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Dynamic Partitioning of a GPI-Anchored Protein in Glycosphingolipid-Rich Microdomains Imaged by Single-Quantum Dot Tracking

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Abstract

Recent experimental developments have led to a revision of the classical fluid mosaic model proposed by Singer and Nicholson 35 years ago. In particular, it is now well established that lipids and proteins diffuse heterogeneously in cell plasma membranes. Their complex motion patterns reflect the dynamic structure and composition of the membrane itself, as well as the presence of the underlying cytoskeleton scaffold and that of the extracellular matrix. How the structural organization of plasma membranes influences the diffusion of individual proteins remains a challenging, yet central question for cell signaling and its regulation. Here we have developed a raft-associated glycosylphosphatidyl Inositol-anchored avidin test probe (Av-GPI), whose diffusion patterns indirectly reports on the structure and dynamics of putative raft microdomains in the membrane of HeLa cells. Labeling with quantum dots (qdots) allowed high-resolution and long-term tracking of individual Av-GPI and the classification of their various diffusive behaviors. Using dual-color total internal reflection fluorescence (TIRF) microscopy, we studied the correlation between the diffusion of individual Av-GPI and the location of glycosphingolipid GM1-rich microdomains and caveolae. We show that Av-GPI exhibit a fast and a slow diffusion regime in different membrane regions, and that slowing down of their diffusion is correlated with entry in GM1-rich microdomains located in close proximity to, but distinct, from caveolae. We further show that Av-GPI dynamically partition in and out of these microdomains in a cholesterol-dependent manner. Our results provide direct evidence that cholesterol/sphingolipid-rich microdomains can compartmentalize the diffusion of GPI-anchored proteins in living cells and that the dynamic partitioning raft model appropriately describes the diffusive behavior of some raft-associated proteins across the plasma membrane.

Keywords

Lipid rafts; Single molecule microscopy; Particle tracking; GM1 lipids; Caveolae; Cholesterol; Membrane diffusion; Cholera toxin B; Live cell; Fluorescence imaging

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Introduction

Over the years, the plasma membrane “fluid mosaic” model proposed by Singer and Nicholson in their 1972 landmark review (1) has been significantly refined. Current models incorporate the notion that membranes are crowded environments (2) having a complex topology, and that they interact strongly with the cytoskeleton and contain microdomains of different sizes and lipid/protein composition (3-6). Several types of plasma membrane microdomains have been proposed: (i) domains delineated by transmembrane proteins attached to the underlying actin cytoskeletal network (7,8); (ii) domains formed by specific protein-protein interactions (9); or (iii) domains formed by the preferential association of certain lipids and proteins into cholesterol- and glycosphingolipid (GSL)-rich liquid ordered (L_o) phases, named lipid rafts (10,11). These various domains are thought to play a role in regulating specific molecular interactions by partitioning the plasma membrane and allowing the rapid assembly or disassembly of specific multi-protein/lipid complexes involved in cellular signaling (12).

Particular attention has been given to lipid rafts because they appear implicated in signal transduction, signal amplification and protein sorting (13,14). From a number of experimental approaches, it has been extrapolated that raft domains are generally enriched in cholesterol, in glycosphingolipids and in specific sets of proteins, notably in glycosylphosphatidylinositol-anchored proteins (GPI-AP) whose saturated fatty acyl anchor favor their packing with cholesterol and association with the L_o lipid phase (15,16). By studying the distribution of cholesterol, glycosphingolipids (such as GM1) or GPI-AP, “raft-like” lipid phases have been observed in model membranes (17-20), and inferred from the isolation of detergent-resistant membranes (DRM) in cells (21). Recent studies have confirmed the existence of lipid domains with different fluidities in cell plasma membranes (22,23). Overall, a growing amount of evidence indicates that lipid rafts are probably small, heterogeneous and highly dynamic (5,24,25). However, despite a multitude of techniques developed over the past 20 years (26), a distinct picture of their structure and dynamics is lacking and it is still unclear how such domains may affect the diffusion and the partitioning of membrane proteins in live cells. Part of the problem is that such small and elusive structures require the use of high-temporal and spatial resolution observation techniques capable of correlating the location of different molecular components. Single-molecule spectroscopy and microscopy techniques are in principle well suited for this purpose, since they give access to static and dynamic molecular heterogeneities (27). Among these techniques, image and fluorescence correlation spectroscopy (ICS and FCS) have been employed to study the diffusion and clustering of integrins (28) and to identify lipid and actin-dependent microdomains in live cells (29,30). Hetero- and homoFRET, as well as fluorescence polarization anisotropy, have been used to study the organization of membrane components at the nanometer scale (31). Tracking techniques, such as single-dye tracking (SDT) (32), single-particle tracking (SPT) (33-35) and more recently, photothermal interference contrast imaging of gold nanoparticles (36), have also permitted the detection of membrane compartments and confinement zones of 30-700 nm, for a variety of raft and non-raft lipids and proteins. Unfortunately, standard single-molecule fluorescence methods are often limited by rapid probe photobleaching, which reduces the duration of measurements and limits the observation of complex dynamics. On the other hand, non-fluorescent particle tracking, which does not suffer from this drawback, is not easily amenable to signal multiplexing, preventing the correlation of multiple parameters.

Quantum dots (or qdots) overcome most of these limitations. They are bright and extremely photostable fluorescent nanoparticles, which, once solubilized and functionalized, make good probes for single-molecule imaging and tracking in live cells (37). Qdots have previously been used for high-resolution membrane tracking of single-proteins such as

glycine receptors (38), AMPA and NMDA receptors (39), integrins (40) and CFTR channel proteins (41,42) among a growing list of many others.

Here we used biotinylated peptide-coated qdots to track single GPI-anchored avidin test probes expressed in HeLa cells. Using dual-color TIRF imaging and single-qdot tracking (SQT), we quantified their lateral diffusion and studied their interactions with glycosphingolipid GM1-rich microdomains of the plasma membrane and with caveolae. Our study reveals that GPI-anchored avidin dynamically partition in and out of GM1-rich microdomains, which are in close proximity to, but distinct, from caveolae. This partitioning is characterized by changes in diffusion coefficients and is cholesterol-dependent. Overall, these observations provide direct evidence that membrane microdomains having the accepted composition of lipid rafts can induce molecular compartmentalization in the cell plasma membrane, consistent with their putative function as protein sorting and signaling platforms.

Results

GPI-anchored avidin tetramers are found in DRM and associate with GM1-rich lipid domains in HeLa cells

To investigate the structure and dynamics of membrane microdomains by single-molecule techniques, we designed a minimal GPI-anchored test probe having no biological functions, no specific interactions with other membrane components, and a binding domain allowing its detection at extremely low concentration of fluorescent probes (<5 pM). This test probe consists of the full-length chicken avidin fused with the GPI-anchoring signal peptide of the human CD14 receptor (43) (Fig. 1A). CD14 is a raft-associated GPI-anchored receptor for lipopolysaccharide normally expressed in monocytes (44-46) while avidin is absent from mammalian cells and has a very high affinity for biotin ($K_D \sim 10^{-15}$ M (47)). The post-translational modification of this avidin fusion protein with a GPI anchor (Av-GPI) resulted in its anchoring to the plasma membrane. When stably expressed in HeLa cells, at expression levels comparable to those of a standard housekeeping gene (GAPDH, data not shown), Av-GPI was efficiently targeted to the outer membrane leaflet, where it was homogeneously distributed with no mislocalization or aggregation (Fig. 1B). Analysis of its oligomeric state from cell membrane preparations and comparison to native chicken avidin indicated that Av-GPI exist essentially as stable tetramers in the plasma membrane and do not form higher-order oligomers as previously reported for a similar LDL receptor-avidin fusion protein (48) (Fig. S2).

In cells, GPI-anchoring sorts CD14 receptors to detergent-resistant membranes (DRM) consistent with their association with lipid rafts as defined biochemically (21,45,46). We therefore examined whether, the GPI-anchoring signal peptide of CD14 promoted a similar sorting of avidin tetramers. In DRM preparations, Av-GPI was found enriched in the buoyant/light fraction while the endogenous transferrin receptor (TfR), used as a non-raft associated protein control, was localized in the denser, non-raft fraction (Fig. 1C). As often observed for native GPI-AP, the association of Av-GPI with DRM was cholesterol-dependent (49). Inhibition of cholesterol synthesis with lovastatin led to a partial redistribution of Av-GPI into the non-raft fraction, but did not affect the distribution of TfR (Fig. 1C and Table S1). This repartition was accompanied by a specific reduction in the recovery of Av-GPI.

The apparent association of Av-GPI with putative membrane rafts was further confirmed by co-clustering with glycosphingolipids GM1 which are often found associated with raft domains in cells (10,21,50). Upon cross-linking with anti-avidin antibodies, Av-GPI redistributed into patches (Fig. 1 & additional data available at

http://fpinaud.bol.ucla.edu/index_files/Traffic.htm). Staining with fluorescent cholera-toxin B sub-unit (CTxB), a high affinity marker of GM1 (51), revealed GM1 patching and co-clustering with Av-GPI. In contrast, cross-linking of Av-GPI did not induce the patching of TfR, which remained homogeneously distributed and showed no specific colocalization with GM1 patches (Fig. 1B). Hence, Av-GPI and GM1 can occupy common membrane lipid domains while TfR appear to reside in separate domains.

Thus, the avidin GPI-anchored test probe is properly expressed and sorted in HeLa cells. While native GPI-AP are generally monomeric, Av-GPI tetramers are representative of native raft-markers because they associate in a cholesterol-dependent manner with DRM and with glycosphingolipid GM1-rich domains of the outer plasma membrane.

Long-term single-molecule tracking of Av-GPI using qdots

We then studied the dynamic properties of Av-GPI in more details by TIRF and single-qdot tracking (SQT). We used biotin and polyethylene glycol (PEG)-modified CdSe/ZnS qdots previously developed for single-molecule imaging in live cells (37,43). These small qdots (diameter: 13.0 ± 1.1 nm) could readily target Av-GPI in the ventral membrane of HeLa cells (Fig. 2).

The diffusion trajectories of Av-GPI were studied for up to 2 min, and high-resolution localization of individual Av-GPI was done frame by frame (100 ms/frame), by fitting the diffraction-limited spot image of each qdots with a Gaussian function (52,53) (Fig. 2B). Each tracked qdot displayed a characteristic on/off blinking behavior (54), confirming that we were following individual Av-GPI (Fig. 3D and Movie 1). The typical position accuracy for the localization of Av-GPI was ~ 30 nm. The diffusion of each qdot-labeled Av-GPI was quantified by analyzing the mean square displacement (MSD) curve (33) and the probability distribution of square displacements (PDSD) for different time lags (55). PDSD analysis was often preferred over MSD analysis because it allows the unbiased detection of multiple diffusion regimes within single trajectories and the quantitative characterization of each regime as verified on Monte Carlo simulated trajectories (Fig. S5).

Using PDSD analysis, we determined whether each Av-GPI experienced a single or multiple diffusion regimes. For each detected diffusion regime, we measured a diffusion coefficient and classified the regime into one of four categories (Fig 2D): (i) pure Brownian diffusion, (ii) restricted diffusion, for diffusions limited by corrals or impaired by obstacles, (iii) directed diffusion, for diffusion dominated by a velocity component, and (iv) no diffusion, for static Av-GPI with diffusion below a cut-off value $D_{\min} = 4.8 \cdot 10^{-5} \mu\text{m}^2/\text{s}$ (see Material and Methods). The diffusion coefficients from all regimes, evaluated for all qdot-labeled Av-GPI on multiple cells, were then reported in distribution histograms having logarithmically-spaced bins. The resulting histograms were fitted by one or two Gaussian, identifying as many populations of diffusion coefficients. In the following, we report the position of the peak (or mode) of these Gaussians (noted $\widehat{D}$) as the characteristic diffusion coefficient of each population. Note that $\widehat{D}$ is smaller than the mean value $\bar{D}$ of the distribution, yet $\widehat{D}$ and $\bar{D}$ values differed at most by 40 %, and our conclusions are unaffected by this choice.

For simplicity, we will henceforth talk about “immobile”, “slow” and “fast” diffusing Av-GPI, when referring to Av-GPI trajectories whose analysis yields immobile, slow and/or fast diffusion regimes respectively.

Av-GPI exhibit multimodal diffusion

The diffusion coefficients of Av-GPI were distributed in two sub-population of fast diffusing $\widehat{D}_{fast}^{SQT}$ and slow diffusing molecules $\widehat{D}_{slow}^{SQT}$ differing by about 40-fold in their modal diffusion coefficient (Fig. 3A). We observed very few immobile Av-GPI ($D < D_{min}$, 3 %). Within the duration of our observation, a majority of slow Av-GPI diffused with pure Brownian motion, while fast Av-GPI often experienced restricted diffusion (Fig. 3C, -CT×B). Of all Av-GPI ($n = 118$), 66 % had a single diffusion regime, while 31 % exhibited two diffusion regimes and only 3 % had three diffusion regimes. During tracking, we observed that 22 % of all Av-GPI switched between fast and slow diffusion regimes (or vice versa). Other Av-GPI (12 %) had two diffusion coefficients both belonging to the same distribution (either fast or slow), an indication that diffusion was more complex than a mere partitioning between fast and slow sub-populations.

The multimodal diffusion of Av-GPI and the values $\widehat{D}_{fast}$ and $\widehat{D}_{slow}$ were further confirmed by global analysis of the ensemble PDSs (data can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm, $D_{ens,1} = 3.8 \cdot 10^{-2} \mu m^2/s$ and $D_{ens,2} = 6.4 \cdot 10^{-4} \mu m^2/s$). Thus, despite the wide diversity of diffusion coefficients demonstrated by Av-GPI, two predominant and very different diffusion regimes are observed in the membrane of HeLa cells.

To verify that this bimodal distribution of diffusion coefficients was not a consequence of transient or permanent clustering of Av-GPI by qdot-induced cross-linking, we performed tracking with quasi-monovalent biotinylated qdots (Fig. S3). With these qdots, the same two sub-populations of fast and slower diffusing Av-GPI were detected, an indication that qdot valency has no effect on diffusion. We also confirmed the absence of cross-linking by SDT and FRAP of Av-GPI labeled with monovalent Alexa 488 biocytin. As described in more details in Supporting Information, the analysis of single dye labeled-Av-GPI trajectories revealed a broad distribution of diffusion coefficients that included the same two prevalent diffusion regimes previously detected using qdots (Fig. S4 and Movie M2). The diffusion coefficients of Av-GPI were about twice larger than for qdots, suggesting that the larger size of qdots (~13 nm compared to ~2 nm for Alexa biocytin) induces a two-fold decrease in diffusion coefficient. Two population of diffusing Av-GPI were again detected by FRAP at room temperature (RT ~27 °C). Characteristic diffusion coefficients were: D_{fast}^{FRAP} and D_{slow}^{FRAP} , comparable to those obtained by SDT and about twice those from SQT experiments (Fig. S1).

For both fast and slow Av-GPI sub-populations, the diffusion measured by SQT or FRAP at 37 °C were three time faster than at RT and well within the range expected for GPI-AP in HeLa cells (56) (data can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm & Fig. S1). In addition, the respective fractions of each regimes were unchanged, an indication that working at RT did not significantly affect the membrane of HeLa cells.

SQT, SMT and FRAP indicate that, in the absence of probe-induced clustering, Av-GPI still experience two distinct diffusion regimes in the plasma membrane of HeLa cells, with a significant fraction (22 %) switching from one diffusion regime to the other.

Interaction with stationary GM1-rich microdomains induces the slow diffusion of Av-GPI

Since Av-GPI and GM1 can occupy common membrane lipid domains (Fig. 1B), we tested whether these domains were responsible for the observed changes in diffusion regime, using dual-color TIRF imaging of qdot-labeled Av-GPI and Alexa 488-CT×B labeled GM1 (Fig. 2 and Fig. 3 and Movie 3).

As before, SQT analysis yielded a bimodal distribution of diffusion coefficients. However, the addition of CT×B resulted in an approximately four-fold reduction of the diffusion coefficients for slow Av-GPI ($\widehat{D}_{slow}^{CT \times B}$), while fast Av GPI were unaffected ($\widehat{D}_{fast}^{CT \times B}$) (Fig. 3). CT×B also induced changes in diffusion of the slow population, consistent with a more restricted mobility. Namely, (i) the fraction of immobile molecules increased from 3 % to 10 %; (ii) the number of slow directed diffusions (possibly immobile Av-GPI located on slowly stretching or retracting patches of membrane) increased from 3 % to 14 % and (iii) the fraction of pure Brownian diffusion was reduced by half (Fig. 3C). Concomitantly we found that the characteristic confinement radius of slow Av-GPI decreased from 152 nm (standard error or SE: 133-169 nm, n = 13) to 83 nm (SE: 69-100 nm, n = 30, data can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm).

In the CT×B detection channel, we detected the presence of mobile GM1, as well as membrane regions where GM1 diffused very slowly and appeared enriched (Movie M3). In mean intensity projection images ($\Sigma I_{mean} GM1$), these stationary/slow diffusing GM1-rich regions formed a discontinuous phase comprised of domains with size varying from a few microns to diffraction-limited spots (Fig. 2C). When overlaid on CT×B intensity projection images, the majority of slow diffusing Av-GPI (~70 %) colocalized with these stationary GM1-rich microdomains. Fast diffusing Av-GPI were mainly restricted to the continuous unstained phase, sometimes appearing to traverse stained GM1 regions (Fig. 3D, bottom panel). Among fast Av-GPI, those undergoing restricted diffusion appeared more confined after addition of CT×B, with a typical confinement radius decreasing from 640 nm (SE: 577-710 nm, n = 57) to 409 nm (SE: 356-470 nm, n = 62, data can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm). The addition of CT×B also reduced the number of Av-GPI switching from the fast to the slow regime or vice versa (11 % vs. 22 % when CT×B was omitted). However, we were still able to observe few cases of Av-GPI entering and exiting stationary GM1-rich microdomains (Fig. 4 and Movies M4, M5 and M6). In these trajectories, entry in and exit from the microdomains correlated with marked changes in instantaneous diffusion coefficients, which clearly pertained to either the fast or the slow sub-population. These rare events clearly demonstrated that interactions with stationary GM1-rich microdomains induced the slowing down of Av-GPI.

As seen from this analysis, GM1-rich microdomains in the membrane of HeLa cells are capable of compartmentalizing the diffusion of Av-GPI in two ways: (i) by inducing a reduction of the diffusion of Av-GPI by one to two orders of magnitude upon entry in microdomains, and (ii) by acting as obstacles to fast Av-GPI diffusing outside these domains. Overall, these observations suggest that, in the plasma membrane, Av-GPI dynamically partition in two different lipid phases, that can compartmentalize their diffusion.

Av-GPI diffusion is slowed down in the proximity of caveolae

Next, we examined how membrane caveolae influenced the diffusion of Av-GPI. Caveolae are small (~50- 100 nm diameters) plasma membrane invaginations having a lipid composition similar to that of lipid rafts and characterized by the presence of the scaffolding proteins caveolin-1 and 2 (25,57,58). GM1 has been shown to partially concentrate in caveolae in many cell types (59,60) and the addition of CT×B seems to further enhance its sequestering into these structures (61,62). The similar characteristics of caveolae and GM1-rich microdomains led us to investigate whether the changes of Av-GPI diffusion regimes were correlated with the location of these invaginations.

For this purpose, we developed a second HeLa cell line stably co-expressing both Av-GPI and caveolin-1- EGFP (Cav1-EGFP) (Fig. 5A). This cell line exhibited a larger number of

caveolae compared to our initial cell line with most caveolae containing Cav1-EGFP (data can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm). Despite a ten-fold lower membrane expression of Av-GPI (data can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm), good membrane staining was obtained with biotinylated probes (Fig. 5A). Both Cav1-EGFP and Av-GPI were enriched in DRM and cross-linking induced the formation of membrane patches in which Av-GPI and GM1 were co-clustered (data not shown). These cross-linked Av-GPI/GM1 patches colocalized only poorly with caveolae ($16 \pm 6 \%$), but were found in their proximity more frequently than expected by chance (Fig. 5B and Supporting Information).

To study the spatial correlation between Av-GPI and caveolae in more details, we used dual-color TIRF and SQT (Fig. 5C and Movie 7). As previously reported (63,64), some caveolae diffused over short distances but the majority were essentially immobile ($\bar{D}_{\text{cav}} = 7.7 \cdot 10^{-5} \mu\text{m}^2/\text{s}$, Fig. 5D, green histogram) and easily identified in mean intensity images ($\Sigma I_{\text{mean}} \text{Cav1-EGFP}$). Qdot-labeled Av-GPI, on the other hand, were much more mobile and distributed as before into two diffusing sub-populations (Fig. 5D and Movie 7). The

diffusion coefficients of fast $\widehat{D}_{\text{fast}}^{\text{cav1-EGFP}}$, and slow Av-GPI $\widehat{D}_{\text{slow}}^{\text{cav1-EGFP}}$, still differed by more than one order of magnitude, but were about twice as large as in cells expressing Av-GPI only. The characteristic radius of both fast (646 nm, SE: 562-741 nm, $n = 49$) and slow confined diffusions (131 nm, SE: 107-159 nm, $n = 29$) were unchanged.

When overlaid on $\Sigma I_{\text{mean}} \text{Cav1-EGFP}$ images, fast Av-GPI were mainly found diffusing in between caveolae (Fig. 6A), whereas slow ones were often located in regions containing many caveolae and diffused in domains adjacent to these structures (Fig. 6B). For Av-GPI having two diffusion regimes (fast and slow), periods of slow diffusion frequently occurred in the proximity of caveolae (Fig. 6C and Fig. S6). Only 15 % ($n = 27$) of all the Av-GPI tracked, exhibited confinement in caveolae as determined by colocalization (Fig. 6D-E and Fig. S6). Half of these colocalized Av-GPI (8 %) had diffusion coefficients comparable to that of caveolae themselves, indicating possible immobilization in the cavities (Fig. 6E and Fig. S6). The other half (7 %) had diffusion coefficients larger than the associated caveolae and might simply have been confined (sometime transiently) within the invaginations (Fig. 6D).

Despite a reduced membrane expression and the expression of Cav1-EGFP, Av-GPI maintained two main diffusion regimes. In addition, despite the high number of caveolae, entry and trapping into these cavities were too infrequent to account for the large number of diffusions pertaining to the slow regime. Clearly, slowing down of Av-GPI appears to be mainly induced by interaction with non-caveolar GM1-rich domains located in close proximity to caveolae, yet clearly distinct from caveolae themselves.

Acute cholesterol depletion slows down Av-GPI and restricts them to GM1-rich domains

We then studied how cholesterol, which promotes the formation of microdomains and controls lipid dynamic in membranes (30,65), influences the lateral diffusion of Av-GPI. Since the physical and functional properties of raft-and caveolae-associated molecules can be affected by a variety of cholesterol depleting drugs (see (66) and references therein) we repeated our SQT experiments after treatment of HeLa cells with lovastatin or methyl-beta cyclodextrin (m β CD), two drugs that reduce cell cholesterol levels.

Lovastatin treatment (10 μM for 30 hr) resulted in a $\sim 14 \%$ reduction in free cholesterol, but did not significantly decrease plasma membrane cholesterol levels compared to control cells ($86 \pm 22\%$, filipin staining, Fig. S7). A partial disorganization of the cortical actin network was also observed (Fig. S8) as previously reported for similar treatments of epithelial and

endothelial cells (67,68). Av-GPI and GM1 appeared homogeneously distributed in the cell membrane, although CT×B staining after fixation and cell permeabilization revealed the presence of a small perinuclear pool of GM1 not present in untreated cells (Fig. S8). The latter is consistent with the redistribution of sphingolipids to the Golgi apparatus upon cholesterol depletion (69). Despite these various effects, lovastatin treatment did not markedly influence the diffusion of Av-GPI or their interaction with GM1-rich domains (Movie M8). When studied by SQT, both sub-populations of fast $\widehat{D}_{fast}^{+lova+CT\times B}$ and slow $\widehat{D}_{slow}^{+lova+CT\times B}$ Av-GPI diffused with characteristics similar to untreated cells labeled with CT×B (Fig. S9). The fractions of fast and slow diffusions were unchanged despite a broader distribution for fast Av-GPI.

mβCD (10 mM for 1 hr) was a much more potent cholesterol-depletion agent and resulted in a ~60 % reduction in free cholesterol and a dramatic decrease in plasma membrane cholesterol levels compared to control cells ($17 \pm 7\%$, filipin staining, Fig. S7). Cells slightly rounded up after treatment (Fig. 7A). To a lesser extent than with lovastatin, the cortical actin network was affected with the appearance of microspikes and some actin-rich foci (Fig. S8). However, depletion of GM1 from the plasma membrane and redistribution to perinuclear compartments were significantly higher than for the lovastatin treatment (Fig. S8). A four-fold reduction in Av-GPI surface density was also detected by FACS (data not shown). Confocal imaging of live mβCD-treated HeLa cells confirmed the low membrane levels of Av-GPI and GM1, but also revealed punctuated domains enriched in both Av-GPI and CT×B-labeled GM1 (Fig. 7A) (70).

Contrary to the lovastatin experiment, mβCD treatment induced a significant reduction of Av-GPI lateral mobility. SQT analysis showed a single distribution of diffusion coefficients (Fig. 7C) with a modal value nearly three times smaller than the slow Av-GPI sub-

population in untreated cells $\widehat{D}_{slow}^{+m\beta CD-CT\times B}$. Concomitantly the typical corral radius of confined diffusions was reduced from 152 nm to 72 nm (SE 64-80 nm, n = 17, data can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm). This reduction in lateral mobility was confirmed by FRAP, but mobility could be restored to pre-treatment levels after incubation in serum-supplemented media for 20 hr (Fig. 7B).

The reduction in Av-GPI surface density, together with the loss of the fast diffusing population and the presence of a single and slow regime of diffusion suggested that most Av-GPI in the membrane might be associated with GM1-rich domains after mβCD treatment. To test this hypothesis, we repeated the SQT experiments in the presence of Alexa 488-CT×B. The number of GM1-rich microdomains in mβCD-treated cells was much lower than observed for untreated cells and the mobility of CT×B-labeled GM1 appeared reduced (Movie M9). More than 50% of all qdot-labeled Av-GPI colocalized with stationary GM1-rich microdomains (Fig. 7D), and CT×B labeling further reduced their mobility, essentially bringing them to a halt $\widehat{D}_{slow}^{+m\beta CD+CT\times B} = 2.7 \cdot 10^{-5} \mu\text{m}^2/\text{s} < D_{\min} = 4.8 \cdot 10^{-5} \mu\text{m}^2/\text{s}$, Fig. 7C). This observation is therefore consistent with a majority of Av-GPI being associated with GM1-rich microdomains after cholesterol depletion by mβCD.

We then repeated the mβCD treatments on HeLa cells co-expressing Av-GPI and Cav1-EGFP. As before, a single population of slow Av-GPI was observed (data not shown). Av-GPI mostly localized in close proximity to immobile Cav1-EGFP-labeled membrane domains (Fig. 7D). Since we previously showed that Av-GPI diffusing in GM1-rich microdomains tend to localize close to caveolae, finding Av-GPI in close proximity to Cav1-EGFP labeled structures after mβCD treatment further supports our observation that Av-GPI are mainly associated with GM1-rich regions of the membrane after acute cholesterol depletion.

Actin cytoskeleton disruption results in faster Av-GPI diffusion but does not suppress slow diffusion

The cortical actin meshwork is essential in maintaining cell shape and structure by providing a dynamic and elastic scaffold to the plasma membrane (71). The actin cytoskeleton can also influence the surface topology of the plasma membrane and affect the diffusion of raft associated lipids and proteins involved in signaling (72,73). To evaluate the role of the actin network, we studied the diffusion of Av-GPI and GM1 after treatments of HeLa cells with a high concentration of latrunculin-A, a potent actin-disrupting agent (74) (Fig. S10 and discussion in Supporting information). Despite extensive disruption of F-actin with 10 μ M latrunculin-A, we still observed a fast and a slow diffusing populations of Av-GPI (Movie M10). However, membrane retraction and ruffling induced by extensive actin depolymerisation prevented us from studying the correlation between slow Av-GPI diffusion and the proximity of GM1-rich domains. Because of this altered topology of the membrane, we only studied trajectories that did not get close these perturbations and limited our analysis to reporting the diffusion coefficient of fast Av-GPI only. The diffusion

coefficient of fast Av-GPI ($\widehat{D}_{fast}^{lat-A} = 1.7 \cdot 10^{-1} \mu\text{m}^2/\text{s}$, SE: $1.4 - 2.1 \cdot 10^{-1} \mu\text{m}^2/\text{s}$) increased nearly five-fold compared to untreated cells suggesting that the cortical actin cytoskeleton can impend the diffusion of fast Av-GPI in normal conditions. This diffusion coefficient was similar to that of qdot-labeled Av-GPI diffusing in the L_d phase of 1:1 DPPC:DOPC + 0.01 % Fluorescein-DPPE supported lipid bilayers within which the GPI-test probes were re-inserted after extraction from the membrane of HeLa cells (data not shown). Interestingly however, the observation that many Av-GPI (~ 50 % of traces) still diffused slowly in live cells indicated that an intact actin cytoskeleton was not necessary for the existence of slow diffusion domains in the membrane.

Discussion

Considering the complex, crowded and dynamic architecture of cell plasma membranes, it can be challenging to assess how different membrane components affect the diffusion of endogenous proteins or lipids. The task is even more complicated without a complete map of existing interactions between these molecules. In this respect, tracking exogenous probes can be advantageous to decipher the respective influence of protein- or lipid-based microdomains, of membrane heterogeneous scaffolding or of the extracellular matrix on the lateral mobility of biomolecules. By designing a raft-associated GPI-anchored test probe (Av-GPI) having no biological function and no specific interaction with other proteins (but possibly non-specific ones) we aimed to study the organization of the plasma membrane and in particular the influence of lipid raft microdomains on the diffusion of raft-associated proteins in HeLa cells. Using targeted qdots and single-molecule imaging techniques, we studied the long-term diffusion of Av-GPI and revealed the existence of non-caveolar, cholesterol- and GM1-rich microdomains within which these GPI-anchored probes can dynamically partition and be compartmentalized.

Av-GPI as a raft-associated GPI-AP model probe

We showed that Av-GPI is efficiently expressed and targeted to lipid rafts as defined biochemically (Fig. 1). As commonly observed for native GPI-AP, (i) Av-GPI associates with DRM in a cholesterol-dependent manner (Fig. 1C), (ii) colocalizes with GM1 patches induced by antibody cross-linking (Fig. 1B), and (iii) diffuses with diffusion coefficients typical of GPI-AP in HeLa cells (56). Thus, Av-GPI appears to be an appropriate model probe to study the dynamics of raft-associated GPI-AP in this cell type.

Since avidin form tetramers in solution, the choice of Av-GPI as a model GPI-AP may appear problematic, because it could exist in various multimeric forms in the membrane

(monomer, dimer, etc), each characterized by a different diffusion behavior. We have shown however, that Av-GPI is displayed in the cell membrane as a tetramer, rather than as a mixture of monomers, dimers, trimers or higher-order oligomers (such as multi-tetramers). In addition, we minimized the probability of targeting hypothetical non-tetrameric configurations of Av-GPI by using concentrations of biotinylated qdots ($\sim 10^{-12}$ M) well below the K_D of the monomeric ($K_D \sim 10^{-7}$ M) and dimeric ($K_D \sim 10^{-8}$ M) forms of avidin (75,76). Finally, the size variation between a monomer and a tetramer (or for that matter multi-tetramers) cannot account for the observed 1-2 orders of magnitude variation in diffusion coefficients (77). Thus, heterogeneous multimerization of Av-GPI, even though it is in principal possible, cannot explain the two main diffusion regimes we observed.

Small peptide-coated qdots have limited effects on the intrinsic diffusion of Av-GPI

We further demonstrated that the valency of biotinylated qdots had little effect on the diffusion of Av-GPI and did not induce their cross-linking and slowing down in the membrane, even when 50 % of the coated peptides are biotinylated (Fig S3). We did not test higher valency since we previously showed that excessive amount of biotin on the qdot surface can inhibit the binding to Av-GPI because of steric hindrance (43). This valency-independent behavior is in stark contrast with that of antibody-coated gold particles commonly used for SPT, where diffusion differs depending on whether quasi-monovalent or multivalent particles are used (78,79) or whether free ligand is added to cells during tracking (80). In the case of multivalent particles, cross-linking of several proteins to a single gold probe is possible due to the particle large size and the recognition of surface epitopes, two problems eliminated by the small size of peptide-coated qdots and the reduced accessibility to the deeply buried biotin-binding pockets of GPI-anchored avidin. The absence of qdot-induced transient or permanent clustering of Av-GPI was further confirmed by SDT and FRAP with monovalent biotinylated probes. As a result, we deem it unlikely that the slow diffusing population of Av-GPI corresponds to transient anchorage (81) or to stimulation-induced temporary arrest of lateral diffusion (80).

Comparison between SQT, SDT and FRAP experiments also revealed a two-fold reduction in the diffusion coefficient of Av-GPI when labeled with qdots. Similar differences have been observed for SPT with gold nanoparticle probes (33). Although, these differences have been attributed to the valency and size of SPT probes (33), our data with quasi-monovalent qdots point toward a size effect only. Despite their small size (~ 4 times smaller than antibody-labeled gold nanoparticles), peptide-coated qdots may still interact with the immediate membrane environment, especially when imaging is performed in the ventral membrane. However, the characteristic multimodal diffusion of Av-GPI was not an artifact of qdots, since quantitatively similar changes in diffusion were still observed with smaller and monovalent fluorescent probes. Despite carrying a qdot, diffusing Av-GPI were still able to access very small membrane structures such as caveolae. Using small peptides to functionalize qdots minimizes their final size and cross-linking issues and thus provides a very advantageous alternative to streptavidin and antibody-functionalized qdot or gold probes.

Av-GPI dynamically partition in stationary GM1-rich domains in the membrane of HeLa cells

Our analysis showed that the diffusion of Av-GPI in the membrane of HeLa cells is heterogeneous and characterized by a broad, bimodal distribution of diffusion coefficients (Fig. 3). The diffusion coefficients determined here by SQT, SDT and FRAP are well within the range reported for various GPI-AP (10^{-4} - $25 \cdot 10^{-2} \mu\text{m}^2/\text{s}$) (33). The presence of diffusion regimes differing by 1-2 orders of magnitude has also been previously reported for other GPI-AP such as Thy-1 (82,83), NCAM120 (84), or GPI-modified MHC class II proteins

(85), but the origin of the observed slow diffusion has remained unclear. Here, using CTxB as glycosphingolipid GM1 marker and Cav1-EGFP as a caveolae marker, we provide direct evidence that slow Av-GPI diffusion correlates with entry into or interaction with stationary, non-caveolar GM1-rich microdomains. Fast Av-GPI diffusion, however, mainly takes place in a what appears to be a continuous phase where GM1 also diffuses rapidly. The simultaneous colocalization and slow diffusion of two “raftophilic” molecules (Av-GPI and GM1) in membrane domains separated from an apparently more fluid membrane phase strongly suggest that GM1-rich microdomains might harbor putative lipid rafts, regardless of their actual size. The existence of two main diffusion regimes for Av-GPI correlating with these different membrane domains further indicates that, in HeLa cells, GM1-rich microdomains are capable of influencing the partitioning and the diffusion of membrane proteins, independently of protein multimerization or probe cross-linking.

It is worth mentioning here, that the existence of these two different diffusion regimes is not an artifact of the 100 ms/frame rate used in these experiments. Although long duration fast diffusions observed at slow frame rates may result in blurring of the point spread function (PSF), the centroids of these (potentially but not necessarily) blurred PSFs can still be accurately determined. Indeed, it has been previously demonstrated by Monte Carlo simulations that frame rate does not affect the determination of diffusion coefficients for uniform Brownian diffusion (35). Our choice of frame rate does not allow us to detect random alternation of very brief bursts of fast diffusion (< ms to a few ms) described in much faster tracking studies (35). It is thus possible that either or both the fast and slow diffusion regimes of Av-GPI identified in this study may in fact comprise very short phases of very rapid diffusion. However, such a behavior would only increase the measured average diffusion coefficients for each regime, without changing the fact that there are two typical diffusion regimes. As proposed in the lipid raft hypothesis (25), we observed that GM1-rich microdomains compartmentalize the diffusion of Av-GPI, at both room temperature (~27°C) and 37°C (SQT, Fig 3A and data available at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm). Although our analysis did not distinguish between corralled diffusion and diffusion limited by obstacles, the colocalization of slow diffusion regimes with CTxB-labeled regions suggests an effective compartmentalization in GM1-rich microdomains (radius: 152 nm). On the other hand, GM1-rich regions also appear in some case to act as barriers to the diffusion of fast Av-GPI. The observation of trajectories grazing without entering these regions suggests that the characteristic length scale of restricted diffusion for fast Av-GPI (radius: 640 nm) reflects the characteristic distance between discontinuous GM1-rich microdomains. As emphasized previously, this compartmentalization effect is independent of CTxB labeling (Figure 3A) but appear accentuated in the presence of the toxin sub-unit.

The observation of stationary GM1-rich microdomains and mobile GM1 sphingolipids both labeled with CTxB, is consistent with several previous studies. FRAP measurements on cell membranes have shown that an immobile fraction of CTxB-labeled GM1 co-exists with rapidly diffusing GM1-associated CTxB (86). FRET measurements in live cells also demonstrated that CTxB can bind both clustered and non-clustered GM1 (87). The existence of stable GM1-rich microdomains in the absence of CTxB has also been confirmed by freeze fracture electron microscopy (88). These experiments provided initial evidence that GM1 clustering is not simply a function of CTxB binding, and that CTxB can effectively be used to detect pre-existing GM1-rich microdomains.

However, CTxB can induce subtle changes in GM1-rich microdomains, which are consistent with GM1 clusters being stabilized upon binding of the pentameric toxin subunit in these domains (87). We observed that CTxB labeling leads to (i) a ~ 3-fold increase in immobile Av-GPI, (ii) a ~ 4-fold reduction of the diffusion coefficients of the slow

population and (iii) a ~ 2-fold reduction of the corral size for the slow confined subpopulation. These changes might stem from partial cross-linking of small GM1-rich structures by CTxB (29,88). Alternatively, they may simply reflect a contraction of preexisting large microdomains due to the replacement of free GM1 molecules by denser GM1-CTxB clusters. In both cases, clustering of GM1 lipids creates an obstacle-rich environment for slow Av-GPI and leads to even slower diffusions or immobilization. CTxB-stabilized structures also appear less permeable to the diffusion of fast Av-GPI as observed from the ~ 1.5-fold reduction in characteristic confinement size for the fast confined population. Both effects can explain the reduced dynamic partitioning of Av-GPI in and out of GM1-rich microdomains observed after addition of CTxB (11% of all Av-GPI vs. 22% when CTxB is omitted). Indeed, obstructed diffusion and slowing down of Av-GPI within CTxB-labeled GM1-rich microdomains could result in a lower escape probability (transition from slow to fast), while the reduced permeability could lead to a lower probability to enter them (transition from fast to slow).

Thus, CTxB induces subtle changes in the organization of GM1-rich microdomains, which can then influence the diffusion of GPI-AP. It is then important to consider these effects when studying the organization of cells plasma membrane. The subtle influence of CTxB on the diffusion of Av-GPI and its recently demonstrated ability to induce the coalescence of raft-like membranes microdomains (89) can be exploited to highlight pre-existing putative raft microdomains, but also clearly limit its use to study the diffusion of GM1 lipids.

Two additional observations may give clues about the organization of GM1-rich domains: (i) some fast Av-GPI were observed to diffuse through CTxB-labeled domains without slowing down (Fig. 3D and S9C) and (ii) the largest fraction of slow Av-GPI did not exhibit any confinement. Highly dynamic barriers delimitating GM1-rich microdomains could account for these characteristics. Alternatively, they fit well into a lipid raft model that predict them to be small (10-200 nm, below optical resolution) (25) and capable of forming high-density nanodomains (< 10 nm) stabilized by cholesterol and sphingolipids (6,90). GM1-rich microdomains might thus be interpreted as regions with higher local density of nanodomains than the rest of the plasma membrane. In these regions, slowing down of Av-GPI could be the result of increased interaction with such nanodomains (91,92).

The fact that Av-GPI transiently interact with glycosphingolipid-rich microdomains indicates that the dynamic partitioning raft model (83,86) is appropriate to describe the diffusive behavior of this GPI-AP in HeLa cells. This partitioning in and out of GM1-rich microdomains was similar for two HeLa cell lines having ten-fold differences in Av-GPI membrane expression levels (data can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm). This suggests that the membrane partitioning of GPI-AP is maintained independently of the protein expression levels. As discussed below, membrane lipids and, in particular, cholesterol and glycosphingolipid GM1 might be important regulators of this partitioning.

Caveolae and GM1-rich domains are distinct membrane structures

Two independent observations indicate that GM1-rich domains and caveolae, despite their known similar raft-like composition, are related but distinct structures of the membrane in HeLa cells. First, two-color imaging and SQT of Cav1-EGFP and qdot-labeled Av-GPI showed that, in most cases, transition from fast to slow diffusion was not due to entry into caveolae. However, many SQT trajectories exhibited a fast to slow transition within a few hundred nanometers of caveolae. Second, although confocal imaging of caveolae and cross-linked Av-GPI indicated a statistically significant proximity of the GPI probes with caveolae, they did not significantly colocalize. This observation might be cell-type specific, as the literature contains results supporting as well as contradicting it. For instance, our

confocal microscopy observations seem in agreement with a study of BHK cells showing that cross-linked GPI-anchored placental alkaline phosphatase does not result in accumulation into caveolae (50), but in contradiction with other studies reporting enrichment of GPI-AP in caveolae after cross-linking (93,94).

Although caveolae and GM1-rich domains appear morphologically distinct, their observed proximity (Fig. 6) is consistent with various functions attributed to caveolae. It has recently been suggested that caveolae play a role in lipid regulation, via the interaction of caveolin-1 with cholesterol, by acting as cholesterol and sphingolipid reservoirs and regulating their supply to membrane raft (95). The proximal localization of GM1-rich microdomains with caveolae observed here in live cells is reminiscent of other reports in which GPI-AP-rich membrane microdomains were found adjacent to caveolae (59,96,97). This close proximity could facilitate cholesterol and GM1 exchange between the two compartments.

We did not investigate the relation between GM1-rich microdomains and clathrin-coated pits in this study. Previous reports have indicated that GM1 and some GPI-AP interact with clathrin-coated invaginations (98-100), but a study using FRET and immunofluorescence has clearly shown that CTxB-labeled GM1 clusters are excluded from them (87). Since we showed that slow diffusing Av-GPI are colocalized with GM1-rich domains, it is unlikely that slow diffusion is due to interaction with clathrin-coated pits. Further study will be needed to fully address this question.

Slow diffusion of Av-GPI may also be the result of entry into CTxB-labeled membrane invaginations other than caveolae or clathrin-coated pits, such as clathrin-independent carriers (101,102). Such carriers can transport GPI-AP and GM1 towards GPI-AP enriched endosomal compartments (103). We have not tested this possible relationship, but one observation argues against it. Indeed, caveolin-1 expression was reported to reduce the endocytic efficiency of clathrin-independent carriers (101). Since different expression levels of caveolin-1 (between the cell line not expressing Cav1-EGFP and that expressing it) did not significantly affect the bimodal distribution of Av-GPI in HeLa cells, slow Av-GPI do not seem to interact significantly with such invaginations.

Cholesterol regulates the organization of GM1-rich domains and the dynamics of Av-GPI

We showed that two standard cholesterol-depleting drugs have very different effect on the diffusion of Av-GPI. Lovastatin treatments, which mildly modified the actin cortical network (Fig. S8A) barely changed cellular and plasma membrane cholesterol levels or the distribution of GM1 in the membrane (Fig. S8B). Accordingly, the diffusion of Av-GPI and GM1 was not significantly affected. mβCD had similar effects on the cytoskeleton, but led to markedly reduced cholesterol and GM1 membrane levels. Concomitantly, the fast population of Av-GPI was strongly affected and our test probes appeared mainly associated with GM1-rich microdomains. This mβCD treatment led to a significant reduction of the lateral mobility of Av-GPI, but also of GM1, as observed for other GPI-AP and glycosphingolipids in previous studies (87,104,105). Despite the possible indirect effects of mβCD treatment on the actin cytoskeleton (106), changes in the diffusion of Av-GPI and GM1 observed in mβCD-treated cells but not in lovastatin-treated cells appear mainly due to the altered cholesterol and GM1 membrane levels rather than to a reorganization of the actin cytoskeleton. A similar dependence of the mobility of GM1 and other GPI-AP on membrane cholesterol content but not on actin was recently observed in COS-7 cells using FCS (30) and in CHO cells by single-dye tracking (104). We also observed a reduced mobility of Av-GPI after mβCD treatment by FRAP. These FRAP experiments, which were performed with much higher concentrations of biotinylated probes compared to that used for SQT, showed that the fraction of immobile Av-GPI increased dramatically, but also that a significant fraction still diffused over long distances (Fig. 7B). This apparently contradicts the fact that

we rarely observed trajectories extending beyond 1 μm during SQT. However, the two observations can be reconciled if the apparent enrichment of Av-GPI within GM1-rich domains after m β CD treatment simply reflects higher number of Av-GPI within these domains compared to the rest of the membrane. In this case, random binding of the few qdots used for SQT will preferentially take place in these domains. This hypothesis is in agreement with the punctuated membrane distribution and the colocalization of Av-GPI with GM1 domains observed by confocal microscopy at high concentration of probes (Fig. 7A). If, as it appears, these domains cover only a small fraction of the total membrane surface area after m β CD-treatment, their contribution to the total fluorescence intensity monitored by FRAP is expected to be moderate, as observed in practice.

There could be several causes to such an apparent enrichment of Av-GPI in GM1-rich microdomains upon acute membrane cholesterol depletion. Changes in the lipid composition of microdomains could result in higher affinity of Av-GPI for these regions compared to the rest of the membrane and lead to an effective accumulation by altered partitioning. Alternatively, extending the argument used to interpret the decrease in dynamic partitioning in and out of GM1-rich microdomains after CT $\times$ B labeling, the apparent enrichment could be simply due to longer time spent in GM1-rich domains. Observing even longer trajectories than done here may allow testing this hypothesis in future experiments. Finally, our data might reflect the action of m β CD on different membrane cholesterol pools. It has been shown that m β CD preferentially disrupts glycerophospholipid-rich regions in plasma membranes and spares sphingolipid-rich microdomains (66). In these domains, cholesterol can tightly pack against the hydrocarbon chains of sphingolipids and can be shielded from extraction. The same report also showed that removal of membrane cholesterol by m β CD can indirectly induce the release of GPI-AP not associated with glycosphingolipid-rich domains. Under similar conditions, we also observed a reduction of Av-GPI surface density by FACS and confocal imaging, as well as the presence of a single Av-GPI population associated with GM1-rich microdomains. It is thus possible that Av-GPI not associated with sphingolipid-rich microdomains (fast population) have been preferentially extracted following cholesterol removal by m β CD, while Av-GPI associated with GM1-rich microdomains (slow population) were spared. The unbalanced membrane distribution of Av-GPI induced by such a differential effect of m β CD can explain why the population of Av-GPI associated with GM1-rich microdomains appears enriched after cholesterol depletion. Importantly such an effect supports our finding that Av-GPI can reside and dynamically partition in two plasma membrane lipid phases having different glycosphingolipid GM1 organization and different cholesterol contents.

We also showed that the characteristic size of GM1-rich microdomains, as well as the diffusion coefficients of Av-GPI in these domains were reduced after acute cholesterol depletion with m β CD. Both effects are consistent with an increased ordering of glycosphingolipid GM1 around Av-GPI in these regions and could reflect a transition from a L_o phase towards a gel (solid-like) lipid phase, favored at low membrane cholesterol and GM1 concentrations (107,108). A similar transition was recently reported by Nishimura *et al.* (105). The near complete immobilization of Av-GPI after addition of CT $\times$ B (Fig. 7, ~80-fold decrease of $\bar{D}$ in m β CD-treated cells *vs.* ~3-fold decrease in untreated cells), also suggests that cholesterol depletion induces a high density packing of GM1 around Av-GPI in GM1-rich microdomains, which is compounded by a more efficient cross-linking of GM1 by CT $\times$ B.

Together, these results indicate that an appropriate balance between cholesterol, GM1 and other membrane lipids is essential for the fluidization of GM1-rich microdomains and for the membrane distribution of both Av-GPI and GM1. They attest that cholesterol is a key

element for the dynamic of raft-associated biomolecules and for the organization and maintenance of some sphingolipid-rich microdomains.

Slow diffusion of Av-GPI is not induced by actin-stabilized microdomains

Recent studies have suggested that the cortical actin cytoskeleton modulates the diffusion dynamics of outer membrane protein and lipids, by (i) organizing the plasma membrane in small and juxtaposed domains delineated by transmembrane proteins anchored to the underlying actin network (picket-fence model) (8) and (ii) stabilizing cholesterol-dependent clusters of raft-associated proteins following cross-linking in transient confinement zones (TCZs) (84,109), transient anchorage zones (TAZ) (81) or zones of stimulation-induced temporary arrest of lateral diffusion (STALL) (80). In line with some of the predictions of the picket-fence model, we find that extensive disruption of the actin cytoskeleton results in significantly faster diffusion of fast Av-GPI. Surprisingly however, many slow diffusing Av-GPI could still be observed, an indication that domains of slow diffusion, which our study strongly correlated with GM1-rich microdomains, are not directly maintained by the actin cytoskeleton. A similar observation has been recently reported by Lenne *et al.* (30). These actin-independent microdomains in which Av-GPI experience slow diffusion seem distinct from actin-stabilized membrane domains such as TCZs, which have been observed for a variety of GPI-AP including Thy-1, NCAM125 and CD59 (80,84,109). TCZs, TAZs and STALLs zones are thought to be membrane domains enriched in glycosphingolipids (83), in which GPI-AP can be efficiently cross-linked. In short, they are regarded as “cluster-stabilized” lipid rafts. As observed for TCZs, the confinement size of slow Av-GPI is on the order of 300 nm in diameter, is cholesterol-dependent and is reduced by two-fold after m β CD treatments. As for TCZs, domains of slow diffusion can also be revisited and Av-GPI can diffuse within them for tens of seconds. Thus, there are some parallels between the membrane microdomains where Av-GPI diffuse slowly and TCZs observed for other GPI-AP. However, one major difference is that, in contrast to our measurements, TCZs are detected after cross-linking and clustering of raft-associated molecules by multivalent gold particles (5). Accordingly, the lifetime and diffusion coefficients of TCZs depend on the number of molecules that are cross-linked (78,81). The large size of the gold probes might also lead to non-specific binding to other membrane proteins or lipids, which could result in an overestimation of confinements. Here we have shown that with small and quasi-monovalent probes, a GPI-AP can display zones of slow diffusion that share many characteristics of TCZs, but are detected in the absence of cross-linking. Moreover, we did not find any correlation between the time spent by Av-GPI in GM1-rich domains and the qdot probes valency. Furthermore, Av-GPI with restricted diffusion represent only a small fraction of all slow Av-GPI, as expected for domains that are not induced by cross-linking. Finally, diffusion in TCZs is characterized by diffusion coefficients that are only about twice smaller as those outside TCZs, while we find much larger variation of diffusion coefficients (~ 40-fold) when Av-GPI partition in and out of GM1-rich microdomains. Thus, our data supports the presence of pre-existing membrane microdomains having characteristics similar to that of putative lipid rafts and capable of influencing the partitioning and the diffusion of GPI-AP, independently of probe cross-linking and stabilization by the actin cytoskeleton. It is of course possible that these same microdomains become transient confinement zones after cross-linking. More experiments with better control on decoupling the cytoskeleton from the membrane will be needed to fully elucidate the relationship between GM1-rich microdomains, slow diffusing GPI-AP and the cytoskeleton.

Conclusion

We have developed two HeLa cell models expressing an artificial GPI-AP to address the nature and dynamics of putative raft microdomains in the membrane of living cells using

long-term single-qdot tracking. According to a recent review, lipid rafts are expected to be “small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein/protein and protein/lipid interactions” (25). Using both Av-GPI and CTxB as raft markers and Cav1-EGFP as a caveolae marker, we have shown that the diffusion of raft-associated proteins is indeed highly dynamic and heterogeneous. In particular, the diffusion of Av-GPI was slowed down upon interaction with stationary GM1-rich microdomains, which were often found in proximity to caveolae but distinct from them. The slow and cholesterol-dependent diffusion of Av-GPI in sphingolipids GM1-rich microdomains further indicates that these membrane regions can compartmentalize cellular processes and are potential lipid raft sites. The cross-linking and crowding effects of CTxB also indicates that GM1 is enriched in GM1-rich microdomains and that these domains can be stabilized by specific protein/lipid interaction. Our data further demonstrates that the dynamic partitioning raft model satisfactorily describes the diffusive behavior of some raft-associated proteins into and out of GM1-rich microdomains in live cells.

Although our results are currently limited to a single cell type, they support the raft hypothesis by providing direct evidence that a GPI-AP can dynamically partition and be compartmentalized in non-caveolar, cholesterol- and GM1-rich microdomains in the plasma membrane of living cells.

Materials and Methods

Single-molecule imaging by objective-type TIRF

Custom-built dual-color TIRF microscopes were used to perform two-color imaging of qdot-labeled Av-GPI and CTxB-Alexa 488 labeled-GM1 or Cav1-EGFP (schematics can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm). In both cases, the ventral plasma membrane of HeLa cells was imaged. The diffusive behavior of Av-GPI was similar in the ventral or dorsal membrane (data not shown). However, we preferred ventral imaging by TIRF to limit the probability of imaging membrane regions containing microvilli or other membrane corrugations thus guaranteeing that the diffusion of the Av-GPI was essentially confined in two dimensions (110). A detailed description of the setups is available in Supporting Information. Unless stated, imaging was done at room temperature (~27 °C).

Typically, 2 to 3 cells per field-of-view were imaged continuously for 90-120 s, using an integration time of 100 ms per frame. Faster acquisitions were also performed (7 ms/frame using only a subset of the EMCCD camera, data not shown). However, because of software limitations that prevented from storing movies with large numbers of frames, such faster frame rate would have resulted in trajectories with shorter duration (and limited field of views), which would have undermined our ability to detect the existence of long-term changes in diffusion regime in the cell membrane. In addition, because CTxB-Alexa 488 and qdots have different brightness, acquisition times had to be optimized so that both signals could be simultaneously detected on each side of the same EMCCD camera. At 100 ms/frame, there was a good balance between signal intensity and acquisition speed.

For single-dye tracking (SDT) of Av-GPI labeled with Alexa-488 biocytin (Invitrogen), imaging was performed continuously with an integration time of 60 ms and at a final dye concentration of 75 pM. For all conditions, cells grown at ~70 % confluency on fibronectin coated coverslips were first starved for 3-4 hr in serum-free DMEM at 37 °C to free Av-GPI from biotin present in the serum supplement. Cells were then imaged in HBSS + 1 % BSA for a maximum of 30-45 min after which they were replaced.

Biotinylated CdSe/CdS/ZnS qdots emitting at 620 nm and coated with 50 % biotinylated peptides and 50 % of 600 Da polyethylene glycol (PEG) peptides were synthesized and prepared as previously described (43,111). Alternatively, 620 nm-emitting CdSe/ZnS in toluene (Evident Technologies, Troy, NY) were used and coated with peptides as above. The final diameter of the biotinylated qdots was 13.0 ± 1.1 nm (112). Quasi-monovalent biotinylated qdots were obtained by reducing the amount of biotinylated peptides to 1-2 % during the coating procedure and using 49-48 % of a third, lysine terminated peptide (peptide # 7 in Pinaud *et al.* (43), Fig. S3). Qdots were added to the cells at a final concentration of 2-3 pM directly in the imaging media to label only few Av-GPI (~ 10 per cell). A low-level labeling facilitated tracking for long duration, by limiting the probability that two or more qdot-labeled Av-GPI crossed paths or blinked simultaneously within the same area. In addition, low concentrations of qdots limit potential toxic effects (113) and interference with normal cellular metabolism. For dual-color imaging experiments, a final concentration of 0.05-0.1 $\mu\text{g/ml}$ of Alexa 488 cholera toxin B (CT $\times$ B) was added simultaneously with qdots.

Data Analysis

Post-acquisition, each frame of a recorded TIRF movie was split into two images (green and red channels) using Metamorph software (Molecular Device, Sunnyvale, CA). Images of both channels were overlaid after correcting for offsets and spherical and chromatic aberrations as determined from the emission of 40 nm TransfluoSpheres. A software was developed in Labview (National Instruments, Austin, TX) to track and analyze trajectories of qdot-labeled Av-GPI (See Supporting information for details). Briefly, regions of interest in images were selected and a semi-automatic algorithm was used to fit the point-spread-function (PSF) of the tracked qdots with a 2D Gaussian in each frame (52, 53) (Fig. 2). The quality of the fit was verified visually for each frame. When the signal in a frame was lost because of blinking, no fitting was performed until re-appearance of the PSF. When a PSF did not re-appear within 10 s, tracking was aborted. Tracking was also aborted when two qdot-labeled Av-GPI crossed paths. Because of blinking, binding to cell membrane during imaging, or internalization of qdot-labeled Av-GPI, the mean duration of trajectories varied between 50-75 s. After tracking, a trajectory file containing all information was saved for each Av-GPI molecule. Subsequent analysis (described in detail in Supporting Information) included (i) computing the mean-square displacement (MSD) curve for a trajectory, (ii) representing the fluorescence intensity along the diffusion path, (iii) representing the instantaneous diffusion coefficient (38), (iv) calculating the probability distribution of

square displacement $P(\vec{r}^2, \tau)$ (PDSD) for each molecule or for all molecules by global analysis (55) and (v) analyzing PDSD for different time lags in terms of diffusion models (Table S2). The presence of single or multiple diffusive regimes within each trajectory was deduced from PDSD analysis on the first 10 % of time lags t , and by fitting the PDSD with one, two or three exponentials. The quality of each fit was evaluated using the normalized residual curve. Normalized residuals deviating systematically by more than 10 % (from zero) resulted in the rejection of the fitted curve. The fitted exponents obtained were then used to plot $r_1^2(t)$ curves for different time lags t (see Supporting Information). If the PDSD curves were well fitted with a single exponential, the corresponding single $r^2(t)$ curve was plotted (Fig. 2D, Av-GPI with a single diffusion mode). If the PDSD was best fitted with more than one exponential, then as many $r^2(t)$ curves were plotted (Fig. 2D, Av-GPI with multimodal diffusion). For each $r^2(t)$ curve, the diffusion mode and corresponding diffusion coefficient were determined as follows. The diffusion model that best described a $r^2(t)$ curve was selected by fitting the $r^2(t)$ curve with the most likely among diffusion models picked from Table S2. For instance, for a concave $r^2(t)$ curve, we skipped the directed motion model. The respective quality of each fit was assessed visually although a quantitative

method such as normalized residuals analysis could be implemented if needed. Additionally, we used Occam's razor principle, keeping the model with the least parameters if two or more models accounted well for the $r^2(t)$ curve. The fitting parameters for the best fit diffusion model were used as is (see Supporting Information). This approach does not only permit a classification of each mode of diffusion into pure Brownian, restricted or directed motion as does the approach of Wilson et al. (114) but also allows the computation of a diffusion coefficient for each mode. Since the PSD curves are calculated for the first 10 % of time lags, there is ample statistics to obtain reliable $r_i^2(t)$ curves. Monte-Carlo simulations of trajectories of freely diffusing particles, confined or switching between free and confined diffusion, confirmed that PSD analysis could detect multimodal diffusion and correctly recover the diffusion coefficient for each mode of diffusion (Fig. S5). In few cases, no convincing PSD fits or no convincing $r^2(t)$ fits could be obtained. In these cases, the trajectories were rejected.

In addition to the classification into different mode of diffusion, we identified a category of immobile Av-GPI (if $D < D_{\min}$) using a diffusion coefficient threshold $D_{\min} = 4.8 \cdot 10^{-5} \mu\text{m}^2/\text{s}$. This cut off value $D_{\min}$ corresponds to the 95th percentile of the distribution of diffusion coefficients for qdots imaged on fibronectin-coated coverslips, and represents the level of drift in the setups (Fig 3, grey histogram).

The apparent diffusion coefficients measured from fitting each $r^2(t)$ curves were reported in histograms. Because the diffusion coefficients are distributed log-normally over several orders of magnitudes, histograms of the decimal logarithms of diffusion coefficients are reported (D-histograms in the following). The bin size of the D-histograms was determined statistically using an approach introduced by Knuth (115). This method adapts the bin size to the underlying distribution without a priori knowledge of its nature. After evaluating the optimal bin size for each experimental condition, we chose twice the largest bin size and applied this modified optimal bin to all data. With this common bin size, a direct comparison between all distributions is possible and sub-populations in histograms are sufficiently separated.

In the text, the diffusion coefficients reported correspond to the peak position(s) of single (or multiple) Gaussian fit(s) of the D-histograms. Standard errors values (SE) were determined using 1,000 bootstrap replica of the distributions (116), and are given in the form of an upper and lower diffusion coefficient. Because the distributions of the diffusion coefficients are relatively large, we only considered differences in diffusion coefficients larger than 3 times the SE to be significant.

To evaluate the number of Av-GPI switching between the fast and slow regime (or vice versa), a cut-off value corresponding to the 95th percentile of the Gaussian fitted on the slow distribution was used (e.g. $1.3 \cdot 10^{-2} \mu\text{m}^2/\text{s}$ for Fig. 3A). Av-GPI trajectories having two very different diffusions regimes distributed above or below this cut-off value were classified as switching trajectories.

The sizes of confinement domains are extracted for Av-GPI traces fitted with a restricted diffusion model (Table S2) and are separately determined for fast and slow subpopulations. The confinement/zone geometry is assumed to be circular (corral). As for diffusion coefficient values, corral sizes appeared to be distributed log-normally. We therefore computed histograms of decimal logarithm of sizes. Confinement sizes were determined by Gaussian fitting of the distribution histograms. Standard error of the mean (SE) was determined as before. Differences in confinement size larger than 3 times the SE were considered to be significant.

To study the colocalization of Av-GPI with immobile/slow diffusing CT×B-labeled GM1-rich domains or Cav1-EGFP-labeled caveolae, Av-GPI trajectories were overlaid with the mean intensity projection image of the movie (green channel, ΣI_{mean}). ΣI_{mean} is defined as the image whose pixel value $p(k, l)$ at coordinates (k, l) is the mean value of all pixels $p_i(k, l)$ for N frames of the movie at this location:

$$p(k, l) = \frac{1}{N} \sum_{i=1}^N p_i(k, l).$$

With this approach, diffusing GM1 or Cav1-EGFP are efficiently filtered out from the final image and GM1-rich microdomains and caveolae are easily identified (data can be viewed at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm). Further confirmation of colocalization was done by overlaying the fluorescence intensity time trace of a qdot-labeled Av-GPI along its trajectory (in a 3×3 pixel region centered on the qdot location) with the corresponding fluorescence intensity time trace in the green channel. Periods showing overlap of red and green fluorescence after background subtraction were interpreted as interaction of Av-GPI with GM1 domains or caveolae. However, this approach is limited by the bleaching of organic dyes and EGFP, which make the distinction between labeled and non-labeled regions more difficult at longer times. In few cases, we could correlate changes in diffusion (D_{in} and D_{out}) with the entry or exit from GM1-rich domains or caveolae by performing MSD analysis on sub-trajectories and PSD analysis (Fig. S5). Parts of the trajectory in which Av-GPI is colocalized with a GM1-rich domain or a caveola (identified by fluorescent signal above background in the CT×B or Cav1-EGFP channel) were selected and separated from the non-colocalized parts. The MSD of each sub-trajectory was separately computed. MSD fitting with a simple Brownian motion model results in a D value for each types of region, D_{in} and D_{out} . However, this analysis has several drawbacks: (i) it assumes that the boundaries of these regions (along the trajectory) can easily be identified and (ii) the MSD analysis does not permit an accurate determination of the type of diffusion, and hence the corresponding diffusion constant. At least qualitatively, MSD analysis reveals that diffusion in GM1-rich domains is much slower than outside them ($D_{\text{in}} \ll D_{\text{out}}$ in Fig. S5). To obtain a quantitative measure, we perform a PSD analysis on the same trajectory. In general, the number of diffusion regimes identified in this manner corresponds to that obtained using the more subjective MSD approach (Fig. S5) and provide accurate diffusion coefficients. When combining sub-trajectories MSD analysis, instantaneous diffusion analysis and PSD analysis of the same trajectory, it is then straightforward to assign an accurate D value to regions and/or time periods exhibiting the slowest diffusion (D_{in}) or the fastest diffusion (D_{out}).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

GPI-anchored avidin is targeted to the outer plasma membrane of HeLa cells and associates with lipid rafts as biochemically defined.

(A) Schematic representation of the avidin/CD14 fusion (Av-GPI) construct. The full-length chicken avidin (amino acids 1-153) was fused in frame with the GPI-anchor sequence of CD14 (amino acids 318-376) to target Av-GPI to the outer membrane of HeLa cells.

(B) Distribution of Av-GPI and GM1 in the membrane of HeLa cells with or without cross-linking with anti-avidin antibodies. Cross-linking induces the co-clustering of Av-GPI and GM1 (see also data at http://fpinaud.bol.ucla.edu/index_files/Traffic.htm). Under the same conditions, endogenous transferrin receptors (TfR, non-raft proteins) remain evenly distributed (bottom row). Scale bars: 10 μ m.

(C) Av-GPI are enriched in detergent resistant membranes (DRM). After cold detergent extraction and sucrose gradient separation, most Av-GPI (96 %, see Table S1) are found in the light/DRM-rich fraction while the non-raft transferrin receptors (TfR, 97 %) are in the dense fraction. Cholesterol depletion with lovastatin induces a partial repartitioning of Av-GPI into the dense fraction (36 %) and a reduced recovery of Av-GPI (10 % of untreated). Lovastatin has no effect on the total protein recovery, the recovery of TfR or their distribution.

Figure 2.

Single qdot tracking of Av-GPI by total internal reflection fluorescence (TIRF) microscopy, quantification and classification of diffusion modes.

(A) First frame from a dual-color TIRF movie of a HeLa cell. Av-GPI in the ventral plasma membrane are labeled with qdots (red) and GM1 are labeled with Alexa-488 CT×B (green).

(B) Selected frames from a region of interest (white square in (A)) in which diffusing Av-GPI are tracked. Diffusion trajectories are determined by the series of fitted positions, connected by a straight line. Notice that Alexa-488 CT×B bleaches fast compared to qdots and signal was nearly completely lost after 10 s (Movie M3). To facilitate visualization, the qdot PSF size was intentionally expanded. Tracking was performed on raw images.

(C) Overlay of Av-GPI trajectories with the mean intensity projection image (ΣI_{mean}) for the Alexa-488 CT×B channel (see Materials and Methods). This approach allows colocalization studies of Av-GPI with fixed/slow diffusing GM1-rich domains despite the fast photobleaching of Alexa-488 CT×B (see data at

http://fpinaud.bol.ucla.edu/index_files/Traffic.htm).

(D) Analysis by probability distribution of square displacements (PDS) for various trajectories and classification into diffusion modes. PDS analysis was done on the first 10

% of time lags (t) (see Material and Methods). The resulting $r_i^2(t)$ curves (black dots or square) were fitted with either pure Brownian, restricted or directed diffusion models (red, Table S2). When an Av-GPI experienced changes in diffusion during tracking, multiple $r_i^2(t)$ curves were obtained and the mode of diffusion and the diffusion coefficient for each regime was determined. A representative sample of the various diffusion modes and diffusion coefficients of Av-GPI in HeLa cells are shown together with the corresponding trajectories and their duration. Notice that classification into various diffusion modes could not be accurately performed by simple visual inspection of the trajectories.

Figure 3.

Bimodal diffusion of Av-GPI and interaction with GM1-rich microdomains.

(A) Distribution of Av-GPI diffusion coefficients (red D-histogram) in HeLa cells without GM1 staining (-CTxB). Two Av-GPI diffusion regimes (fast and slow) are recovered after PDSD analysis, with $\widehat{D}_{fast} = 3.8 \cdot 10^{-2} \mu\text{m}^2/\text{s}$ (SE $3.2\text{-}4.5 \cdot 10^{-2} \mu\text{m}^2/\text{s}$, 55 %) and $\widehat{D}_{slow} = 9.1 \cdot 10^{-4} \mu\text{m}^2/\text{s}$ (SE $0.7\text{-}1.2 \cdot 10^{-3} \mu\text{m}^2/\text{s}$, 42 %). A D-histogram of qdots non-specifically bound to fibronectin (gray) is used to define immobile Av-GPI (3 %). The fraction of Av-GPI switching between fast and slow diffusion is 22 % (fraction determined using $1.3 \cdot 10^{-2} \mu\text{m}^2/\text{s}$ as a cut-off diffusion value to separate slow and fast diffusion. This value encompasses 95 % of the diffusion coefficients of the slow population).

(B) Distribution of Av-GPI diffusion coefficients in the presence of the GM1-marker Alexa 488 cholera toxin-B (+CTxB). CTxB specifically induces a four-fold reduction in diffusion for the slow Av-GPI sub-population ($\widehat{D}_{slow}^{+CTxB} = 2.4 \cdot 10^{-4} \mu\text{m}^2/\text{s}$, SE $1.7\text{-}3.4 \cdot 10^{-4} \mu\text{m}^2/\text{s}$, 43 %). Fast Av-GPI are unaffected ($\widehat{D}_{fast}^{+CTxB} = 3.6 \cdot 10^{-2} \mu\text{m}^2/\text{s}$, SE $2.9\text{-}4.6 \cdot 10^{-2} \mu\text{m}^2/\text{s}$, 47 %). Arrows indicate the center of the distributions in the absence of CTxB in (A).

(C) Effect of CTxB on the diffusion modes for slow and fast Av-GPI sub-populations. The increase in directed diffusions and stationary molecules at the expense of pure Brownian diffusion indicates that CTxB restrict the mobility of slow Av-GPI. For the fast diffusing sub-population, CTxB only induces a moderate reduction of Av-GPI with restricted diffusions.

(D) Colocalization studies of Av-GPI with immobile/slow diffusing CTxB-labeled GM1 domains. A majority of slow Av-GPI (~70 %) are found colocalized with GM1-rich microdomains, while fast Av-GPI avoid these domains. Colocalization observed from ΣI_{mean} images are confirmed by correlating the fluorescence intensity time trace of qdot labeled Av-GPI (red) with the CTxB signal (green) along the diffusion trajectory. Because fluorescent signals varied from cell to cell, the background signal (grey) in close proximity to the trajectory is plotted for both red and green detection channels. Scale bars: 500 nm.

Figure 4.

Dynamic partitioning of Av-GPI in and out of GM1-rich membrane microdomains. Example of Av-GPI exiting (A, B) or entering (C, D) cholera toxin B (CTxB) labeled GM1-rich microdomains (trajectory start: ► / stop: *). For Av-GPI exiting GM1-rich domains (A, B), initial signal colocalization and subsequent absence of colocalization between qdot and CTxB is observed from trajectory overlay on ΣI_{mean} images and from fluorescence intensity time traces. Domain exit correlates with an abrupt increase in the diffusion coefficient (instantaneous diffusions and PDSD analysis). Time periods where Av-GPI colocalize with GM1-rich domains are highlighted in green on instantaneous diffusion plots. The diffusion coefficients inside (D_{in}) and outside (D_{out}) GM1-rich domains are determined from sub-trajectories MSD analysis and PDSD analysis (see Material and Methods). These diffusion values fall well within the distribution of diffusion coefficients for fast and slow Av-GPI determined in Fig. 3B.

Detecting the entry of Av-GPI in GM1-rich domains (C, D) relies mainly on trajectory overlay with ΣI_{mean} images because labeled domains are usually bleached at the time of entry. Domain entry is associated with a clear reduction in diffusion coefficient. Notice that domains can be revisited (D). Some of these trajectories are from cells treated with lovastatin but are also representative of untreated cells since no difference in diffusive behaviors, interaction with GM1-rich domains or partitioning of Av-GPI were observed between both conditions. Scale bars: 500 nm.

Figure 5.

Imaging and tracking of Av-GPI and caveolae in HeLa cells.

(A) Confocal images of HeLa cells expressing Av-GPI and Cav1-EGFP. Scale bar: 10 μm

(B) Cross-linking of Av-GPI with anti-avidin antibodies induces the formation of Av-GPI and GM1-rich patches located in caveolae-rich regions of the membrane. Magnified region of interest (white squares) show that there is no extensive colocalization of these clusters with caveolar domains but that they are often contiguous (arrows). Scale bar: 5 μm .

(C) First frame from a dual-color TIRF movie of a HeLa cell co-expressing Av-GPI (red) and Cav1-EGFP (green, left panel). After tracking, Av-GPI trajectories are overlaid on the mean intensity image of Cav1-EGFP (ΣI_{mean}) to detect interactions with caveolae (right panel). Scale bar: 3 μm .

(D) Distribution of diffusion coefficients for Av-GPI (red) and caveolae (green). The two sub-populations of Av-GPI: fast ($\widehat{D}_{\text{fast}}^{\text{cav1-EGFP}} = 6.0 \cdot 10^{-2} \mu\text{m}^2/\text{s}$, SE $5.3\text{-}6.7 \cdot 10^{-2} \mu\text{m}^2/\text{s}$, 54 %) and slow ($\widehat{D}_{\text{slow}}^{\text{cav1-EGFP}} = 1.8 \cdot 10^{-3} \mu\text{m}^2/\text{s}$, SE $0.9\text{-}3.5 \cdot 10^{-3} \mu\text{m}^2/\text{s}$, 36 %) diffuse much faster than caveolae ($\widehat{D}_{\text{cav}} = 7.7 \cdot 10^{-5} \mu\text{m}^2/\text{s}$, SE $6.5\text{-}9.2 \cdot 10^{-5} \mu\text{m}^2/\text{s}$), an indication that Av-GPI are rarely immobilized within caveolae.

Figure 6.

Tracking of Av-GPI reveals rare colocalization with caveolae but slower diffusion in their proximity.

(A) Fast Av-GPI diffuse mainly in caveolae-free part of the membrane. Colocalization with Cav1-EGFP labeled domains sometimes occurs but is not accompanied by apparent changes in diffusion.

(B) Slow diffusing Av-GPI are often found adjacent to caveolae.

(C) Examples of Av-GPI undergoing changes in diffusion during tracking. As in (B), intervals of slow diffusion (white arrows) often localize in close proximity to caveolae.

(D) On rare occasions, changes in diffusion upon direct interaction with caveolae are observed. Slow diffusion domains colocalizing with caveolae are highlighted in red and indicated by white arrows.

(E) Slow Av-GPI colocalized with caveolae have diffusion coefficients that may or may not be similar to that of the caveola itself, suggesting immobilization or simple confinement within the caveolar invagination (see also Fig. S6). Scale bars (A-E): 1 μ m.

Figure 7.

Acute cholesterol depletion by m β CD induces a slowing down of Av-GPI and an apparent re-distribution in non-caveolar GM1-rich microdomains.

(A) Three dimensional projection of live HeLa cells treated with m β CD for 1 hr. Acute cholesterol depletion leads to the rounding up of cells, decreased Av-GPI surface density and redistribution of Av-GPI and GM1 into colocalizing punctuated domains in the membrane. Scale bar: 15 μ m.

(B) FRAP of membrane Av-GPI directly after m β CD treatment (open circle) and after cholesterol replenishment (black circle). The diffusion of Av-GPI is reduced after treatment with m β CD but can be restored to pre-treatment levels after re-incubation into serum-supplemented media for 20 hr. Both FRAP curves are averaged over three cells and acquired at 37 $^{\circ}$ C.

(C) Distribution of Av-GPI diffusion coefficients after m β CD treatment (top panel) and m β CD treatment followed by imaging in the presence of cholera toxin B sub-unit (+CTxB, bottom panel). A single diffusing population of Av-GPI was recovered ($\widehat{D}^{m\beta CD/-CTxB} = 3.5 \cdot 10^{-4} \mu\text{m}^2/\text{s}$, SE $2.5\text{--}4.9 \cdot 10^{-4} \mu\text{m}^2/\text{s}$). The addition of CTxB induces a ~ 80 -fold decrease in diffusion and nearly complete immobilization of Av-GPI ($\widehat{D}^{m\beta CD/+CTxB} = 2.7 \cdot 10^{-5} \mu\text{m}^2/\text{s}$, SE $2.2\text{--}3.3 \cdot 10^{-5} \mu\text{m}^2/\text{s}$).

(D) Correlation between Av-GPI trajectories and GM1-rich domains (top panel) or Cav1-EGFP domains (bottom panel) in m β CD-treated HeLa cells. About 50 % of Av-GPI colocalized with slow/immobile CTxB-labeled GM1-rich microdomains. Fluorescent CTxB signal along the trajectory of Av-GPI indicates that Av-GPI interact directly with these domains. After m β CD-treatment Av-GPI diffused in domains adjacent to but distinct from caveolae, as verified by the absence of Cav1-EGFP fluorescent signal along the trajectory of the AV-GPI (marked by an asterisk). Scale bars: 1 μ m (top) and 2 μ m (bottom).