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Interaction of influenza virus haemagglutinin with sphingolipid–cholesterol membrane domains via its transmembrane domain

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Sphingolipid–cholesterol rafts are microdomains in biological membranes with liquid-ordered phase properties which are implicated in membrane traffic and signalling events. We have used influenza virus haemagglutinin (HA) as a model protein to analyse the interaction of transmembrane proteins with these microdomains. Here we demonstrate that raft association is an intrinsic property encoded in the protein. Mutant HA molecules with foreign transmembrane domain (TMD) sequences lose their ability to associate with the lipid microdomains, and mutations in the HA TMD reveal a requirement for hydrophobic residues in contact with the exoplasmic leaflet of the membrane. We also provide experimental evidence that cholesterol is critically required for association of proteins with lipid rafts. Our data suggest that the binding to specific membrane domains can be encoded in transmembrane proteins and that this information will be used for polarized sorting and signal transduction processes.
Keywords: cholesterol/microdomain/polarized sorting/signal transduction/transmembrane domain

Introduction

Lipids in biological membranes are asymmetrically distributed over the exoplasmic and cytoplasmic leaflets. According to the fluid-mosaic model, the lipid molecules are distributed homogeneously in each leaflet of the bilayer (Singer and Nicolson, 1972). Recently, evidence has accumulated for an additional lateral organization in sphingolipid- and cholesterol-rich microdomains (Simons and Ikonen, 1997). Cells might use this subcompartimentalization of membranes to coordinate signal transduction and membrane trafficking steps (Brown, 1993; Lisanti *et al.*, 1994; Simons and Ikonen, 1997). The microdomains have been termed sphingolipid–cholesterol rafts and can be isolated biochemically by their insolubility in non-ionic detergents such as Triton X-100 (TX-100) or CHAPS at low temperature. After extraction with an excess of cold TX-100, the resulting detergent-insoluble glycolipid-rich complexes (DIGs) can be floated in sucrose gradients and thereby be separated from other detergent-insoluble material such as the cytoskeletal elements (Brown and Rose, 1992; Fiedler *et al.*, 1993). DIGs also contain caveolins, the structural components of plasma membrane invaginations termed caveolae (Kurzchalia *et al.*, 1992; Rothberg *et al.*, 1992). However, as DIGs can be detected in cells lacking caveolins and morphologically recognizable caveolae, they are not equivalent to caveolae and do not require caveolins for their formation (Fra *et al.*, 1994). Recently, a model for the structure of rafts has been proposed (Simons and Ikonen, 1997). Briefly, glycosphingolipids and lipids with long saturated acyl chains would associate laterally and the space in the membrane vacant under the lipid headgroups would be filled with cholesterol molecules. The most important property of the lipid microdomains is that specific proteins associate with them. Glycosylphosphatidylinositol (GPI)-anchored proteins and doubly acylated kinases bind to rafts by their lipid moieties (Brown and Rose, 1992; Rodgers *et al.*, 1994; Casey, 1995). Also several transmembrane proteins have been recovered in DIGs, but by what means they interact with the lipid microdomains is not yet known (Fiedler *et al.*, 1993; Danielsen, 1995; Kundu *et al.*, 1996). One characteristic of lipid rafts which has caused considerable confusion is that they seem to be small and dynamic structures. It has only been possible to visualize these microdomains in cells as large patches generated by antibody clustering of raft proteins or after extraction of 'non-raft' regions by detergent (Mayor *et al.*, 1994; Mayor and Maxfield, 1995).

The function of rafts could be to select and concentrate molecules in a micro-environment of the membrane as kinases and their substrates for signal transduction or cargo molecules and transport machinery for trafficking. This implies that determinants must exist which allow the specific inclusion or exclusion of proteins from the microdomain. For example, it has been demonstrated that these domains maintain a pool of the hyperphosphorylated (inactive) Src-like kinase Lck by exclusion of the corresponding phosphatase CD45 (Rodgers and Rose, 1996). While Lck interacts with rafts by its myristyl and palmitoyl anchor, CD45 does not have raft affinity and therefore cannot dephosphorylate Lck.

The existence of lipid rafts in vesicular transport has been first postulated in polarized epithelial cells where apical and basolateral proteins have to be sorted in the *trans*-Golgi network (TGN). Recent results have extended the role of rafts to non-polarized cells which appear to have apical and basolateral cognate routes (Müsch *et al.*, 1996; Yoshimori *et al.*, 1996). While the basolateral pathway uses cytoplasmic signals and adaptor proteins, the apical route seems to employ *N*-glycosylation as the sorting determinant and sphingolipid–cholesterol rafts as platforms for transport (Rodriguez-Boulan and Powell, 1992; Pimplikar *et al.*, 1994; Scheiffele *et al.*, 1995; Simons and Ikonen, 1997). It has been demonstrated that the apical plasma membrane is enriched in glycosphingolipids and cholesterol, and that TGN-derived apical trans-

port vesicles contain a detergent-resistant subcomplex. Furthermore, while basolateral cargo proteins are excluded from DIGs, several apical cargo proteins such as the influenza virus haemagglutinin (HA) and neuraminidase (NA), brush border enzymes and GPI-anchored proteins are found in DIGs during transport to the cell surface (Simons and Ikonen, 1997). In all cases, the apical proteins are soluble in the endoplasmic reticulum (ER), but become resistant to TX-100 after transport to the Golgi complex (Fiedler *et al.*, 1993; Danielsen, 1995; Kundu *et al.*, 1996; Simons and Ikonen, 1997).

Here we have analysed the mechanism of raft association of HA as a model protein to understand how transmembrane proteins can be coupled to raft lipids. We postulate that determinants required for association with the lipid microdomain will also be important for apical transport and signal transduction processes involving sphingolipid–cholesterol rafts.

Results

Detergent insolubility of HA is an intrinsic property of the molecule

We first analysed whether raft association of HA is only transient during interaction with the apical transport machinery or persists after surface arrival. In a pulse–chase experiment, influenza virus-infected cells were surface biotinylated after different chase times and subsequently extracted with CHAPS at 4°C. HA was recovered in DIGs upon surface arrival and stayed detergent insoluble even after prolonged chase times (Figure 1). Note that the two proteins on the autoradiograph besides HA and NA bind unspecifically to the streptavidin–agarose, as they are already recovered after 2 min of chase, whereas HA and NA reached the surface after 30 min of chase as expected. When TX-100 instead of CHAPS was used, similar results were obtained (data not shown). It is unlikely, therefore, that detergent insolubility of HA reflects a complex formation with the anterograde or retrograde proteinaceous delivery machinery. Like HA, NA also stayed insoluble at the cell surface (Figure 1). In order to find out if raft association requires cellular proteins, we next examined the solubility of HA in isolated influenza virus particles. The viral envelope contains only two major transmembrane proteins, HA and NA, embedded in lipids derived from the plasma membrane. The virus particle therefore provides a simple and homogeneous system, allowing quantitative analysis of lipid and protein interactions. When viruses were extracted with TX-100 at 4°C, ~70% of the HA was found in the pellet whereas it was soluble at 37°C (Figure 2A). To show that the observed insolubility is not due to protein–protein interactions between HA and the nucleocapsid (NP) and matrix protein (M), detergent extracts prepared at 4°C were loaded in the bottom of a TX-100-containing sucrose gradient. After centrifugation, detergent-insoluble HA and NA were recovered in low density fractions, whereas NP and M were found in the pellet because they do not associate with DIGs (Figure 2B). When extracts prepared at 37°C were analysed, HA and NA were found in the bottom fraction (data not shown). These experiments demonstrate that HA is found in DIGs and associates with raft lipids even in the absence of cellular proteins.

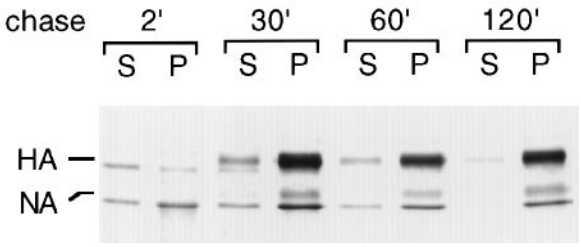

Fig. 1. HA stays insoluble after reaching the plasma membrane. Influenza virus-infected MDCK cells were pulse-labelled with [^{35}S]methionine, surface biotinylated and detergent extracted after different chase times. Subsequently, biotinylated proteins from soluble (S) and insoluble (P) fractions were analysed by electrophoresis.

Role of cholesterol

Cholesterol has been postulated to be a crucial structural component of rafts, and it was demonstrated that choles-

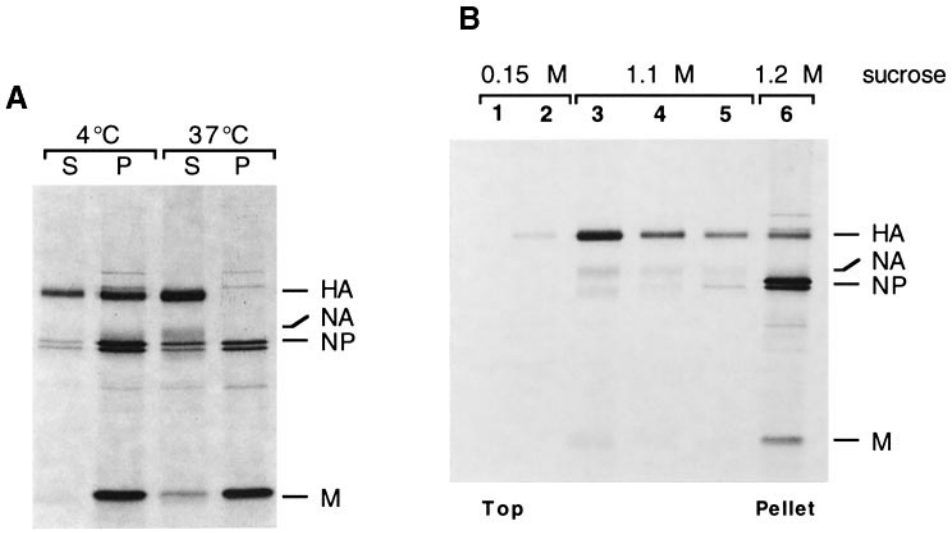

Fig. 2. TX-100 solubility of HA in isolated influenza viruses. (**A**) ^{35}S-labelled influenza viruses were extracted with 1% TX-100 at 4 or 37°C. Soluble (S) and insoluble fractions (P) were separated by centrifugation and analysed by gel electrophoresis. (**B**) Following extraction at 4°C, the extract was adjusted to 1.2 M sucrose, overlayered with 1.1 and 0.15 M sucrose in extraction buffer and centrifuged. Fractions were collected from the top of the gradient and analysed by gel electrophoresis. NP is nucleoprotein and M the matrix protein.

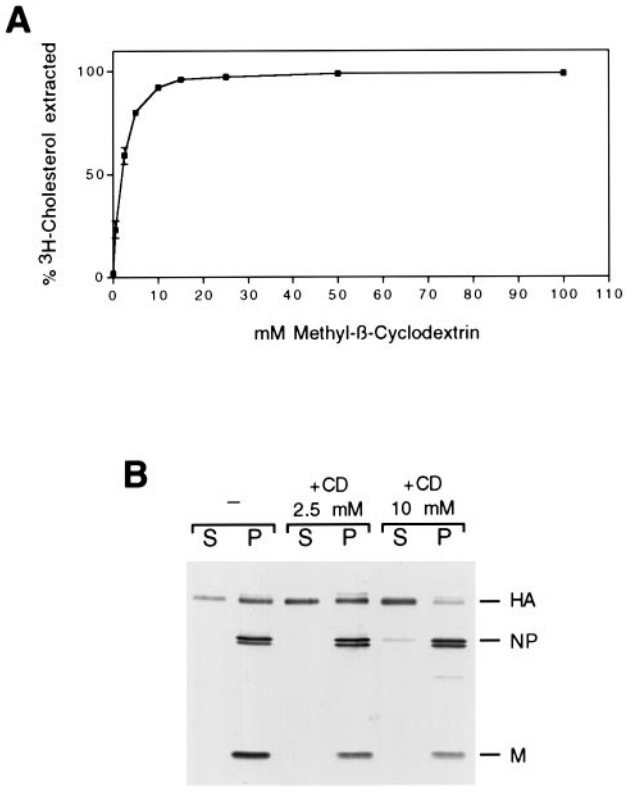

Fig. 3. HA insolubility in influenza viruses requires cholesterol. (**A**) [^{3}H]Cholesterol-labelled viruses were treated with increasing amounts of methyl-β-cyclodextrin (CD). Viruses were pelleted and cholesterol in supernatant and viruses was quantified by scintillation counting (n = 3). Free radioactivity not pelleted without CD treatment (4% of total counts) was subtracted from the other supernatant samples. (**B**) ^{35}S-labelled influenza viruses were pre-treated without (–) or with 2.5 or 10 mM CD, respectively. Subsequently, pelleted viruses were extracted with 1% TX-100 at 4°C and soluble (S) and insoluble fractions (P) analysed.

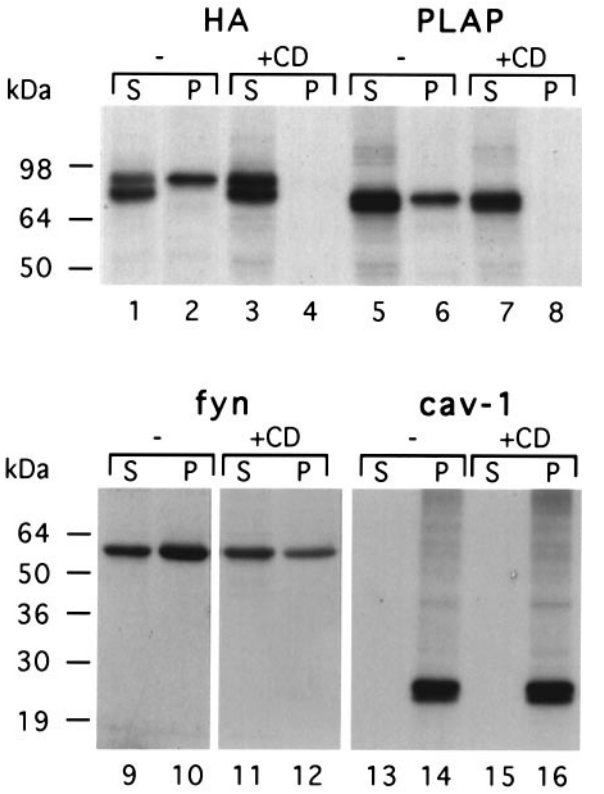

Fig. 4. Raft association in BHK cells requires cholesterol. Cells were transiently transfected, treated with 10 mM CD and then pulse-labelled with [^{35}S]methionine. After a 60 min chase, treated and control cells (–) were extracted with TX-100 and immunoprecipitates from soluble (S) and insoluble (P) fractions analysed. HA, wild-type haemagglutinin; PLAP, placental alkaline phosphatase; cav-1, endogenous caveolin-1.

terol is enriched in DIGs (Brown and Rose, 1992; Simons and Ikonen, 1997). In order to demonstrate directly the involvement of cholesterol, we decided to analyse raft association of HA in cholesterol-depleted membranes. Methyl-β-cyclodextrin (CD) has been shown before to be a useful tool with which to extract cholesterol from biological membranes with high preference over other lipid species (Klein *et al.*, 1995; Yancey *et al.*, 1996). For a titration experiment, [^{3}H]cholesterol-labelled influenza viruses were prepared and treated with increasing concentrations of CD. Subsequently, virus particles were collected by centrifugation and cholesterol was quantified. CD at 2.5 or 10 mM removed 59 ± 4% or 92 ± 1% of the cholesterol from the virus particle, respectively (Figure 3A). The cholesterol-depleted virus particles were then subjected to extraction with TX-100 at 4°C. The solubility of HA increased from 32 ± 9% in the untreated viruses to 54 ± 9% or 85 ± 11% after treatment with 2.5 or 10 mM CD, respectively (Figure 3B). Consistently, no protein was found floating in TX-100 density gradients (data not shown). We next analysed the solubility of HA in cholesterol-depleted cellular membranes. Baby hamster kidney (BHK) cells were transiently transfected with HA cDNA. In a pulse–chase experiment, the terminally modified (Golgi) form of HA was recovered in DIGs whereas the ER form (lower band) was soluble as described before (Skibbens *et al.*, 1989). However, when cells were pre-treated with 10 mM CD, HA could be fully solubilized in TX-100 at 4°C (Figure 4, lanes 1–4). Note that also in cholesterol-depleted cells, HA was transported efficiently to the Golgi complex, as indicated by its terminally glycosylated form (Figure 4, lane 3). The extent of the cholesterol depletion in tissue culture cells under these conditions is 50–65% of the total cellular cholesterol (A.G.Rietveld and K.Simons, unpublished), but as the cellular membranes are heterogeneous this does not allow conclusions about the HA-containing membranes.

In order to find out whether loss of raft association after cholesterol depletion occurs only for HA or is due to disruption of the lipid microdomains, we analysed the solubility of other raft proteins in CD-treated cells. We transiently transfected BHK cells with cDNAs encoding the GPI-anchored placental alkaline phosphatase (PLAP) and the doubly acylated src-like kinase fyn. While in untreated cells both proteins were partially insoluble as described before (Brown and Rose, 1992; Shenoy-Scaria *et al.*, 1994; Gorodinsky and Harris, 1995), PLAP was not found in DIGs after cholesterol depletion, in agreement with previous studies (Cerneus *et al.*, 1993; Hanada *et al.*, 1995) (Figure 4, lanes 5–8). The insolubility of fyn was decreased reproducibly from 70 to 33%, as quantified by phosphor imager analysis (Figure 4, lanes 9–12). The less dramatic effect on raft association of fyn might be due to less efficient removal of cholesterol from the inner leaflet

of the membrane. However, the experiment demonstrates that association with the glycosphingolipid-free cytosolic surface of rafts also requires membrane cholesterol. Cholesterol depletion might generally abolish protein binding to sphingolipid–cholesterol rafts or even disrupt the microdomains. However, endogenous caveolin-1 could not be solubilized in TX-100 using the protocol described (Figure 4, lanes 13–16). This suggests that cholesterol depletion from caveolin-1-containing membrane domains is not achieved under the conditions used, probably because caveolin-1 itself binds tightly to cholesterol (Murata *et al.*, 1995), but also other interactions might confer insolubility to caveolin-1, such as homo- and hetero-oligomerization of the caveolins (Monier *et al.*, 1995, 1996).

Raft association of HA requires a determinant in the TMD

The ability of the HA molecule to associate directly with lipid microdomains must be encoded in the protein structure. The most likely candidate region is the membrane anchor of HA which might allow partitioning into a raft lipid environment, either by its physical properties for packing with lipids in the microdomain or by binding directly to a raft lipid (Murata *et al.*, 1995). We analysed chimeric HA molecules containing lumenal and cytoplasmic domains of HA with transmembrane domains (TMDs) from molecules which should not associate with sphingolipid–cholesterol rafts (Simons and Ikonen, 1997): one with the TMD of vesicular stomatitis virus G protein (HGH, Figure 5A) and another with that of the herpes simplex virus C protein (HCH, Figure 5A) (Srinivas *et al.*, 1986). HCH has been shown to trimerize and to be transported to the plasma membrane with kinetics similar to that of the wild-type HA (wtHA) molecule, whereas trimerization and surface transport of HGH were found to occur with slower kinetics (Lazarovits *et al.*, 1990). The mutants were expressed transiently in BHK fibroblasts and analysed in pulse–chase experiments. After a 60 min chase, the HA mutant proteins were immunoprecipitated and digested with endoglycosidase H to distinguish ER and Golgi forms. Neither chimeric protein partitioned into DIGs, whereas the wtHA acquired TX-100 insolubility in the Golgi complex, as previously observed (Skibbens *et al.*, 1989) (Figure 5B). Therefore, swapping the TMDs caused the protein to lose its ability to interact with sphingolipid–cholesterol rafts. The TMDs of HCH and HGH are significantly shorter than that of the wtHA (23 or 20 amino acids, respectively, versus 27 in wtHA), and it has been proposed that the length of the membrane-spanning domain can determine Golgi localization of transmembrane proteins by excluding them from membranes with increased bilayer thickness (Bretscher and Munro, 1993). In order to find out whether raft association is influenced by the length of the TMD or rather by a more specific feature in the membrane anchor, we analysed additional mutant HA molecules with unaltered TMD length, changing blocks of two or four amino acids in the TMD to alanines (Figure 5A). All mutants were expressed transiently in BHK cells and analysed in pulse–chase experiments, followed by TX-100 extraction and immunoprecipitation of HA (Figure 5B). Some mutants were soluble (4A511, 2A511, 2A514, 2A520) whereas others were recovered in DIGs to a slightly lower or similar

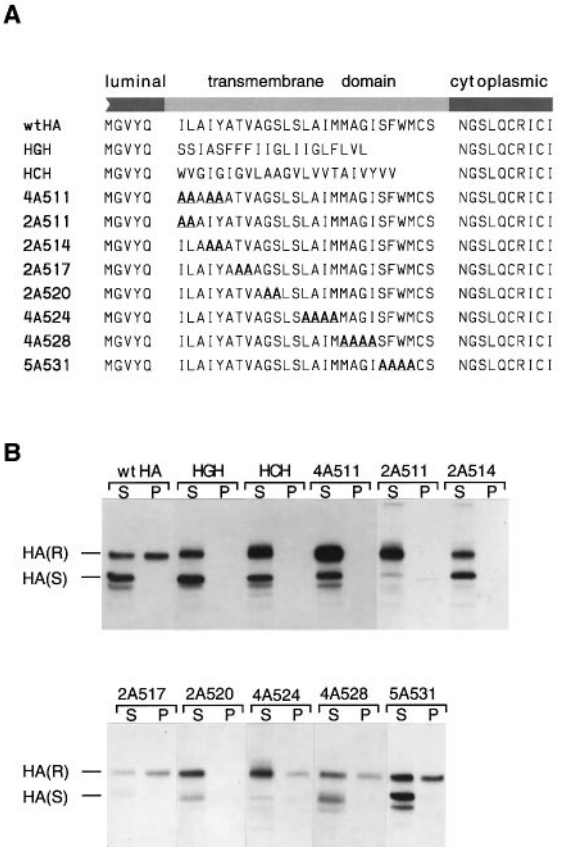

Fig. 5. Raft association of HA depends on amino acids in the transmembrane domain. (**A**) HA mutants were constructed containing luminal and cytoplasmic domains of the wild-type protein (wtHA) and transmembrane sequences of VSV G (HGH) or HSV gC (HCH). In the mutants 4A511–5A531, sets of two or four amino acids in the HA TMD were replaced by alanines (bold and underlined). The three cysteines in the cytoplasmic tail and TMD of wtHA are palmitoylation sites. (**B**) Detergent solubility of endoglycosidase H-resistant HA(R) and -sensitive HA(S) forms of HA mutants. Proteins were transiently expressed in BHK cells and pulse-labelled with [^{35}S]methionine. After a 60 min chase, cells were extracted with 1% TX-100 at 4°C and HA was immunoprecipitated from soluble (S) and insoluble fractions (P). After digestion with endoglycosidase H, proteins were analysed by gel electrophoresis.

extent to the wtHA (2A517, 4A524, 4A528, 5A531) (Figure 5B). There is a strict requirement for amino acids in contact with the exoplasmic leaflet of the membrane, but changes in residues contacting the cytoplasmic leaflet are tolerated. It is therefore not simply the length of the TMD which influences interaction with sphingolipid–cholesterol rafts, but rather a specific determinant in the exoplasmic half of the TMD. The most important residues identified by the mutagenesis are large and hydrophobic, with the glycine/serine in mutant 2A520 as the only exception.

Palmitoylation of the mutant HA 4A511 is not affected

Besides the TMD, HA is also anchored to the membrane by palmitoylation on three cysteine residues (Naim *et al.*, 1992) which might influence raft association. To rule out an indirect effect of the TMD mutations on palmitoylation, we analysed the incorporation of [^{3}H]palmitic acid in wtHA, the mutant 4A511 and HCH, which is lacking one of the palmitoylation sites in the TMD (see Figure 5A).

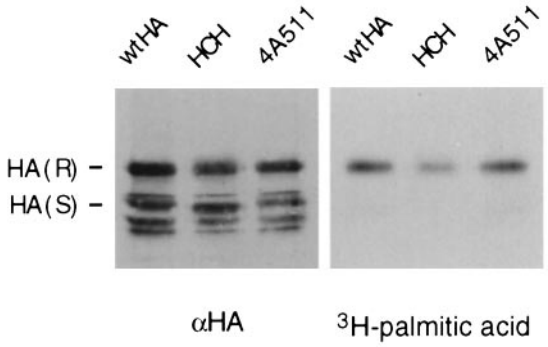

Fig. 6. Solubility of HA 4A511 is not caused by decreased palmitoylation. Proteins were expressed as described in Figure 5, but labelling was performed with [^{3}H]palmitic acid. Cell lysates were treated with endoglycosidase H to differentiate between resistant [HA(R)] and sensitive forms [HA(S)]. Samples were split and either analysed directly by Western blotting with polyclonal HA antibodies (left panel) or immunoprecipitated with the same antibody and analysed by fluorography (right panel). Similar results were obtained when quantification of the expression level was performed by labelling with [^{35}S]methionine on a separate dish instead of Western blotting.

The expression level of the proteins was determined by Western blotting of an aliquot of the samples (Figure 6, left panel). Mutant 4A511 HA incorporated essentially the same amount of [^{3}H]palmitic acid as the wtHA whereas, in agreement with the lack of one palmitoylation site, HCH was labelled significantly less (~25% decrease of palmitic acid signal per protein unit according to densitometric scanning; Figure 6, right panel). Therefore, it is unlikely that the loss of raft association of the TMD mutants is due to a change in acylation, but rather reflects deletion of a TMD determinant required for protein–lipid interactions.

Discussion

In this study, we analysed the interaction of a type I transmembrane protein with sphingolipid–cholesterol rafts. It has been reported previously that HA is found in DIGs after transport to the Golgi complex, and this has been interpreted as interaction with the apical transport machinery (Fiedler *et al.*, 1993). We have demonstrated that incorporation into DIGs is based on direct protein–lipid interactions via the TMD and does not require formation of hetero-oligomeric complexes with cellular proteins (Figure 2). This is confirmed further by the fact that cholesterol depletion from cells or virus particles abolishes incorporation into DIGs and leads to subsequent solubilization of HA in TX-100 at 4°C.

Cholesterol as a raft lipid

The model for raft structure postulates an important role for cholesterol as a spacer to link sphingolipids together in the exoplasmic leaflet of the bilayer (Simons and Ikonen, 1997). Our results confirm that cholesterol is crucial for raft association of a transmembrane and two lipid-modified proteins. Previous studies have already suggested a role for cholesterol in raft association of GPI-anchored proteins (Cerneus *et al.*, 1993; Hanada *et al.*, 1995; Hannan and Edinin, 1996). Interestingly, fyn binds to the lipid microdomain in a cholesterol-dependent manner from the cytoplasmic side. This could indicate that binding to the cytoplasmic surface of rafts occurs by a mechanism similar to binding of GPI-anchored proteins. However, glycosphingolipids are restricted to the exoplasmic leaflet of the bilayer, and the lipids specifying rafts in the intracellular leaflet still remain to be identified.

Cholesterol must also exist in 'non-raft' regions of cellular membranes (Chong, 1994), but in the context of raft lipids, cholesterol appears to be complexed as indicated by its detergent insolubility, whereas cholesterol as such or incorporated in phosphatidylcholine-containing ('non-raft') liposomes is soluble (Schroeder *et al.*, 1994). We propose that raft integrity and protein association depend on the presence of cholesterol and that cholesterol depletion can be used as an additional criterion for testing whether proteins specifically associate with the lipid microdomains. This appears even more important as *in vitro* studies demonstrated that some glycosphingolipids are intrinsically detergent insoluble (Schroeder *et al.*, 1994), but in biological membranes they will require cholesterol to form rafts with the ability to bind specific proteins.

Cholesterol is thought to increase the thickness of the lipid bilayer, and lipids with long acyl chains have been found enriched in DIGs (Bretscher and Munro, 1993; Schroeder *et al.*, 1994). However, so far there is no evidence which would support increased bilayer thickness as an important characteristic for protein association with sphingolipid–cholesterol rafts (see below).

Transmembrane domains as determinants for raft association

Membrane-spanning domains have been implicated in different steps of protein trafficking, such as retrieval and retention of proteins in the ER or the Golgi complex. In many cases, formation of hetero- or homo-oligomers mediated by the TMDs was proposed to be the mechanism for TMD involvement (Machamer *et al.*, 1993; Munro, 1995; Nilsson, 1996). Also, in yeast, protein–protein interactions within the membrane plane have been postulated to be required for ER retrieval. Rer1p, a four transmembrane protein, is employed to retrieve proteins with a TMD signal from the *cis*-Golgi. Crucial amino acids in the TMD regulating these processes are polar residues, consistent with a role in protein–protein interactions (Machamer *et al.*, 1993; Sato *et al.*, 1996). In contrast, it seems that association of transmembrane proteins with rafts employs direct protein–lipid interactions, as HA in isolated virus particles in the absence of cellular proteins is found in DIGs (Figure 2B). Crucial amino acids identified by the mutagenesis were mostly hydrophobic residues in the exoplasmic half of the TMD. These hydrophobic amino acids could shape the TMD in the right way to be accommodated with cholesterol in the microdomain, rather than being employed in direct binding to one lipid species. This is consistent with the dynamic nature of rafts (Harder and Simons, 1997). The structural role of this TMD determinant is underlined by the fact that fusion of the normally trimeric HA TMD and the cytoplasmic tail to a dimeric molecule, PLAP, yields a detergent-soluble protein (Arreaza and Brown, 1995). This could be due to a change from a trimer to a dimer leading to looser intercalation into the microdomain. However, it is possible that the context of the TMD also will be

important, and whereas palmitoylation in HA 4A511 was not changed, we do not exclude that palmitoylation of transmembrane proteins promotes their raft association.

Protein–lipid interactions have also been proposed to be involved in regulating retention of proteins in the Golgi complex (Bretscher and Munro, 1993). In this model, packing of the TMDs in a bilayer of increasing thickness created by the cholesterol gradient from the ER to the plasma membrane would determine if a protein is retained in the Golgi complex or can be transported to the plasma membrane. Our experimental data clearly show that the length of a TMD is not the determining factor for raft association. Neither did comparison of TMD sequences of six raft-associated and eight non-raft plasma membrane proteins show any significant preference for long TMDs in raft proteins (data not shown).

One consequence of our findings is that determinants for raft association will be an important factor specifying function of a protein. TMD signals might be used generally to localize proteins to sphingolipid–cholesterol rafts not only in membrane trafficking but also in cell signalling. In T-cell activation the src family tyrosine kinase fyn at the inner leaflet of the plasma membrane is thought to couple to GPI-anchored proteins in the outer leaflet via transmembrane proteins. These complexes can be co-precipitated from detergent lysates (Stefanova *et al.*, 1991; Brown, 1993; Casey, 1995), and our results suggest that their association depends on cholesterol. All components of such a signalling unit could be concentrated in rafts by their GPI anchors, by their lipidation or by specific properties of their TMDs, respectively. Thereby fast and specific transduction of signals would be ensured within lipid rafts which in turn also would provide sheltered subdomains to avoid unwanted cross-talk and interventions (Rodgers and Rose, 1996). Cholesterol depletion by CD might prove a valuable tool to analyse directly the role of rafts in signal transduction processes.

Apical transport and detergent insolubility

Sphingolipid–cholesterol rafts have been proposed to mediate apical transport in polarized epithelial cells, and we have here identified determinants required for raft association of transmembrane proteins. As the GPI anchor has been shown to have dual functions, linking proteins to rafts and functioning as an apical targeting signal, we propose that determinants for raft association of HA also function in apical transport. Two apical signals have been found in GPI-anchored proteins: besides the lipid anchor, the ectodomain also contains apical targeting information, most likely the *N*-glycans (Brown *et al.*, 1989; Lisanti *et al.*, 1989; Scheiffele *et al.*, 1995). Similarly, apical transmembrane proteins appear to contain two signals which probably act cooperatively: glycosylation in the ectodomain and a TMD signal, which could function together to ensure association with sphingolipid–cholesterol rafts (Scheiffele *et al.*, 1995; Kundu *et al.*, 1996). However, the presence of both signals at the same time appears not to be strictly required for apical sorting because there are apical proteins which are not detergent insoluble (Danielsen, 1995) and others which are not glycosylated (Hughey *et al.*, 1992). Analysis of non-glycosylated apical transmembrane proteins will allow separation of the two potential apical sorting determinants. However, the already existing data demonstrate that apical and basolateral signals operate hierarchically for sorting of cell surface proteins in epithelial cells (Mostov *et al.*, 1986; Matter and Mellman, 1994). This complexity probably evolved to allow regulation of protein sorting in different cell types as they terminally differentiate during development.

Materials and methods

Recombinant DNA techniques

Wild-type HA (A/JAPAN/305/57) and mutant cDNAs constructed by megaprimer PCR mutagenesis (Sarkar and Sommer, 1990; Lazarovits *et al.*, 1996) were inserted in the vector pcDNA3 (Invitrogen) under control of a cytomegalovirus (CMV) and T7 promoter. PLAP and fyn were expressed from Rous sarcoma virus (RSV) or CMV promoter constructs, respectively. Restriction enzymes were from Boehringer Mannheim (Germany) and were used according to the manufacturer's instructions.

Surface biotinylations

MDCK II and BHK cells (strain CCL10; American Type Culture Collection) were cultured as described (Matlin and Simons, 1983). MDCK cells were infected with influenza viruses (A/PR8/8/34) as described (Fiedler *et al.*, 1993) and 5 h post-infection pulse-labelled for 5 min with [^{35}S]methionine (150 μCi/ml). After the indicated chase times in the presence of 20 μg/ml cycloheximide and 150 μg/ml methionine, cells were cooled on ice, washed twice with ice-cold phosphate-buffered saline (PBS) and biotinylated for 30 min with 1 mg/ml *N*-hydrosuccinimido-LC-biotin (NHS-biotin, Pierce) in PBS at 4°C. Cells were then washed with PBS and excess NHS-biotin was quenched with 0.2% bovine serum albumin (BSA) and 0.1 M glycine in PBS. After extraction with 20 mM CHAPS or 1% TX-100 in TNE, pH 7.4 [50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol (DTT), 25 μg/ml each of chymostatin, leupeptin, antipain and pepstatin A (CLAP)], soluble and insoluble material was separated by a 5 min centrifugation at 12 000 *g* and biotinylated proteins were recovered from the supernatant and pellet with streptavidin–agarose (Pierce) in 1% NP-40, 0.2% SDS in TNE, pH 8.0. Proteins bound to streptavidin–agarose were then analysed by polyacrylamide gel electrophoresis.

Virus production

Influenza virus stocks (A/PR8/8/34) and HA polyclonal antisera were prepared as described (Matlin and Simons, 1983). Radiolabelled viruses were produced in MDCK II cells grown on 75 mm polycarbonate filters (Costar). Cells were labelled 1.5 h after infection with [^{35}S]methionine (50 μCi/ml) and viruses were collected overnight. Aggregates were removed by centrifugation at 3000 *g* and viruses subsequently pelleted through a 50% glycerol cushion in 10 mM Tris–HCl, 150 mM NaCl, pH 7.4; 0.5% of the preparation was used per experiment. [^{3}H]cholesterol-labelled viruses were isolated by essentially the same protocol with the following modification: cells were first incubated with 20 μCi [^{3}H]cholesterol/ml in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% ethanol for 4 h. After the label was removed, viruses were applied for infection.

Detergent and CD extractions

Viruses or BHK cells (90% confluent on 3 cm dishes) were extracted on ice for 30 min with 600 μl of 1% (w/v) TX-100 in TNE, pH 7.4, followed by centrifugation at 120 000 *g*. Treatment of viruses with CD (Sigma) was performed in TNE, pH 7.4, 10 μg/ml defatted BSA at 37°C for 15 min with gentle agitation. Subsequently, viruses were recovered by centrifugation at 4°C (120 000 *g*) and TX-100 extracted as above. For treatment of BHK cells with CD, 70% confluent BHK cells on 3 cm dishes were washed twice with PBS and then incubated with 10 mM CD in serum-free medium containing 50 mM HEPES for 30 min at 37°C with agitation. Following two washes in PBS, cells were pulse-labelled as described below. The efficiency of the cholesterol depletion was strongly influenced by the cell density.

Transfection and pulse–chase analysis

Transfections were performed with lipofectin reagent (Gibco). Identical results in the insolubility assay were obtained for HA constructs by

expression from either the CMV promoter or with T7 RNA polymerase-recombinant vaccinia virus (vTF7.3) (Fuerst *et al.*, 1986) 12 or 2 h post-transfection, respectively. Proteins were pulse-labelled for 20 min with 0.3 mCi/ml [^{35}S]methionine and chased for 60 min in the presence of 20 μg/ml cycloheximide and 150 μg/ml methionine. Immunoprecipitations were performed with polyclonal HA antibodies, polyclonal PLAP antibodies (DAKO), polyclonal fyn antibodies (kindly provided by G.Alonso) or polyclonal caveolin-1 N-20 antibodies (Santa Cruz Biotechnology), respectively. Immunoprecipitates were recovered with protein A–Sepharose (Pharmacia), washed four times in 1% (w/v) NP-40, 0.2% SDS in TNE, pH 8.0, and either analysed directly by gel electrophoresis, or eluted with 1.5% β-mercaptoethanol, 0.6% SDS in 150 mM Na-citrate, pH 5.5 at 95°C and, after addition of two volumes of 0.75% NP-40, digested with 5 mU endoglycosidase H (Boehringer Mannheim, Germany) for 12 h at 37°C. Subsequently samples were analysed by polyacrylamide gel electrophoresis and fluorography.

Palmitoylation analysis

BHK cells were transfected as described above and labelled with 1 mCi/ml [^{3}H]palmitic acid for 20 min. After a 60 min chase, cells were lysed in 1% NP-40, 0.2% SDS in TNE, pH 8.0 on ice. After immunoprecipitation, incorporation of [^{3}H]palmitic acid was detected by fluorography. To quantify the expression level, an aliquot of the samples was analysed by Western blotting with HA antibodies. Alternatively, cells on a separate dish were labelled with [^{35}S]methionine and processed identically to the [^{3}H]palmitic acid sample. The extent of palmitoylation was quantified after densitometric scanning of autoradiographs (for the [^{3}H]palmitic acid signal and the Western-blotted control samples) or by phosphor imager analysis (for the ^{35}S-labelled control samples) with similar results.

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