Quantitative electron microscopy and fluorescence spectroscopy of the membrane distribution of influenza hemagglutinin

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Although lipid-dependent protein clustering in biomembranes mediates numerous functions, there is little consensus among membrane models on cluster organization or size. Here, we use influenza viral envelope protein hemagglutinin (HA₀) to test the hypothesis that clustering results from proteins partitioning into preexisting, fluid-ordered “raft” domains, wherein they have a random distribution. HA₀ expressed in fibroblasts was visualized by electron microscopy using immunogold labeling and probed by fluorescence resonance energy transfer (FRET). Labeled HA coincided with electron-dense, often noncircular membrane patches. Poisson and K-test (Ripley, B.D., 1977. J. R. Stat. Soc. Ser. B. 39:172–212) analyses reveal clustering on accessible length scales (20–900 nm). Membrane treatments with methyl-β-cyclodextrin and glycosphingolipid synthesis inhibitors did not abolish clusters but did alter their pattern, especially at the shortest lengths, as was corroborated by changes in FRET efficiency. The magnitude and density dependence of the measured FRET efficiency also indicated a nonrandom distribution on molecular length scales (~6–7 nm). This work rules out the tested hypothesis for HA over the accessible length scales, yet shows clearly how the spatial distribution of HA depends on lipid composition.

Introduction

The landmark discovery that enveloped viruses can acquire membranes with differential composition, as they bud out of different regions of the plasma membrane (van Meer and Simons, 1982), forms some of the earliest evidence that enveloped viruses exploit glycosphingolipid and cholesterol-enriched domains known as lipid rafts as part of the viral life cycle (Suomalainen, 2002). Subsequent studies have shown that there are two different lipid domains in the viral envelope (Bukrinskaya et al., 1987), and that HA, the major envelope protein of influenza, is insoluble in cold nonionic detergent, now recognized as a biochemical fingerprint of raft-resident proteins (Skibbens et al., 1989). Infectivity of HIV, Ebola, and influenza, as well as budding of influenza, depend on sphingolipid and cholesterol-rich membrane domains (Scheiffele et al., 1999; Nguyen and Hildreth, 2000; Ono and Freed, 2001; Bavari et al., 2002; Sun and Whittaker, 2003) that are putatively disrupted by removal of cellular cholesterol by methyl-β-cyclodextrin (Kilsdonk et al., 1995). HA-mediated cell–cell fusion, viral infectivity, and budding were also found to depend on HA (H3 subtype) transmembrane domain sequence, and correlated with the clustering of HA in the plasma membrane as detected by EM (unpublished data and Takeda et al., 2003). These biological effects are thought to result from redistribution of the HA and lipid after disruption of putative “raft” domains that presumably restrict lateral diffusion and thereby maintain a high local concentration of HA (Takeda et al., 2003).

The proposed functions of lipid rafts are not limited to viral entry and exit from cells. A crescendo of publications on membrane microdomains suggests they are important in a variety of cell signaling and membrane trafficking pathways. Despite this, there is considerable controversy on the existence, size, life time, and physiological importance of putative domains (Simons and Ikonen, 1997; Edidin, 2001, 2003; Anderson and Jacobson,
2002; Fujiwara et al., 2002; Nakada et al., 2003; Kenworthy et al., 2004; Sharma et al., 2004). Here, we use HA to test some of the predictions of raft models, focusing on the idea of a fluid lipid domain. We expect fluid domains to have distinct, testable properties, including rounded boundaries and preferential partitioning of certain proteins and lipids into those domains. In this work, we set out to test whether HA is found in fluid domains that are ideally mixed. One hypothesis for HA clustering would be a favorable partition coefficient between domain and nondomain membrane. This hypothesis predicts that within each domain the lateral distribution of protein will be random, but at a concentration that is proportional to the average membrane density. Thus, another prediction is that the distance between HAs will decrease as average membrane density increases. On the other hand, an alternate hypothesis is that the assembly of domains is due to self-assembly of molecular complexes, in which case there need not be any fixed size, as this will depend on the relative on- and off-rates of dynamic cluster assembly and disassembly. Self-assembled complexes would also allow for average HA–HA distances that do not depend on the average HA density. Self-assembly of protein–lipid complexes due to specific intermolecular interactions would also provide a diversity of distinct membrane domains with different protein and lipid compositions, each serving potentially different biological functions. In agreement with such a mechanism, raft markers such as Thy-1, GM1, and palmitoylated LAT have been observed previously to be found in distinct membrane clusters that are not colocalized (Wilson et al., 2004). In contrast, work by Shvartsman et al. (2003) shows that GPI-anchored BHA-PI and GPI-GFP can coexist in microdomains with wt-HA. Thus, there are presumably selective mechanisms that determine the partitioning of specific components into a given type of microdomain. Finally, there is the set of hypotheses that includes all types of membrane domains other than ideally mixed fluid domains and self-assembled complexes.

We determined the lateral organization of HA on plasma membranes of stably transfected fibroblast cells (the HAb2 cell line) (Ellens et al., 1990) at length scales that span three orders of magnitude. Our results for influenza HA (H2 subtype) are inconsistent with partitioning of trimers into domains with random intra-domain distributions. We hypothesize that the longer and shorter length scale regimes for HA mediate two of its crucial biological functions: viral assembly and viral entry, respectively. We further hypothesize that lipid-dependent assembly of HA achieves these functions by first creating pre-envelope domains of specific lipids and proteins on the plasma membrane before interacting with influenza matrix protein M1 for budding of complete virions; this assembly may be important for fusion.

Results

Thin sections and membrane sheets imaged en face show a nonrandom distribution of influenza HA as visualized by immunogold EM

HAb2 fibroblast cell plasma membrane ultrathin (50 nm) sections were prepared by thin section, and membrane sheets were
prepared by the “rip-flip” method of Sanan and Anderson (1991). Treated membranes from cells grown in culture were prepared by incubation with compounds that alter the membrane lipid composition before sectioning or rip-flip. Treatments include methyl-β-cyclodextrin (MβCD) (Kilsdonk et al., 1995), used to remove cholesterol from the cell membranes; 10 µM 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP); and 10 µM 1-phenyl-2-palmitoyl-amino-3-morpholino-1-propanol (PPMP) (Abe et al., 1992, 1996). PDMP and PPMP inhibit glycosphingolipid synthesis, but at 25 µM also are reported to reduce sphingomyelin in cells (Rosenwald et al., 1992; Chen et al., 1995) and in hippocampal neurons (Hisaki et al., 2004).

Membrane sections were labeled with α-HA primary antibody, followed by 10-nm colloidal gold-conjugated secondary antibody, and were then imaged by EM (Fig. 1). Membranes fixed before labeling (Fig. 1 a), membranes labeled before fixing (Fig. 1 b), and membranes cut in 50-nm serial sections (Fig. 1 c) all showed a highly nonrandom distribution of HA. Note that the membrane bilayer frequently appears darkened in regions near where a gold particle is bound. Both membranes treated with MβCD, PDMP, and PPMP, and untreated membranes showed a clustered distribution of HA (unpublished data). Frequently, electron-dense material was visible between the gold particles and the immediately adjacent plasma membrane.

To increase the number of gold particles observed and to visualize their distribution more easily in two dimensions, HAb2 cell membrane sheets prepared by the method of Sanan and Anderson (1991) were labeled with the same α-HA primary and 10-nm colloidal gold-conjugated secondary antibodies and imaged en face (Fig. 2 and Fig. 3). Clustering was visible on many length scales, and generally, clusters coincided with electron-dense membrane regions that were observed in the presence or absence of immunogold but not in grid regions without membrane or where the membrane had locally torn away (unpublished data). Generally, these electron-dense regions had irregular boundaries resembling small dark islands in a light sea or other self-similar shapes, although occasionally they showed rounded boundaries containing clusters of HA. Gold particles were correlated with the electron-dense regions (see image analysis method 6) on length scales roughly less than correlation length \( r_0 \sim 92 \pm 16 \) nm in selected untreated membranes and \( r_0 \sim 136 \pm 80 \) nm in MβCD-treated membranes. In some cases the clusters appeared to have triangular or hexagonal symmetry (Fig. 2, arrow) reminiscent of packing of circles or spheres. To better quantify the HA distribution, the x-y coordinates of each immunogold particle were tabulated and analyzed by a number of methods.

**Poisson analysis**

As a test of randomness of the membrane distribution of HA, we performed Poisson analysis of the frequency distribution of the number of immunogold particles within boxes of a given size spaced uniformly across imaged areas. This analysis revealed quantitatively \( \chi^2 > 10^2; P < 0.00001; 7 \) degrees of freedom) that the distribution of gold-labeled HA trimers was nonrandom (Fig. 4 A). As a control, identical analysis of a computer-generated random distribution of particles in a planar surface with area and overall particle density equal to those of the analyzed cellular membrane resulted in agreement between the observed and expected frequency distribution of particles per box (Fig. 4 B).

Next, the nonrandomness of the particle distribution on different spatial scales was investigated. Using the same Poisson analysis, the box size was varied from 50–350 nm and the observed frequency distribution of particles per box was fitted with Eq. 1, resulting in the \( \chi^2 \) values that are shown in Fig. 4 C. The large values of \( \chi^2 \) indicate a vanishing probability (p value) that the observed membrane distribution of labeled HA is consistent with the null hypothesis that HA is distributed randomly on the length scales investigated; rather, HA was distributed nonrandomly on every accessible length scale. Contrary to the result expected if partitioning into fluid domains were the cause of HA clustering, treatments with MβCD, PPMP, or PDMP, which are expected to disrupt membrane microdomains, did not result in a random distribution of HA trimers. Furthermore, the order of fixation and labeling did not affect whether the particle distribution was nonrandom on any of the length scales we investigated.
Distribution of distances between all pairs of HA trimers

To further examine the particle distribution for characteristic cluster size and inter-cluster distance, we calculated the frequency histogram of distances, \( H(r) \), between each labeled HA and every other, as a function of treatment (Fig. 5 A), where \( r \) denotes distance between gold particles. The comparison of \( H(r) \) for a random distribution and the observed \( H(r) \) for all treatments reveals differences: HA–HA distances less than \( \sim 1.3 \) \( \mu \)m were observed more frequently than expected for a random distribution, whereas distances longer than \( \sim 1.3 \) \( \mu \)m were less frequent than expected. The measured \( \phi \) frequency of HA–HA distances between \( \sim 0.5 \) and \( 0.9 \) \( \mu \)m was significantly higher than expected (simulated, \( \phi \)) for a random distribution; this increase is due to the clustered distribution of HA and likely corresponds to distances between trimers within clusters. This increase is expected to occur in a clustered HA distribution because (compared with a random distribution) HAs within a cluster will have relatively more neighbors that are closer to each other and fewer that are far away, at least on length scales near the cluster size. Hence the length scales where the observed \( H(r) \) is larger than expected (0.5–0.9 \( \mu \)m) give one estimate of cluster size.

Ripley’s K-test

The Ripley’s K-test provides a quantitative measure of the deviation of the observed HA distribution from a random distribution, as a function of the length scale examined. To further test the spatial HA distribution for characteristic cluster sizes, the modified Ripley’s K-test (Eq. 2) was performed on the gold particle coordinates as a function of treatment. Fig. 5 B shows Ripley’s K-test metric (\( L(r) - r \)) for position data from 9 to 14 images of untreated and treated membrane sheets. Note a range of consistently positive values for \( L(r) - r \) that are significantly larger than the 99% confidence level (CI\textsubscript{99}) (data are normalized such that the CI\textsubscript{99} is equal to unity) over most length scales accessible to our analysis, indicating clustering. Note also that the K-test statistic appears to increase monotonically over the range of length scales considered (<700 nm), again indicating there was no single, well-defined size of an HA cluster.

The average \( L(r) - r \) for untreated images and M\&\textsubscript{3}CD-, PPMP-, and PDMP-treated images (Fig. 5 B) show significant decreases in the K-test amplitude (a measure of the degree of clustering) after treatment. However, the amplitude was not reduced below the CI\textsubscript{99} in any case. Thus, clusters were partially disrupted but not abolished by the lipid treatments. Furthermore, the effects of PPMP, PDMP, and M\&\textsubscript{3}CD on clustering appear to be similar, despite the numerous postulated side-effects of these drugs.
Nearest neighbor distance distribution

The frequency distribution of nearest neighbor distances, \( N(r) \), provides a measure of the shortest distance between a labeled HA trimer and any other, and thus reports on the intra-cluster environment as a function of the various membrane treatments (Fig. 6 A). The observed \( N(r) \) has a maximum, indicating a most common nearest neighbor distance, \( r_{\text{nn}} \) that depends on treatment, as shown in Table I.

However, changes in \( N(r) \) upon treatment were described completely by a reduced antibody binding probability or capture ratio (\( k \)) for the treated membranes, which is also consistent with the reduced average density of gold particles upon treatment. The fraction of clustering (\( f_{\text{clust}} \)) that gave the best fit between simulation and the observed \( N(r) \) was for all treatments in the range 80–100%, whereas the best-fit capture ratio \( k \) changed more significantly, ranging from 5–7% for untreated membranes and fitting best with \( k \sim 2\% \) for treated membranes. Attempting to force the model to describe the observed \( N(r) \) with a fixed \( k \) by instead using \( f_{\text{clust}} \) and minimum distance between trimers as free parameters was unsuccessful, resulting in significantly larger values for the goodness of fit test (Eq. S4, available at http://www.jcb.org/cgi/content/full/jcb.200412058/DC1; unpublished data). Fluorescence measurements of primary antibody binding to HAb2 cells under the same conditions using the same antibody (except for the presence of fluorophores) did not show a significant difference in binding as a function of membrane treatment (unpublished data). Furthermore, the capture ratio was not significantly improved by immunolabeling with 6-nm gold-conjugated antibody (Fig. 3, E and F). These results are again consistent with only minor effects of lipid treatments on the clustering of HA on the length scales accessible in these experiments (20–900 nm).

If the labeled HA molecules are clustered with a characteristic nearest neighbor distance within the membranes we imaged, the nearest neighbor distance should not depend on the density of labeled HA molecules. Fig. 6 B shows the most probable nearest neighbor distance \( r_{\text{nn}} \) in a given image field, as a function of the average labeled HA density in that field. The best-fit slope of \( r_{\text{nn}} \) vs. density was zero within uncertainty in all cases, except possibly PDMP treatment, where the best-fit slope was 1.3 \( \pm \) 0.7 \( \times 10^{-3} \) nm\(^{-1}\). The independence of \( r_{\text{nn}} \) on HA density is strong evidence for the clustering of HA in HAb2 cell membranes.

Angular distribution of two nearest neighbors

Next, we examined the question of whether the clustering of HA in the membrane would result in angular biases of the two neighbors nearest to a given HA trimer. In particular, fluid membrane domains would be expected to have uniform density within a domain and relatively little orientational or radial order, whereas a highly packed domain with hexatic or possibly quasi-crystalline properties would be expected to show a highly biased distribution of angles, \( A(\theta) \). Between a given particle (at point P) and its two nearest neighbors (say at O and Q), the angle \( \theta \) is defined as the angle between the lines OP and OQ. In fact, the HA distribution in the electron micrographs occasionally showed clear examples of triangular or hexagonal packing of the gold particles at nearest neighbor distances larger than the gold particles themselves (Fig. 2). The observed \( A(\theta) \) for untreated membranes and MB\(_{\text{CD}}\)-treated membranes were plotted with control \( A(\theta) \) from a simulated random distribution of the same particle density within the same area (Fig. 7).

The uncertainty \( \pm \sigma_{\theta} \) in the frequency \( A \) was estimated as \( \sigma_{\theta} = \sigma / \sqrt{A} \). The difference between the observed and expected \( A(\theta) \) was significant: 207 \( \pm \) 49 and 130 \( \pm \) 30 more particles than expected were observed to have nearest neighbor angles of \(<90^\circ \) in untreated and MB\(_{\text{CD}}\)-treated membranes, respectively, indicating that these biases are significant, independent of membrane cholesterol content.
Fluorescence resonance energy transfer

We have used fluorescence resonance energy transfer (FRET) to probe the lipid dependence of the HA distribution on molecular length scales. Cy3- and Cy5-labeled α-HA mAbs were used as a FRET pair with nominal $r_0 \approx 5.3$ nm (Ishii et al., 1999). The FRET efficiency was measured by donor dequenching upon acceptor photobleaching in fixed HAb2 cells as a function of lipid treatments (applied before fixation) and antibody concentration. Fig. 8 shows the measured FRET in untreated cells and cells treated with MβCD, PDMP, and PPMP. Significant decreases in FRET occurred upon all lipid treatments and at all antibody concentrations. Measurements made on two different microscopes (confocal and widefield) are presented and show similar effects. Monte-Carlo modeling of the expected FRET efficiency for a clustered distribution of HA trimers (see supplemental material for details; available at http://www.jcb.org/cgi/content/full/jcb.200412058/DC1) describes the observed FRET efficiency (Fig. 8), whereas FRET from a simulated random distribution did not describe the measured FRET as well. The strong dependence of the FRET efficiency on treatments that altered membrane lipid composition indicates that relative distance and orientation of HA trimers is being altered on the molecular length scales (6–7 nm) by such treatments. This corroborates the findings of Henis and colleagues using FRAP that show HA diffusion changes with cholesterol depletion (Shvartsman et al., 2003). Our results are a complement to the EM results, which indicate relatively small effects of treatments on the HA distribution on longer (≥20 nm) length scales.

Discussion

We have shown that the two-dimensional distribution of influenza HA is highly nonrandom in fibroblast plasma membranes on all accessible length scales, from 6 nm to 900 nm. Nearest neighbor distance was independent of HA density over a wide range. FRET, which measures clustering on length scales <10 nm, showed that the shortest distances between trimers were increased by agents that remove cholesterol and inhibitors of glycosphingolipid synthesis. The amount of FRET was too high for a random distribution and the density dependence is weaker than expected for random. These results are inconsistent with the hypothesis that clustering results from proteins partitioning into preexisting, fluid-ordered raft domains, wherein they have a random distribution. The results are consistent with the idea that specific proteins and lipids self-assemble in the plane of the membrane to form nonrandom microscopic or nanoscopic domains.

Two-dimensional analysis of HA distribution: comparison with related methods and raft markers

The analysis presented here using a number of methods shows decisively that HA is clustered in the plasma membrane of HAb2 cells, consistent with previous results (Takeda et al., 2003). Our results analyze a large number of gold particles, providing improved statistics, and to our knowledge provide for the first time the two-dimensional distribution of HA within...
Figure 6. Effect of treatments on the observed nearest neighbor distance distribution (points) can be explained by changes in antibody binding efficiency, or capture ratio, \( \kappa \). (A) The frequency distribution of nearest neighbor distances \( N(r) \) between 10-nm immunogold-labeled HA trimers on HAb2 cell membranes shows a maximum value from 25–30 nm with little dependence on treatment and fixation. Samples were fixed and then labeled unless noted otherwise. The observed distributions were least-squares fitted with Monte-Carlo simulations (solid lines) of clustered distributions of HA trimers (see Materials and methods) that were least-squares fitted to the observed \( N(r) \) as a function of capture ratio \( \kappa \) and fraction of clustered trimers \( f_{\text{clus}} \). Best fit between observed and simulation occurred in untreated membranes (black squares) for \( \kappa = 7\% \), \( f_{\text{clus}} = 80\% \) (black line), in untreated membranes labeled before fixation (open green circles) for \( \kappa = 5\% \), \( f_{\text{clus}} = 90\% \) (green line), in M\( \beta \)CD-treated membranes (open red squares) for \( \kappa = 2\% \), \( f_{\text{clus}} = 100\% \) (red line), in PPMP-treated membranes (black circles) for \( \kappa = 2\% \), \( f_{\text{clus}} = 90\% \), and in PDMP-treated membranes (orange triangles) for \( \kappa = 2\% \), \( f_{\text{clus}} = 100\% \) (orange line). Nearest neighbor histogram expected for a random distribution is shown for comparison [RAND; black line]. (B) Peak nearest neighbor distance \( r_p \) does not depend strongly on the average gold density per square micron, strong evidence for a clustered membrane distribution of HA, independent of treatment.

The plane of the membrane. Takeda et al. (2003) analyze in one linear dimension 363 gold particles labeling wild-type trimeric HA and 1,298 gold particles labeling nonraft mutant trimeric HA; we analyze in two dimensions 8,245 gold particles labeling trimeric HA in untreated membranes, 1,988 in M\( \beta \)CD-treated membranes, 2,585 in PDMP-treated membranes, and 2,198 in PPMP-treated membranes.

A second significant observation apparent from the images in Figs. 1–3 is the colocalization of the HA with electron-dense membrane regions, which bears some similarity in appearance to the colocalization of immunogold-labeled FcεRI and IgE–FcεRI complexes with osmiophilic membrane regions reported previously (Wilson et al., 2000, 2002). More work is needed to interpret this finding.

Our results, which show clustering on many length scales, are in contrast to the findings of Prior et al. (2003) for the protein Ras, whose clusters are apparently less extended and thus have a much better defined distribution of sizes. Such differences likely relate in part to the relative strength of specific protein–lipid and protein–protein interactions within the plane of the membrane, which may derive from differences in modes of attachment to the membrane.

Treatments altering lipid composition minimally change observed HA distribution

The effect of cholesterol removal and glycosphingolipid synthesis inhibition on the degree and size of clusters was surprisingly minor: based on the K-test (Fig. 5), clusters were somewhat broken up, but they were not abolished. A Kolmogorov-Smirnov test using area-normalized \( L(r) = r \) for untreated and M\( \beta \)CD-treated membranes resulted in a significant difference between the two distributions (\( P < 10^{-6} \)). The Poisson analysis (Fig. 4 C) of number of particles per box also shows a highly nonrandom distribution of labeled HA after each of the three treatments. These results indicate that clusters are only partially disrupted by the treatments. On the other hand, M\( \beta \)CD treatment reduces the probability of FRET (sensitive to length scales of \( \approx 3–9 \) nm) between antibody-labeled HA trimers (Fig. 8), consistent with lipid-dependent disruption of HA packing at molecular length scales. Perhaps the large-scale clusters depend more on cytoskeletal interactions.

One well-established example of protein–protein interactions that results in membrane structures of a similar size is the clathrin-coated pit (Mahaffey et al., 1989). However, our EM results do not show gold-labeled HA in any of the features of clathrin-coated pits observed by similar methods (Mahaffey et al., 1989; Lin et al., 1991), and this may correlate with the relative stability of HA on the surface of the cell membrane (Roth et al., 1986). Membrane domains such as caveolae could also be highly relevant to the internalization dynamics of HA.

Table I. Summary of nearest neighbor distances and gold density vs. membrane treatment

| Treatment | Peak nearest neighbor distance (nm ± SD) | Average nearest neighbor distance (nm ± SD) | Average gold density (#/\( \mu \)m\(^2 \) ± SD) |
|-----------|----------------------------------------|---------------------------------------------|-----------------------------------------------|
| UN-PF     | 17.0 ± 0.5                             | 48 ± 5                                      | 65 ± 34                                       |
| UN        | 18.5 ± 0.6                             | 46 ± 5                                      | 82 ± 46                                       |
| M\( \beta \)CD | 24.8 ± 0.5                             | 77 ± 10                                     | 31 ± 21                                       |
| PPMP      | 28.1 ± 0.5                             | 75 ± 7                                      | 33 ± 18                                       |
| PDMP      | 28.7 ± 0.7                             | 71 ± 5                                      | 41 ± 20                                       |
Models for lipid domains

HA clustering on many length scales.

Our results show clustering of HA on many length scales. A central finding of an important review of membrane microdomain models (Anderson and Jacobson, 2002) is that different methods yield different sizes for rafts. Our results suggest that for proteins such as HA, clustering will be observed on the length scales accessible to a method. Should a method only be able to access a narrower range of length scales than does the protein, one may erroneously conclude that this narrow range defines the size distribution of the cluster. A similar situation occurs in the measurement of the distribution of fusion pore conductances (Zimmerberg, 1993). Measurement of cluster size by different methods may result in different sizes for the same clusters. This is the situation today in the field of lipid rafts, which finds no consensus on domain size but a plethora of models. Second, the presence of nonrandomness on nearly all observed length scales precludes the possibility of a uniform distribution within domains, except on length scales we do not have access to (<6 nm, which is 2 nm less than the diameter of the smallest cylinder that could enclose the HA trimer ectodomain). Despite nonrandomness on all accessible length scales, clusters may occur preferentially over a certain range of length scales. Indeed, we find a peak in the pair–distance distribution (Fig. 5) and discuss this below in terms of the size of the viral envelope. In fact, we think that one important achievement of this work is to avoid any arbitrary definitions of domains that would be necessary to obtain a size distribution of clusters. Thus, our statement on the nonrandom nature of the distribution at all length scales is model independent. Some raft models of membrane domains propose that proteins partition preferentially into fluid phase (liquid ordered) lipid domains wherein proteins are miscible and lateral diffusion is unrestricted. Such models that would predict a random distribution within such a domain are inconsistent with our observations. Therefore, it is possible that HA does partition into preexisting domains, but is immiscible in the lipid environment of that domain. In that case, the HA would aggregate to minimize its exposure to the domain lipids. Finally, as stated above, in a partitioning system one would expect a dependence of the peak nearest neighbor distance on density, and in our system the observed dependence is insignificant.

Density independence of HA nearest neighbor distance. Although the finding that local HA density within domains is constant is inconsistent with a miscible partitioning model, this feature is not unique to HA clusters. Density independence of the homoFRET characterizing GPI-linked proteins is also not explained by a miscible partitioning model (Sharma et al., 2004). Again, condensed phases of cholesterol and sphingolipids have been proposed to explain this phenomenon (Sharma et al., 2004). It may be useful to divide the universe of raft models into those that feature partitioning into preexisting domains, and self-assembly of proteins and lipids into specific biological structures.

Significance for viral assembly and fusion

It is clear that cellular lipids have the propensity to form coexistence regions of liquid-ordered and liquid-disordered phases,
as revealed by macroscopic domain formation in phospholipid bilayers formed from lipid mixtures (Korlach et al., 1999; Feigenson and Buboltz, 2001; Veatch and Keller, 2002, 2003; Baumgart et al., 2003). Given the ability of cell membrane lipids to self-organize discrete domains, it is compelling to suggest that domains are used by cells to organize the plasma membrane (Simons and Ikonen, 1997).

The link between membrane microdomain association, clustering, and biological function merits further investigation. Some studies find no effect of MβCD on cell–cell fusion mediated by HA (Melikyan et al., 1999; Armstrong et al., 2000). The finding that viral infectivity is dependent on membrane cholesterol content (Takeda et al., 2003) is consistent with our observations that membrane fusion catalyzed by the major envelope protein of influenza, HA, is MβCD sensitive (unpublished data), but this may reflect nonraft aspects of cholesterol action because VSV is also inhibited by MβCD (Takeda et al., 2003). Our results here, together with electron microscopic data on viral–liposome fusion (Kanaseki et al., 1997) and our previous work on membrane fusion (Chernomordik et al., 1998; Frolov et al., 2000), are consistent with (but not proof of) the formation of a fusion “rosette,” where a high local concentration of HA, cholesterol, and sphingolipids may be the crucial quantity for membrane fusion (Markovic et al., 2001). Disruption of this local molecular ordering by removal of cholesterol is consistent with fluorescence mobility measurements that generally show an increase in mobility of raft-associated membrane proteins such as HA when membrane cholesterol is depleted (Shvartsman et al., 2003). Lipid-dependent trimer–trimer interactions may be more relevant to fusion activity and may likewise control clustering on the length scale of individual trimers (~8 nm).

With respect to domain size distribution and viral budding sites, the frequency distribution $H(r)$ of distances between HA molecules was clearly nonrandom, with various maxima and minima indicating that certain intermolecular distances were favored (see Fig. 5). The combined distributions for all images with the same treatment each showed some bias toward 0.5–0.9 μm, irrespective of treatment. These distances can be interpreted as a characteristic size of clusters (Vereb et al., 2000), but because HA clusters span many size scales we view this interpretation with caution. Such a cluster size is potentially consistent with the use of microdomains as viral budding sites, as suggested previously for clusters of HA in cells infected with influenza virus (Takeda et al., 2003) and vesicular stomatitis virus (Brown and Lyles, 2003). It seems larger than the usually given size of a single influenza virus (~0.1–0.2 μm), but there is heterogeneity in the size and shape distribution of the influenza virus (Ruigrok et al., 1985). If the entire concentration shows significant reduction in FRET efficiency in MβCD-, PDMP, and PPMP-treated membranes compared with untreated membranes, suggesting that whereas lipid-dependent redistribution of HA trimers on long length scales (>20 nm) appears to be minor, that significant redistribution occurs on molecular (~6 nm) length scales. (C) Measured energy transfer probability vs. HA density is more closely the simulated energy transfer vs. HA density for a clustered HA distribution (Sim. C) than with a random distribution (Sim. R).
Materials and methods

Cell culture, fixation, and immunogold labeling

Fibroblasts expressing the Japanese strain of HA, here referred to as HA (HAb2 cells), were grown as a monolayer on glass coverslips (22 mm square) to 80% confluence. The growth medium was washed with PBS (w/ Ca2+/Mg2+) at RT (~23°C), twice. The cells were washed twice in 0.1 M Hendry’s phosphate buffer (HPB; see supplemental material), then fixed in 2% PFA and 0.05% glutaraldehyde (followed eventually by 4% glutaraldehyde; please see supplemental material), then labeled with anti-HA mAb, followed by gold-conjugated secondary antibody.

Membrane retrieval and preparation for EM

After gold labeling, whole membrane segments were retrieved according to the procedure adapted from Sanan and Anderson (1991), with minor modifications.

Cell preparation for thin sectioning

Cells for thin sectioning were grown, fixed, and immunostained according to the above protocol, then prepared for EM essentially as described by Neale and colleagues (Fernando et al., 1989), with minor modifications. Ultrathin and serial cross sections (50-nm thick) were cut.

Imaging

En face and serial sections were imaged using a transmission electron microscope (100 CX; JEOL) at 50,000× and 80 kV.

Image analysis

Horizontal and vertical coordinates of gold particles were recorded from the images manually using NIH ImageJ. Coordinate assignments assumed the imaged membrane to be a planar surface with negligible height variation out of plane. Membrane serial sections of HAb2 cells expressing HA constituted indicate that out-of-plane invaginations and buds had heights of up to 100 nm over lateral distances of several micrometers. The en face membrane preparation method brings the sample through the air-water interface and results in considerable flattening of the membranes as revealed by tomographic reconstruction.

Membrane treatments

Before fixation and gold labeling, some cells received one of three types of treatment: (1) methyl-β-cyclodextrin (MβCD), which removes cholesterol from membranes (Klein et al., 1995; Niu et al., 2002), incubated with cells in PBS at final concentration 10 mM for 30 min at 4°C; (2) glucosylceramide synthase inhibitor PDMP, or (3) glucosylceramide synthase inhibitor PPMP ( Abe et al., 1992) [Matreyek, Inc.]. PDMP and PPMP were incubated with cells in Dulbecco’s minimum essential medium at a final concentration of 10 μM for 48 h at 37°C. These cells were then processed to produce either membrane sheets or material for thin sections.

Numerical image analysis

(1) Poisson analysis of clustering. The field of each image was divided uniformly into square areas (boxes) and the particles in each box were counted. The frequency distribution of the number of particles per box was then fit using a Poisson distribution:

\[
P(x,n) = \frac{A_0 e^{-x/n}}{n^x} \]

where \(x\) is the mean of the frequency distribution (the mean number of particles per box), \(n\) is the number of particles per box, and \(A_0\) is a constant. The number of degrees of freedom was determined from the histogram, taking into account the number of bins within the histogram that had a count of 6 or higher; bins with fewer than five counts were not used for determination of the \(x^2\) value.

(2) Pair–distance frequency distribution. The distribution of distances between each labeled HA particle and all other labeled HA particles was calculated for each image and binned by 5 nm into a histogram. The distribution for a random field of particles with the same density per unit area and same total area was calculated and compared with the experimental distribution.

(3) K-test for clustering. The Ripley’s K-test (Ripley, 1977, 1979; Prior et al., 2003) was performed, which measures the number of particles within a given radius \(r\) of any given labeled HA molecule as follows:

\[
L(r) = \frac{N(r)}{\pi D r^2}
\]

where \(N(r)\) is the number of particles within a distance \(r\) of a given particle, and \(D\) is the average particle density per unit area. Positive values of \(L(r)\) indicate clustering on length scale \(r\).

(4) Nearest neighbor distance distribution. The frequency distribution of nearest neighbor distances \(N(r)\) between labeled HA molecules was calculated using a fixed bin size of either 1 nm or 6.35 nm, then compared with results for a random or simulated clustered field of particles at the same density per unit area and the same total area. Replica experiments (various images of membranes given the same treatment) were summed to yield a global histogram.

(5) Angular neighbor distribution. The angle between a given trimer (as the vertex of the angle) and its two nearest neighbors was calculated for all trimers within each image. A global histogram A(θ) as a function of the angle \(θ\) was generated from the sum of the histograms for each image with a given membrane treatment.

(6) Colocalization of HA trimers with electron-dense membrane patches. Images were scanned from negatives at high resolution and read into custom software that identifies gold particles and dark membrane regions. Gold particles were located by scanning the image in a raster pattern into custom software that identifies gold particles and dark membrane regions.
tern and finding all points with intensity darker (pixel value lower) than a fixed threshold. For each identified gold particle, the average pixel intensity as a function of radius r from the center of the particle was calculated, averaged for all images with the same treatment, and fitted to the function \( A + Be^{-r^2/2\alpha} \) to obtain a characteristic length scale \( \alpha \) over which the gold particles and electron-dense membrane regions were correlated.

FRET microscopy

FRET microscopy was performed essentially as described in Kenworthy and Edidin (1999). Anti-HA mAb FC125 was conjugated with succinimidylic ester derivatives of Cy3 or Cy5 (Amersham Biosciences) as indicated by the manufacturer, to an average level of 2 fluorophores per IgG molecule. Hab2 (unintegrated or cholesterol/SM/GSL depleted) cells were grown on 4-well coverglass chambers (#1.5; Nalge Nunc), fixed in 4% paraformaldehyde/HCl (pH 7.4) for 20 min followed by labeling with a mixture of Cy3- or Cy5-conjugated mAb (50 \( \mu \)g/ml concentration) and imaged directly.

Digital images were collected using a 20\( \times \), 0.75 NA objective on a microscope (Diaphot 300; Nikon) using a 12-bit scientific CCD camera (model CH250; Photometrics). FRET was excited with an xenon arc lamp and visualized using Cy3 and Cy5 filter sets (Chroma Technology Corp.). Images of Cy3 and Cy5 before (Cy3pre and Cy5pre) and after (Cy3post and Cy5post) bleaching the Cy5 were collected using identical exposure times, typically 10 s. Cy5 was >95% photobleached by continually exciting it for 7–8 min. Custom-designed software (available upon request) was used to analyze the FRET data. This program tabulates the average fluorescence intensity from identical regions of interest from each of the four images in each sample after background subtraction. Energy transfer probabilities were then calculated as their efficiencies (E) from the background-corrected values of Cy3 fluorescence as

\[
E_{\text{exp}} = \left( \frac{F_{\text{post}}^{\text{Cy5}} - F_{\text{pre}}^{\text{Cy5}}}{F_{\text{pre}}^{\text{Cy3}}} \right) / F_{\text{post}}^{\text{Cy3}}
\]

where \( F_{\text{pre}}^{\text{Cy3}} \) and \( F_{\text{pre}}^{\text{Cy5}} \) are the measured fluorescence Cy3 intensities before and after acceptor photobleaching. Typically data were collected from 4–5 fields of cells for each treatment. For further analysis, data were pooled by treatment and the average energy transfer efficiency compared across treatments. The average fluorescence intensity of the acceptor was also monitored, to confirm that the average surface densities of labeled HA molecules were constant across treatments.

Online supplemental material

Online material includes further details of cell culture and sample preparation for immunogold EM, numerical modeling of the FRET efficiency as a function of experimental parameters, and details on methods of analysis of protein distributions: Poisson, and Ripley's K test. Next are descriptions of methods for Monte-Carlo simulation of membrane HA and immunogold distributions and fitting of the observed distributions using results from the simulations, followed by estimates of the range of accessible length scales in these experiments. Finally, we discuss possible artifacts due to antibody labeling, fixation, sample drying, and projection of gold particle positions from three dimensions into a two-dimensional plane. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200412058/DC1.

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