be competed by pre-incubation of the antiserum with 1 μg/ml VIP21-C peptide (Figure 2B) and not by pre-incubation with the VIP21-N peptide (not shown). Since VIP21 was originally identified as a protein of TGN-derived transport vesicles, the relationship between this labelled structure and the TGN was examined further. After

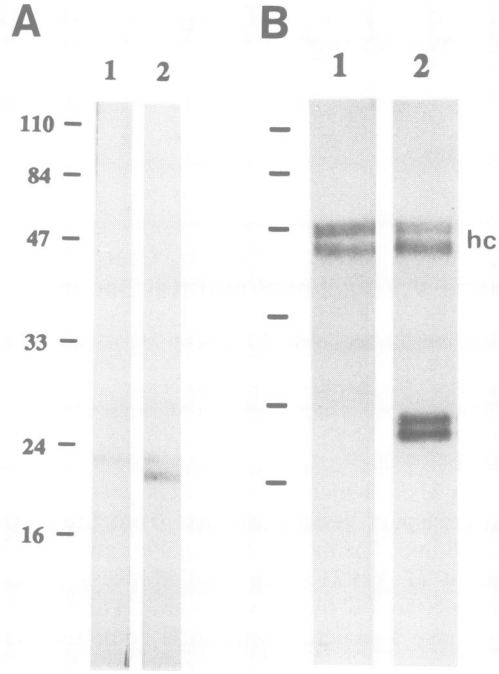

Fig. 1. Both VIP21-N and VIP21-C antisera recognize VIP21 alone. **A.** Western blot of MDCK cell membranes probed with affinity-purified VIP21-N (lane 1) and VIP21-C (lane 2) antisera. **B.** Immunoprecipitation followed by Western blotting. Extracts from MDCK cells were precipitated with ~10 μg of pre-immune serum (lane 1) or affinity-purified VIP21-N antibody (lane 2) and a blot was probed with affinity-purified VIP21-C antibody. hc−, heavy chain of the precipitating antibody revealed by the conjugated secondary anti-rabbit antibody. Molecular weight standards are as in panel A.

infection of cells with influenza WSN ts061, protein transport was blocked in the TGN by incubation at 20°C (Matlin and Simons, 1983). In these cells, VIP21 and the influenza HA co-localized (Figure 2C and D), in good agreement with the finding that they are both components of the detergent-insoluble structures in TGN-derived vesicles (Kurzchalia *et al.*, 1992). Although non-viral protein synthesis had been inhibited in this experiment by viral infection, VIP21 was still localized in the Golgi. Indeed, it was found that VIP21 was still present in the Golgi after 10 h of viral infection at 37°C (data not shown). Since it continually leaves the TGN in transport vesicles together with the viral protein, this observation suggests that VIP21 might recycle back to the Golgi apparatus.

In contrast to the labelling of the Golgi complex by the VIP21-C antiserum, the VIP21-N antiserum labelled punctate structures on or close to the plasma membrane in baby hamster kidney (BHK) cells, with very little internal staining (Figure 2F). Labelling was present on the apical as well as the basolateral membranes of polarized MDCK cells grown on filters (Figure 2E, and not shown), an observation consistent with the presence of VIP21 in both apical and basolateral vesicles (Wandinger-Ness *et al.*, 1990). The labelling could be competed by pre-incubation of the antisera with the VIP21-N peptide (data not shown). The reason for the low internal labelling by the VIP21-N antiserum is not clear, but the post-translational modification that prevents recognition on a protein blot might prevent this antibody recognizing the Golgi-localized VIP21.

To determine the precise location of VIP21 at the plasma

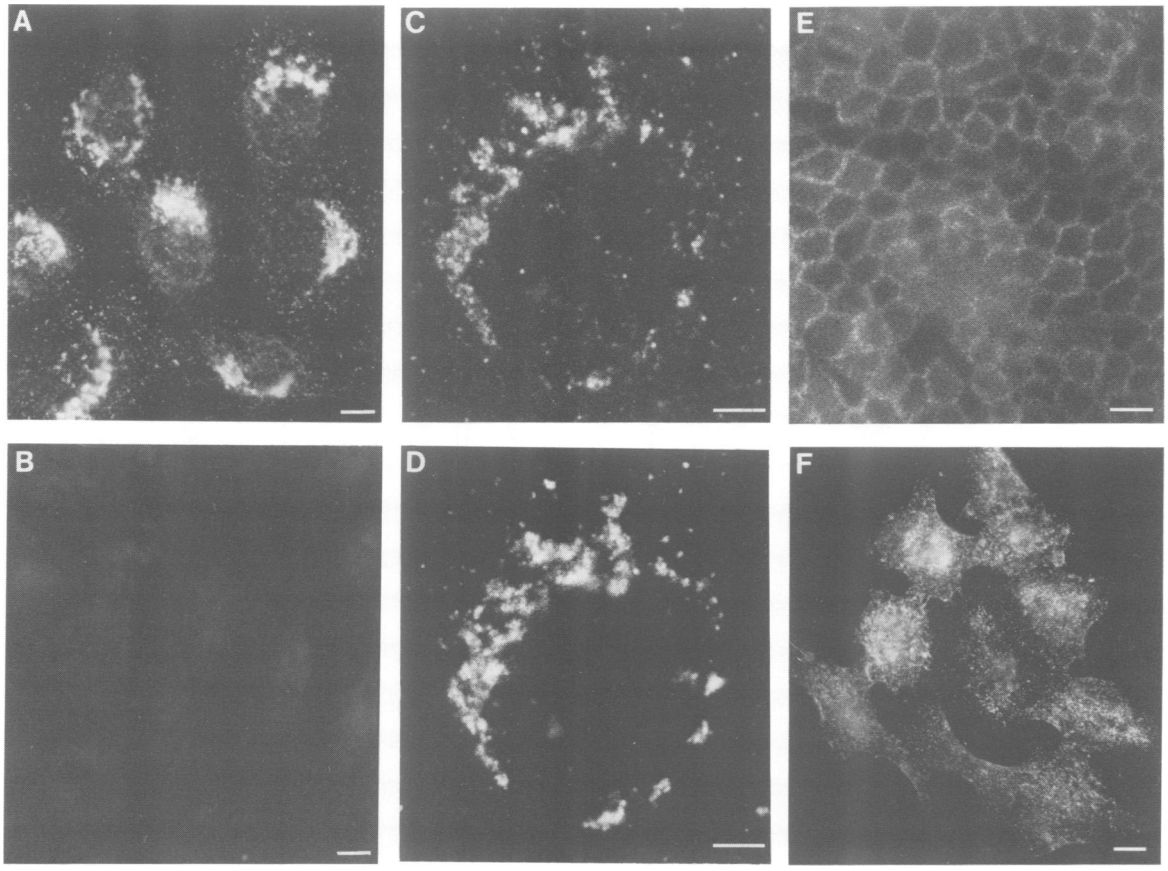

Fig. 2. Immunofluorescence of MDCK (A–E) and BHK cells (F). Labelling of the TGN with VIP21-C antiserum (**A**) can be competed by the specific peptide 1 μg/ml (**B**). VIP21-C labelling of the TGN (**C**) co-localizes with the influenza HA protein (**D**) blocked in transport by incubation of the cells at 20°C. Punctate staining of plasma membrane structures of filter-grown MDCK cells (**E**) and BHK cells (**F**) with the VIP21-N antiserum. Bars, A and B, 5 μm; C and D, 2 μm; E, 15 μm; F, 10 μm.

membrane, immunoelectron microscopy was performed on ultra-thin cryosections. Surprisingly, labelling for VIP21-N was found in non-clathrin-coated plasma membrane invaginations in BHK cells (Figure 3A and B). Whereas up to five gold particles labelled a non-clathrin-coated invagination, clathrin-coated pits and vesicles showed negligible labelling. More than 90% of the plasma membrane labelling was over non-clathrin-coated invaginations, whereas they occupied only 3.4 ± 0.7% (mean ± SEM) of the plasma membrane surface area (see Materials and methods) (Table I). An estimate for the density of VIP21 in the caveolar membranes is 8000 molecules/μm^2 (see Materials and methods). Of the very low labelling that was not clearly in the plasma membrane area, half was over endosome- and TGN-like structures. Consistent with the immunofluorescence data, labelling of both the apical and basolateral membranes of MDCK cells was observed (not shown).

VIP21 is localized to caveolae on the cell surface

The non-clathrin-coated plasma membrane invaginations labelling with VIP21 are similar to those named plasmalemmal vesicles (Palade and Bruns, 1968) and caveolae (Anderson *et al.*, 1992). These structures are especially abundant in endothelial cells (see Severs, 1988). We therefore examined whether such structures were labelled by the VIP21-N antiserum using ultrathin cryosections of endothelial cells from mouse intestine. As shown in Figure 3C, heavy labelling was evident on the caveolar membranes, confirming that the non-clathrin-coated membrane invaginations in the BHK and MDCK cells were caveolae, and therefore that VIP21 is abundant in these structures.

Subsequent to the preparation of this manuscript, the amino acid sequence of a 21 kDa caveolae protein named caveolin (Rothberg *et al.*, 1992) has been published (Glenney and Soppet, 1992). The amino acid sequence of VIP21 is 86% identical to that of the chicken caveolin. The differences probably reflect species variation. It therefore appears that caveolin is the chicken homologue of VIP21, confirming the plasma membrane localization of VIP21 to caveolae. These workers also saw labelling of the Golgi apparatus, but did not comment on the observation (Rothberg *et al.*, 1992).

β-adrenergic receptors redistribute into caveolae

Can VIP21-caveolin be used as a marker for studying segregation events in the membrane? β-adrenergic receptors provide an interesting example in this respect. β-adrenergic receptors have been reported to segregate into non-clathrin-coated invaginations on the surface of epidermoid A431 cells after incubation with a monoclonal antibody against the receptor and anti-mouse IgG–gold (Raposo *et al.*, 1989). We repeated these experiments using thin frozen section immunoelectron microscopy to find out whether or not the receptors co-localized with VIP21-caveolin under these conditions. As shown in Figure 4, after antibody binding

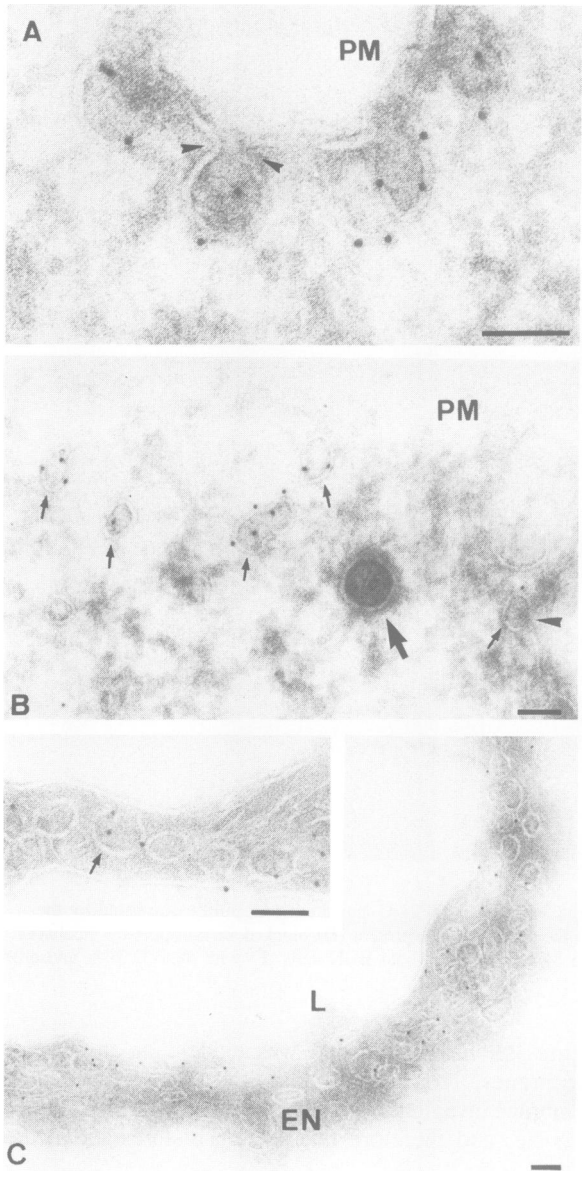

Fig. 3. VIP21-N labels non-clathrin-coated invaginations (caveolae). (**A** and **B**) Thawed frozen sections of formaldehyde-fixed BHK cells were labelled with anti-VIP21-N followed by 9 nm protein A−gold. In panel A the cell was sectioned almost perpendicular to the plasma membrane, whereas panel B shows a grazing section across the cell surface. Invaginations and possible vesicles (small arrows) of ~60−80 nm diameter close to the plasma membrane (PM) are labelled. Clathrin-coated vesicles (large arrow in panel B) are unlabelled. Arrowheads in panel A indicate the neck of a labelled flask-shaped invagination. A connection between a labelled structure and the plasma membrane is also evident in panel B (arrowhead). **C**. Section of mouse intestine labelled as above. An endothelial cell (EN) borders the capillary lumen (L). Heavy labelling is apparent on caveolae as shown at higher magnification in the inset. Bars, 100 nm.

and incubation at 37°C for 30 min, the receptors were present in sites containing VIP21-caveolin. A quantitative analysis of the plasma membrane distribution of the β-adrenergic receptors at this time point showed that 64 ± 7% of the gold label for the receptor was associated with VIP21-N-positive caveolae. The remainder was associated with VIP21-N-negative caveolar-like structures (17 ± 5%) and featureless plasma membrane (19 ± 6%). No labelling was evident in clathrin-coated pits. The receptors must

Table I. Quantification of cell surface VIP21-N labelling

	% associated with feature (± SEM)
Clathrin-coated pits and vesicles[a]	0.05 ± 0.15
Non-clathrin-coated invaginations and vesicles[a]	92 ± 5
Featureless plasma membrane	8 ± 5

The number of gold particles within 1 μm of the plasma membrane associated with the various features was determined.
[a]For the purpose of the quantification, plasma membrane attached and apparent free vesicles were not distinguished.

acquire affinity for caveolae after antibody modulation, since after labelling at 4°C they are more uniformly distributed in the plasma membrane (Raposo *et al.*, 1989).

Membrane topology of VIP21

Based on sequence information, we have previously speculated that VIP21 is a type II membrane protein containing a single transmembrane domain of 33 amino acids (Kurzchalia *et al.*, 1992). The N-terminal domain would be cytosolic and consist of 100 amino acids preceding the signal anchor sequence that is required to direct the nascent polypeptide to the membrane. In the type II membrane orientation, the 40 amino acid C-terminal domain would be lumenal in the TGN and extracellular at the plasma membrane, and therefore available for antibody binding. In addition to the sequence information, the protein has been classified as an integral membrane protein owing to its partitioning into the detergent phase of TX114 (Kurzchalia *et al.*, 1992), and the fact that it was very effectively labelled by the hydrophobic labelling agent iodonaphthylazide (INA) (K.Fiedler, T.Kobayashi, T.V.Kurzchalia and K.Simons, in preparation). To support this classification, MDCK membranes were extracted with sodium carbonate, pelleted and then VIP21 was analysed by Western analysis of the supernatant and pellet fractions. Figure 5 shows that the protein is not extracted by this treatment, confirming that it behaves as an integral membrane protein by this criterion in addition.

For further studies on the localization and routing of VIP21, it is essential to know the membrane topology. The bacterial toxin streptolysin-O (SLO) can be used to permeabilize the plasma membrane (Bhakdi and Tranum-Jensen, 1987; Gravotta *et al.*, 1990), allowing access of antibodies to the cytosolic domains of membrane proteins. It binds to cholesterol in the membranes at 4°C, but holes are made in the membrane only after the excess has been washed away and the temperature raised to 37°C (Hugo *et al.*, 1986). Thus, under these conditions internal membranes are not permeabilized. After partial SLO permeabilization of BHK cell plasma membranes, only those cells that were permeabilized, as judged by access of the mictrotubule antibody, gave positive labelling of the caveolae by the VIP21-N antiserum (Figure 6A−C). Neither antibody labelled unpermeabilized cells. This clearly demonstrates that the VIP21-N epitope is intracellular as originally proposed. An experiment was carried out using the VIP21-C antiserum to determine the membrane orientation of the C-terminal domain. Since the plasma membrane even of saponin permeabilized cells was not strongly labelled (see

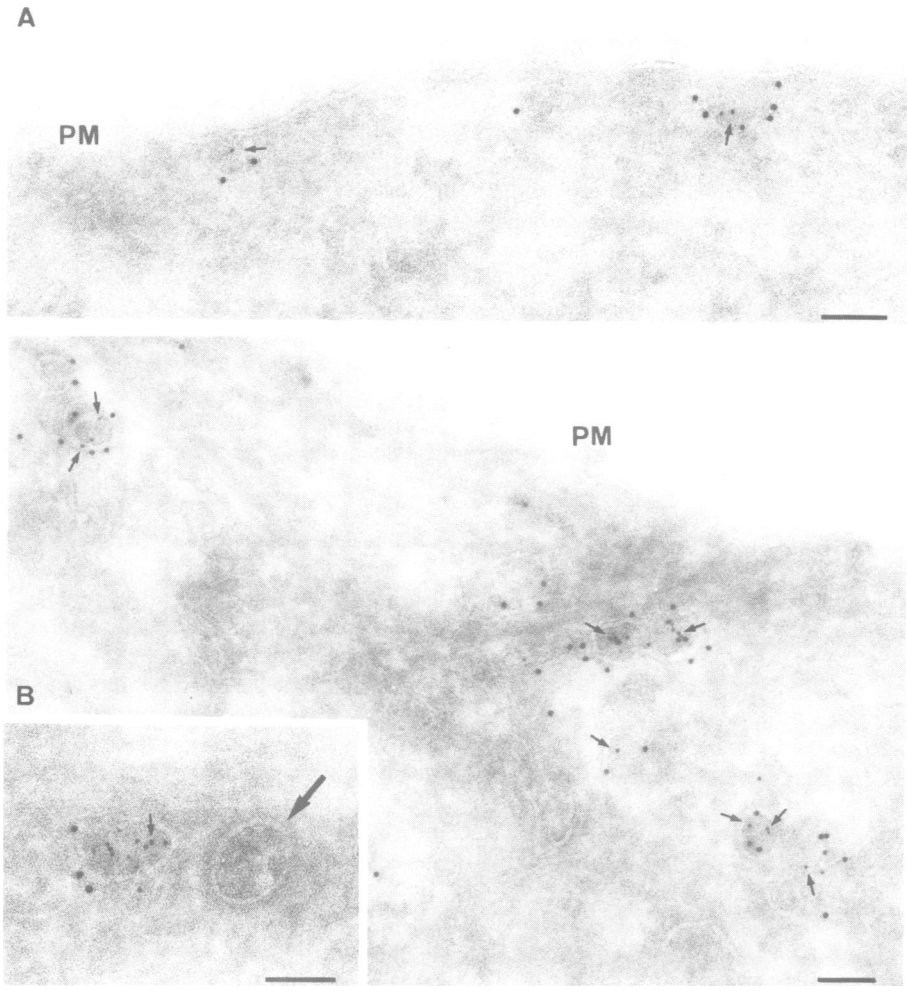

Fig. 4. Co-localization of the β-adrenergic receptor with VIP21-N in A431 cells. A431 cells were surface-labelled at 4°C with antibody and 5 nm gold particles. The cells were then warmed to 37°C for 30 min. After fixation thin frozen sections were prepared and labelled with VIP21-N antiserum followed by 9 nm protein A−gold. Labelling for the receptor (5 nm gold particles, small arrows) is evident within invaginations and vesicular profiles which are positive for VIP-21N (9 nm gold particles). In the inset an unlabelled clathrin-coated profile is indicated (large arrow). PM, plasma membrane. Bar, 100 nm.

Figure 2A), the accessibility of the Golgi-localized epitope was of interest. If the epitope were lumenal, there should be no labelling after SLO permeabilization of the plasma membrane. However, after permeabilization, the typical Golgi labelling was observed (Figure 6D−F). This indicates that like the N-terminal domain, the C-terminal domain is cytosolic. The unlikely possibility that the TGN was permeabilized in this experiment was ruled out, since a monoclonal antibody against a lumenal VSV-G epitope did not label the viral protein after accumulation in the TGN under the same conditions as in Figure 6 (data not shown). Thus both the N- and C-termini of the protein are available for antibody binding in the cytosol. Furthermore, after trypsinization of MDCK microsomal membranes, neither antibody detected a protected fragment (data not shown). Together with the data that VIP21 is an integral membrane protein, this suggests that the protein has two membrane spanning domains. However, the hydrophobic region of 33 amino acids is not divided by a helix−turn motif and is rather short for two such helices because they require on average 24 amino acids to traverse the membrane. The length would be sufficient for two membrane-spanning helices with the inclusion of further residues including arginine and lysine (possible since these amino acids contain long hydrophobic chains) at each end of the hydrophobic region. Analysis of the sequence suggests that the helices formed may be amphipathic in nature. Since VIP21-caveolin may oligomerize (see above and Glenney 1989), one can speculate that the polar or less hydrophobic residues of the helices interact with other VIP21 molecules, whereas the hydrophobic side faces the lipid. The possibility of other structures including a hairpin-like structure not composed of helices cannot yet be excluded.

Discussion

The hypothesis of co-sorting of apically-destined proteins and glycolipid microdomains in the TGN of epithelial cells led to the identification, purification and molecular cloning of VIP21 as a component of a detergent-insoluble complex from vesicles (Kurzchalia *et al.*, 1992). There are strong indications that the resistance to detergent extraction of some components in these apical vesicles reflects the existence of glycolipid microdomains. The apical influenza HA protein can be solubilized by Triton X-100 when it is in the endoplasmic reticulum of MDCK cells, but becomes

insoluble during its passage through the Golgi when it encounters glycolipids (Skibbens *et al.*, 1989). Similarly, we have shown that this HA protein becomes incorporated into a CHAPS-insoluble complex in the TGN, whereas the basolateral marker, the VSV-G protein, does not (Kurzchalia *et al.*, 1992; K.Fiedler, T.Kobayashi, T.V.Kurzchalia and K.Simons, in preparation). Furthermore, Brown and Rose (1992) have demonstrated that GPI-linked proteins, which are transported apically in MDCK cells, become incorporated into Triton X-100-insoluble complexes in the Golgi complex and these are enriched in glycosphingolipids. The similarity between the CHAPS- and the Triton X-100-insoluble material has recently been confirmed (K.Fiedler, T.Kobayashi, T.V.Kurzchalia and K.Simons, in preparation). The relationship between detergent insolubility and the glycolipid microdomains is thus quite firmly established (Brown, 1992).

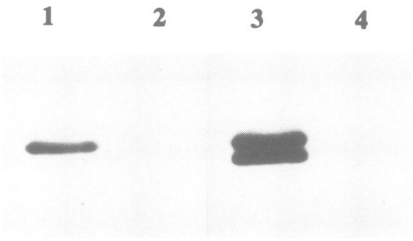

Fig. 5. VIP21 cannot be extracted by sodium carbonate. A sodium carbonate extract of MDCK cell membranes was separated into pellet (non-extracted proteins, lanes 1 and 3) and supernatant (extractable proteins, lanes 2 and 4) fractions. After PAGE and blotting to nitrocellulose membranes of duplicate gels, the membranes were probed with affinity-purified VIP21-N (lanes 1 and 2) or VIP21-C antiserum (lanes 3 and 4).

The surprising finding that VIP21 is abundant in caveolae strongly links the glycolipid domains in the TGN to these plasma membrane structures. Recent sequence data demonstrating that canine VIP21 is homologous to chicken caveolin (Glenney and Soppet, 1992) confirms the caveolar localization of VIP21. What is most interesting in this context is that caveolae are plasma membrane invaginations with a specialized lipid composition (Anderson *et al.*, 1992). Although it has not been demonstrated directly that they are enriched in glycosphingolipids, they are probably enriched in the gangliosides to which cholera and tetanus toxins bind (Montesano *et al.*, 1982; Parton *et al.*, 1988), and it has been shown that cholesterol is essential for their integrity (Rothberg *et al.*, 1990; Chang *et al.*, 1992). Moreover, GPI-linked proteins, which lack a transmembrane domain and are anchored to the membrane by their lipid modification, are often localized in caveolae (Rothberg *et al.*, 1990; Anderson *et al.*, 1992). Thus, caveolae have many circumstantial but intriguing similarities to the glycolipid rafts of apical carrier vesicles (Rothberg *et al.*, 1990; Lisanti and Rodriguez-Boulan, 1991; Brown, 1992). The presence of VIP21-caveolin in both structures now makes a functional connection between them.

We must now consider the rafts as a general phenomenon, whereby certain proteins can be segregated within a lipid bilayer. The original proposal was that they would be involved in protein sorting in the Golgi complex of epithelial cells (Simons and van Meer, 1988). The similarity of this process to that apparently occurring in the generation of caveolae suggests that the phenomenon is more widespread. However, as yet unidentified proteins must determine the specific association with either caveolae or apical vesicles that distinguishes these processes. Since VIP21 is also routed to caveolae on the basolateral side, specificity for apical

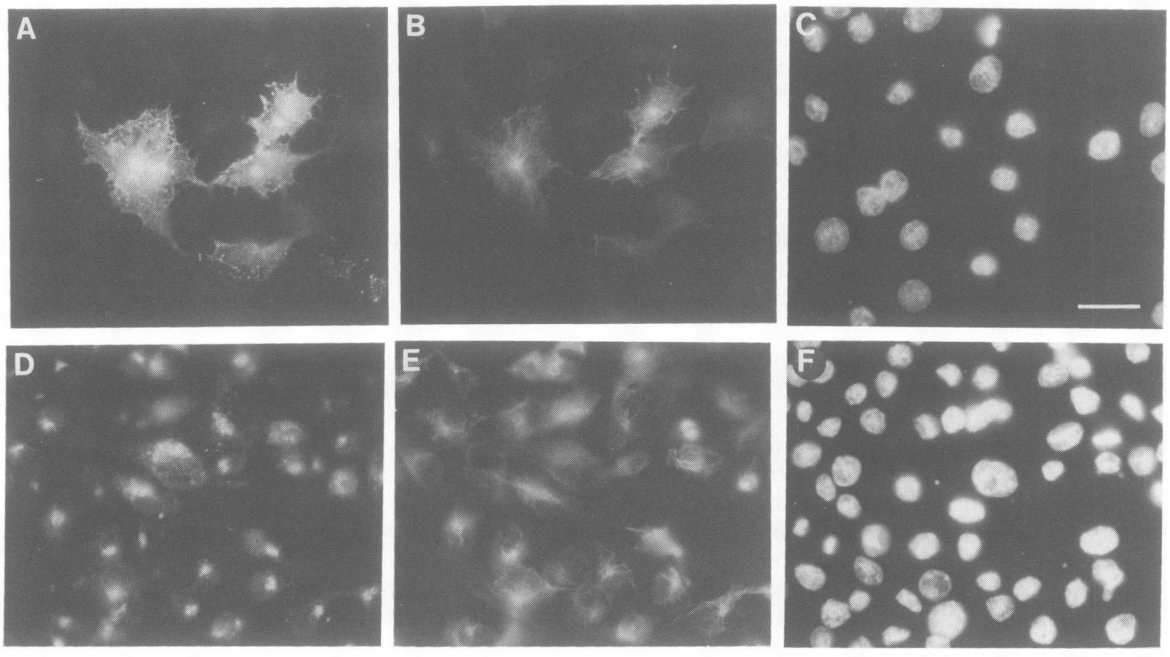

Fig. 6. Topology of VIP21 N- and C-terminal domains revealed by indirect immunofluorescence with domain-specific antibodies. BHK cell plasma membranes were partially permeabilized using SLO. (A–C) The VIP21-N antiserum labels the plasma membrane (**A**) only when the cells are permeable, as judged by accessibility of the anti-microtubule antibody (**B**). The nuclei of many unpermeabilized cells not stained with either antibody are visible after Hoechst staining (**C**). (D–F) The VIP21-C antiserum labels the Golgi complex (**D**) from the cytosolic side of the membrane. The plasma membranes of most cells were permeabilized, as judged from microtubule staining (**E**) and nuclei (**F**). The diffuse microtubule staining is a consequence of the PFA fixation. Bar, 20 μm.

sorting must involve other proteins. Just as VIP21 is present in non-polarized cells and the basolateral membrane of epithelial cells, so caveolae are also present in these locations. Indeed, although glycolipids are sorted preferentially to the apical membrane, some glycolipids are routed basolaterally (Simons and van Meer, 1988). It is therefore not surprising that VIP21, which seems to be a more general marker of these domains, is also present in the basolateral membrane.

In the processes of sorting into apical vesicles and into caveolae, the association of many proteins with these membrane domains may be transient and regulated. Thus in epithelial cells, apically-destined proteins associate with the rafts in the TGN, but dissociate on reaching the plasma membrane. This concept is further demonstrated by the clustering of β-adrenergic receptors after the addition of antibodies to the receptor on the cell surface. On the one hand, these receptors are known to be localized on the basolateral membrane of epithelial cells (Simmons *et al.*, 1984) and should therefore be excluded from the membrane patches that form apical vesicles in the TGN. On the other hand, the receptors can redistribute into caveolae, co-localizing with VIP21-caveolin. What brings about this clustering of β-adrenergic receptors to the caveolae remains to be defined. It is probable that there are specific components interacting with the glycolipid rafts that reversibly bind the antibody-modulated receptors. It is interesting that VIP21 seems to be undergoing a structural change or a post-translational modification that can be monitored by antibodies at the cell surface and intracellularly. Such changes may control the direction of similar binding events.

Signal transduction events may also involve glycolipid microdomains. Antibodies against several GPI-anchored proteins can activate T-cells via *src* kinases (see Robinson, 1991), an observation without an immediately obvious explanation since these proteins have no transmembrane domains to interact with each other. However, together with evidence that these GPI-linked proteins can be immunoprecipitated with the *src*-family tyrosine kinases and glycolipids after plasma membrane solubilization (Stefanova *et al.*, 1991; Cinek and Horejsi, 1992), one can propose that the interaction is similarly mediated by detergent-insoluble lipid domains (Cinek and Horejsi, 1992; Thomas and Samelson, 1992).

If the sorting into apical vesicles is dependent on glycosphingolipid rafts, it is unlikely that *de novo* lipid synthesis would satisfy the requirements, and it was originally proposed that these might have to recycle back to the TGN (Simons and van Meer, 1988). The observation that VIP21-caveolin is abundant in the TGN-derived vesicles, and yet a pool is maintained in the TGN, suggests that the protein might recycle from the plasma membrane. It is also not consistent with the model that VIP21-caveolin is a resident component of caveolar membrane coats (Rothberg *et al.*, 1992). We have shown that the protein was absent from clathrin-coated pits and vesicles, but very abundant in the caveolae. Do VIP21-caveolin and glycolipid rafts recycle to the TGN via caveolae? If this were the case caveolae could be involved in endocytosis (Moya *et al.*, 1985; Sandvig *et al.*, 1987; Van Deurs *et al.*, 1989). Although this possibility is supported by our observations, our data do not directly address this question. Some recent evidence has favoured a role of caveolae in potocytosis, a process in which these structures would not be released from the plasma membrane into the cell (Anderson *et al.*, 1992). However, other studies have demonstrated that cholera and tetanus toxins are internalized by a non-clathrin-mediated pathway (Montesano *et al.*, 1982; Tran *et al.*, 1987). The β-adrenergic receptors, shown here to co-localize with VIP21, have been found to internalize into endosomes after antibody addition (Raposo *et al.*, 1989). Furthermore, both a CD4 construct with a GPI anchor (Keller *et al.*, 1992) and recently the GPI-anchored 5′ nucleotidase (Schell *et al.*, 1992) were found to be internalized by an alternative endocytic route, observations that would also suggest that these glycolipid domains are endocytosed. As we have found that VIP21-caveolin has no external domain that could be used for recycling studies to prove that this protein returns to the TGN, such experiments must await the isolation of further molecules from these structures. Whether or not caveolae are directly involved in endocytosis, we believe that glycolipid domains will soon be seen to be an important feature of several membrane processes.

Materials and methods

Materials

Unless otherwise stated, all chemicals were obtained from the sources described previously by Wandinger-Ness *et al.* (1990). Streptolysin-O was from Wellcome Diagnostics. The mouse monoclonal antimicrotubule 1AL was a gift from T.Kreis (EMBL). The mouse monoclonal anti-VSV G 17.2.21.4 was previously prepared in this laboratory.

Cell culture and viral infection

Growth media compositions, cells, viruses and cell culture protocols were described previously by Wandinger-Ness *et al.* (1990), Kurzchalia *et al.* (1992) and Raposo *et al.* (1989). Viral infection with WSN ts61 and accumulation of HA in the TGN by a temperature block at 20°C were as described by Matlin and Simons (1983).

Preparation of antibodies

Polyclonal sera were raised against synthetic peptides covalently coupled to keyhole limpet haemocyanin using the N-terminal residues 1–20 of canine VIP21 (MSGGKYVDSEGHLYTVPIRE, single letter code) (VIP21-N) or C-terminal residues 161–178 (EAVGKIFSNIRINMQKET) (VIP21-C) according to Kreis (1986). Sera were collected after the sixth injection of antigen. For affinity purification, the N-terminal peptide was linked directly to CNBr-activated Sepharose 4B according to the manufacturer (Pharmacia). The C-terminal peptide showed low solubility in aqueous buffers and therefore was linked to Affigel-10 (Bio-Rad) by incubation for 4 h at room temperature in 100% dimethyl formamide. Serum (2 ml) was passed continuously over the matrices overnight at 4°C. Bound antibody was eluted with 0.2 M glycine pH 2.8 and the fractions neutralized with unbuffered Tris.

Carbonate extraction, immunoprecipitation and Western analysis

Total MDCK cellular membranes were prepared from a post-nuclear supernatant (Kurzchalia *et al.*, 1992) by centrifuging at 100 000 *g* for 30 min. To determine the extent of membrane association by carbonate extraction, a membrane pellet was extracted by a freshly prepared solution of 0.1 M Na_2CO_3 (final concentration) for 15 min on ice. The membranes were recovered by centrifugation at 110 000 *g* for 10 min at 4°C and the extraction repeated. The pellet was analysed directly, whereas the supernatant was neutralized with HCl before further analysis.

For immunoprecipitation, MDCK membranes were solubilized in buffer (50 mM Tris pH 7.4, 2 mM EDTA, 150 mM NaCl, 1% NP-40, 0.4% sodium deoxycholate and 0.4% SDS) at 4°C and BSA added to 1 mg/ml. After removing insoluble material by microcentrifugation, antibody was added to 15 μg/ml. After incubation overnight at 4°C, protein A–Sepharose (Pharmacia) was added to allow precipitation of antibody–protein complexes by microcentrifugation.

Samples were separated by reducing SDS–PAGE (15%) (Laemmli, 1970) before transfer to nitrocellulose in a blotting buffer consisting of 25 mM Tris, 190 mM glycine and 20% methanol. Blots were incubated with 1:50

dilutions of the affinity-purified sera overnight at 4°C and then in 1:5000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad) as the second antibody for 1 h, using a blocking a solution of 5% non-fat dried milk, 0.2% Tween-20 (Sigma). Bands were detected using diaminobenzidine (0.6 mg/ml) in 50 mM Tris pH 7.6 and hydrogen peroxide (0.03%).

Immunofluorescence microscopy

Cells grown on coverslips were washed twice with phosphate-buffered saline (PBS) and permeabilized with 0.05% saponin in 80 mM PIPES pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$ for 5 min. The cells were fixed in 3% paraformaldehyde in PBS for 15 min, rinsed in PBS and the aldehyde groups quenched with 50 mM NH_4Cl in PBS for 15 min. After a further rinse in PBS the cells were incubated in 10% heat-inactivated fetal calf serum (FCS) for 30 min and the first antibody diluted in 5% FCS−PBS was added. The affinity-purified N-terminal antiserum was used at a dilution of 1:50, whereas the VIP21-C serum was diluted 1:200. The cells were incubated for 30 min at 37°C and washed effectively with PBS before visualizing the primary antibody with pre-adsorbed donkey anti-rabbit rhodamine-conjugated or donkey anti-mouse FITC-conjugated antibodies (Dianova, Hamburg, Germany). However, MDCK cells on coverslips were permeabilized with 0.01% saponin when incubated with the VIP21-C antiserum alone, and MDCK cells grown on filters were permeabilized with 0.2% Triton X-100 for 5 min. The coverslips were viewed and photographed with an Axiophot photomicroscope (Carl Zeiss).

Immunoelectron microscopy

Cells were removed from the culture dishes according to Green *et al.* (1981). They were then fixed with 8% paraformaldehyde in 250 mM HEPES pH 7.35, prepared for cryosections and labelled with antibodies and protein A−gold as described previously by Griffiths *et al.* (1984, 1985). Mouse intestine was fixed by immersion and processed as described above. For quantification of VIP21-N labelling >2500 immunogold particles were counted within 1 μm from the plasma membrane. This criterion was used based on the apparent distance of surface-connected caveolae from the plasma membrane of cells in a pellet after labelling the cell surface for 30 min at 4°C with 25 μg/ml wheat germ agglutinin-HRP (Sigma). The percentage of these gold particles associated with non-coated invaginations or apparent vesicles, with featureless plasma membrane or with clathrin-coated structures, was determined. The maximum density of labelling associated with the caveolar membrane was ~400 gold/μm^2 calculated from the maximum observed number of gold particles per caveolar profile of mean diameter 64 ± 12 nm (mean ± SEM of measurements on 42 vesicles). Assuming a labelling efficiency of 5%, in the range of previous estimates (Griffiths, 1993) an estimate of 8000 VIP21 molecules/μm^2 was obtained. The percentage of the plasma membrane occupied by caveolae (defined morphologically as uncoated surface invaginations) was determined by intersection counting on epon sections of vertically cut cells as described for coated pits previously by Griffiths *et al.* (1989) and Bucci *et al.* (1992). The estimated area of the plasma membrane occupied by caveolae was 3.4 ± 0.7%, compared with 1.6 ± 0.3% for clathrin-coated pits (Griffiths *et al.*, 1989).

A431 cells were incubated with the monoclonal antibody BRK2 raised against the β-adrenergic receptor followed by 5 nm gold-labelled second antibodies at 4°C as described previously by Raposo *et al.* (1989). The cells were then incubated at 37°C for 30 min before fixation in 0.5% glutaraldehyde−250 mM HEPES and processing for frozen sections. Sections were treated for 10 min with free protein A (0.1 mg/ml) prior to labelling to block any available protein A binding sites. They were then incubated with VIP21-N antiserum and 9 nm protein A−gold. Negligible labelling was observed when the primary antibody was omitted. For quantification of β-adrenergic receptor labelling, the number of 5 nm gold particles associated with VIP21-N-positive and VIP21-N-negative caveolae, clathrin-coated pits or featureless plasma membrane was determined. More than 800 immunogold particles were counted.

Streptolysin-O permeabilization of cells

BHK cells grown on coverslips for 36 h were washed twice in PBS (4°C) and then a solution of SLO (1 U/ml) in SLO buffer (50 mM potassium−PIPES pH 7.0, 5 mM $MgCl_2$, 50 mM KCl, 2 mM EGTA and 1 mM DTT) was added. After 10 min incorporation of SLO into the plasma membrane at 4°C, the excess SLO was removed by two rinses with SLO buffer. After addition of further SLO buffer plus 0.2% BSA, 1 μM taxol (SLO+ buffer) to stabilize the microtubules, the cells were warmed to 37°C for 5−15 min to achieve variable permeabilization. The cells were then cooled by two rinses in cold SLO+ buffer. The cells were then fixed by 3% PFA for 5 min at 4°C plus 20 min at room temerature, and then aldehyde groups quenched and immunofluorescence continued as described above. In addition, a PBS−saponin (0.05%) permeabilization step was included before the second antibody incubation to allow more effective washing. The cells were incubated in Hoechst 33258 (3.5 μg/ml) to visualize nuclei before the final washes and mounting.

Acknowledgements

We thank Klaus Fiedler and Vesa Olkkonen for discussions, Ed Hurt for critically reading the manuscript, Hank Lane of Costar, Cambridge, M.A. for Transwell filters. We thank also Hilkka Virta and Sigrun Brendel for technical assistance. This work was supported by The Royal Society (P.D.), The European Molecular Biology Organization (T.V.K) and SFB 352 of the Deutsche Forschungs Gemeinschaft.

References

Anderson,R.G.W., Kamen,B.A., Rothberg,K.G. and Lacey,S.W. (1992) *Science*, **255**, 410−411.

Bennett,M., Wandinger-Ness,A. and Simons,K. (1988) *EMBO J.*, **7**, 4075−4085.

Bhakdi,S. and Tranum-Jensen,J. (1987) *Rev. Physiol. Biochem. Pharmacol.*, **107**, 147−223.

Brown,D. and Rose,J. (1992) *Cell*, **68**, 533−544.

Brown,D.A. (1992) *Trends Cell Biol.*, **2**, 338−343.

Bucci,C., Parton,R.G., Mather,I.H., Stunnenberg,H., Simons,K., Hoflack,B. and Zerial,M. (1992) *Cell*, **70**, 715−728.

Chang,W.-J., Rothberg,K.G., Kamen,B.A. and Anderson,R.G.W. (1992) *J. Cell Biol.*, **118**, 63−69.

Cinek,T. and Horejsi,V. (1992) *J. Immunol.*, **149**, 2262−2270.

Glenney,J.R. (1989) *J. Biol. Chem.*, **264**, 20163−20166.

Glenney,J.R. and Soppet,D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 10512−10517.

Gravotta,D., Adesnik,M. and Sabatini,D.D. (1990) *J. Cell Biol.*, **111**, 2893−2908.

Green,J., Griffiths,G., Louvard,D., Quinn,P. and Warren,G. (1981) *J. Mol. Biol.*, **152**, 663−698.

Griffiths,G. (1993) *Fine Structure Immunocytochemistry*, Springer-Verlag, Berlin, pp. 371−445.

Griffiths,G., McDowell,A., Back,R. and Dubochet,J. (1984) *J. Ultrastruct. Res.*, **89**, 65−78.

Griffiths,G., Pfeiffer,S., Simons,K. and Matlin,K. (1985) *J. Cell Biol.*, **101**, 949−964.

Griffiths,G., Back,R. and Marsh,M. (1989) *J. Cell Biol.*, **109**, 2703−2720.

Hugo,F., Reichwein,J., Arvand,M., Kramer,S. and Bhakdi,S. (1986) *Infect. Immunol.*, **54**, 641−645.

Keller,G.A., Siegel,M.W. and Caras,I.W. (1992) *EMBO J.*, **11**, 863−874.

Kreis,T.E. (1986) *EMBO J.*, **5**, 931−941.

Kurzchalia,T.V., Dupree,P., Parton,R.G., Kellner,R., Virta,H., Lehnert,M. and Simons,K. (1992) *J. Cell Biol.*, **118**, 1003−1014.

Laemmli,U.K. (1970) *Nature*, **227**, 680−685.

Lisanti,M.P. and Rodriguez-Boulan,E. (1991) *Cell Biol. Int. Rep.*, **15**, 1023−1049.

Matlin,K.S. and Simons,K. (1983) *Cell*, **34**, 233−243.

Matlin,K.S. and Simons,K. (1984) *J. Cell Biol.*, **99**, 2131−2139.

Montesano,R., Roth,J., Robert,A. and Orci,L. (1982) *Nature*, **296**, 651−653.

Moya,M., Dautry-Varsat,A., Goud,B., Louvard,D. and Boquet,P. (1985) *J. Cell Biol.*, **101**, 548−559.

Palade,G.E. and Bruns,R.R. (1968) *J. Cell Biol.*, **37**, 633−649.

Parton,R.G., Ockleford,C.D. and Critchley,D.R. (1988) *Brain Res.*, **475**, 118−1127.

Raposo,G., Dunia,I., Delavier-Klutchko,C., Kaveri,S., Strosberg,A.D. and Benedetti,E.L. (1989) *Eur. J. Cell Biol.*, **50**, 340−352.

Robinson,P.J. (1991) *Immunol. Today*, **12**, 35−41.

Rodriguez-Boulan,E. (1983) In Satin,B.H. (ed.), *Modern Cell Biology*. Alan Liss, New York, vol 1, pp. 119−170.

Rodriguez-Boulan,E. and Nelson,J. (1989) *Science*, **245**, 718−725.

Rodriguez-Boulan,E. and Pendergast,M. (1980) *Cell*, **20**, 45−54.

Rothberg,K.G., Ying,Y.-S., Kamen,B.A. and Anderson,R.G.W. (1990) *J. Cell Biol.*, **111**, 2931−2938.

Rothberg,K.G., Heuser,J.E., Donzell,W.C., Ying,Y.-S., Glenney,J.R. and Anderson,R.G.W. (1992) *Cell*, **68**, 673−682.

Sandvig,K., Olsnes,S., Petersen,O.W. and van Deurs,B. (1987) *J. Cell Biol.*, **105**, 679−689.

Schell,M.J., Maurice,M., Steiger,B. and Hubbard,A.L. (1992) *J. Cell Biol.*, **119**, 1173–1182.
Severs,N.J. (1988) *J. Cell Sci.*, **90**, 341–348.
Simmons,N.L., Brown,D.A. and Rugg,E.L. (1984) *Fed. Proc.*, **43**, 2225–2229.
Simons,K. and Fuller,S.D. (1985) *Annu. Rev. Cell Biol.*, **1**, 243–288.
Simons,K. and van Meer,G. (1988) *J. Biochem.*, **27**, 6197–6202.
Simons,K. and Wandinger-Ness,A. (1990) *Cell*, **62**, 207–210.
Skibbens,J.E., Roth,M.G. and Matlin,K.S. (1989) *J. Cell Biol.*, **108**, 821–832.
Stefanova,I., Horejsi,V., Ansotegui,I.J., Knapp,W. and Stockinger,H. (1991) *Science*, **254**, 1016–1019.
Thomas,P.M. and Samelson,L.E. (1992) *J. Biol. Chem.*, **267**, 12317–12322.
Tran,D., Carpentier,J.-L., Sawano,F., Gorden,P. and Orci,L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7957–7961.
Van Deurs,B., Petersen,O.W., Olsnes,S. and Sandvig,K. (1989) *Int. Rev. Cytol.*, **117**, 131–178.
Wandinger-Ness,A., Bennett,M.K., Antony,C. and Simons,K. (1990) *J. Cell Biol.*, **111**, 987–1000.

Received on November 6, 1992; revised on January 7, 1993