Multimerizable HIV Gag derivative binds to the liquid-disordered phase in model membranes

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Introduction

Lipid rafts are commonly seen as assemblies of saturated lipids, cholesterol and sphingomyelin that accumulate a subset of membrane proteins (Simons and Ikonen, 1997). Rafts are highly dynamic, have a size on the nm scale and can be stabilized or recruited by external factors to become bigger domains. Lipid raft stabilization or recruitment is functionally important in various processes (Lingwood and Simons, 2010). It can occur if multivalent or multimerizing proteins specifically bind raft components and thereby cross-link them. This has been shown in cells, in vitro, and in simulations as, for example, in the case of cholera toxin binding to the ganglioside GM1 (Wolf et al., 1998; Hammond et al., 2005; Putzel and Schlick, 2009).

So far, lipid rafts have mainly been discussed in the context of the outer leaflet of the plasma membrane. Nevertheless, a functional role of membrane domains has been established for several processes on the intracellular side of the plasma membrane (Hancock and Parton, 2005; Ono and Freed, 2005; Yanez-Mo et al., 2009). However, it is difficult to investigate if these kinds of domains are linked to or even identical with lipid rafts, because they usually cannot be purified without artefact-prone disruptive techniques. HIV assembly is ideally suited to study this process, because it involves the inner leaflet of the plasma membrane, it is functionally dependent on membrane microdomains, and the subsequent release of viral particles provides means for non-invasive purification of these membrane domains (Aloia et al., 1988; Brugger et al., 2006; Chan et al., 2008; Ono, 2010). Lipidomic analysis of purified HIV-1 particles has revealed their raft-like lipid composition (Brugger et al., 2006; Chan et al., 2008), suggesting that assembly either occurs at pre-existing membrane microdomains or induces microdomain formation.

The assembly of HIV particles takes place at the plasma membrane of host cells (Bieniasz, 2009). This process is governed by the structural polyprotein Gag. When expressed alone, Gag induces the formation of virus-like particles, which are structurally very similar to complete virions (reviewed by Sundquist and Krausslich, 2012). Gag only binds to membranes when they contain phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] (Ono et al., 2004; Dalton et al., 2007; Chukkapalli et al., 2008; Alfadhi et al., 2009) and when the matrix domain (MA) is

Summary

During HIV assembly, a protein coat on the inner leaflet of the plasma membrane drives the formation of virus particles, and appears to induce the preferential accumulation of ‘raft’ lipids in the viral envelope, although the lipid raft concept mainly proposes microdomains of these lipids in the outer leaflet. The common hypothesis is that Gag preferentially associates with, and thereby probably induces, raft-like domains, because the protein is multimerized and specifically linked to two saturated acyl chains. To test this hypothesis, we constructed a minimal in vitro system in which we analysed the interaction of a Gag derivative, which could be triggered to multimerize, with a domain-forming model membrane resembling the inner leaflet of the plasma membrane. Confirming studies with authentic Gag, this Gag derivative only bound to membranes when it was multimerized, myristoylated and when phosphatidylinositol 4,5-bisphosphate was present in the membrane. Unexpectedly, however, the multimerized Gag derivative was largely excluded from ordered domains in model membranes. This suggests that the mechanism of membrane reorganization during HIV assembly does not simply result from a higher affinity of the clustered Gag membrane binding domain to ordered membrane domains, but involves more complex biophysical interactions or possibly also an additional protein machinery.
myristoylated (Gottlinger et al., 1989; Zhou et al., 1994). Furthermore, multimerization-deficient Gag mutants fail to bind membranes (Zhou et al., 1994; Scarlata et al., 1998; Liang et al., 2003). At the membrane, Gag assembles into higher-order multimers. Multimerization is promoted by protein interactions between the Gag capsid (CA) domains and nucleic acid binding by the Gag nucleocapsid (NC) domains (Ganser-Pornillos et al., 2008). Nuclear magnetic resonance spectroscopy analysis of MA in complex with a PI(4,5)P₂ derivative with truncated acyl chains suggested that PI(4,5)P₂ is specifically bound by MA in a way that one saturated hydrocarbon chain remains in the membrane, while the second unsaturated hydrocarbon chain is flipped out of the membrane (Saad et al., 2006). This would mean that each MA domain is linked to two saturated lipid anchors. Together with the high degree of Gag multimerization, this could trigger an accumulation of raft lipids at the HIV assembly site (Lori­zate and Krausslich, 2011). However, direct experimental evidence supporting this hypothesis is currently lacking.

We tested the core aspect of this hypothesis, whether multimerized Gag by itself is preferentially targeted towards more ordered membrane domains. This can strictly only be addressed in vitro, because influences of other factors cannot be excluded in a living cell. Furthermore, the actual state of the membrane in live cells can neither be determined nor manipulated without side-effects. In vitro, lateral inhomogeneity of membranes is usually modelled by phase-separated membranes consisting of liquid-ordered (Lo) raft-like domains and complementary liquid-disordered (Ld) domains. In contrast to lipid rafts, Ld and Lo domains in model membranes are in thermodynamic equilibrium, µm-sized, and additional factors are absent. Despite the apparent differences in mechanisms that govern the existence of rafts and Lo domains, the mechanisms that determine the association of components with Lo domains and lipid rafts seem to be similar. This is supported by several in vitro studies, which showed sorting of putative raft-proteins into Lo phases in model membranes (Hammond et al., 2005; Kahya et al., 2009; Windschiegl et al., 2009). Here, we devised a minimal system based on a phase-separated model membrane, which mimics the composition of the inner leaflet of the plasma membrane. This extends earlier studies, which mainly focused on modelling the outer leaflet. In this system, we analysed the membrane binding of an HIV-1 Gag-derived protein, which retains its membrane binding function, but can be induced to multimerize. The results of these experiments directly confirmed that membrane binding of Gag is dependent on myristoylation, multimerization and specific binding of PI(4,5)P₂, but did not show evidence for the preferential binding to or recruitment of Lo membrane domains by the model Gag protein.

Results

A multimerizable Gag derivative for use with model membranes

HIV Gag is a multifunctional polyprotein. The N-terminal MA domain directly interacts with the membrane via a patch of positively charged amino acids, an N-terminal modification with the saturated fatty acid myristate, and binding of PI(4,5)P₂ (Fig. 1A, scheme). The CA and NC

![Fig. 1. MA-EGFP-Fv3 multimerizes into planar patches upon addition of dimerizer if it is myristoylated and PI(4,5)P₂ is present.](image-url)

A. HIV Gag is a multidomain protein. Positive charges and myristoylation in the MA domain, its binding to PI(4,5)P₂ as well as intermolecular interactions of the CA and NC domains, which promote multimerization (arrows), all contribute to membrane binding and assembly. Upon cotransfection of Gag and Gag-EGFP, 293T cells exhibit sub-diffraction fluorescent spots at the plasma membrane.

B. In the Gag derivative MA-EGFP-Fv3, the domains CA, NC and p6 have been replaced by three FKBPV domains, each of which can be cross-linked by the bifunctional artificial ligand dimerizer (dashed arrows). Upon addition of dimerizer to 293T cells expressing MA-EGFP-Fv3, planar micrometre-sized fluorescent domains are observed at the plasma membrane. These domains do not form if cells are depleted of free PI(4,5)P₂, a myristoylation-deficient mutant is used (D), or dimerizer was omitted (E). Transfected live 293T cells were imaged by laser scanning microscopy. Projections of image stacks are shown. Scale bar: 10 µm.
domains direct the assembly of Gag into a highly ordered hexameric lattice by protein–protein (CA) and protein–nucleic acid (NC) interactions. This multimerization is required for efficient membrane binding (Ganser-Pornillos et al., 2008). However, the ability of the multifunctional Gag protein to induce high local curvature on a sub-diffraction scale and subsequently bud away from the membrane severely complicates quantitative analysis of membrane binding by optical methods. In addition, topology-induced membrane reorganization can hardly be uncoupled from lipid-specific non-steric effects by cross-linked saturated acyl chains serving as raft anchors. Moreover, full-length Gag is biochemically largely incompatible with studies on model membranes for practical reasons. Concentrated Gag is only monomeric at high salt concentrations, and its assembly into multimers in the presence of membranes would have to be triggered by reducing the salt concentration. The associated changes in osmolarity destroy free-standing model membranes such as giant unilamellar vesicles (GUVs). Furthermore, its high aggregation potential hampers purification of myristoylated full-length Gag in sufficient amounts for biochemical studies.

To overcome these limitations, we constructed a Gag derivative that contains MA, EGFP and three artificial multimerization domains. MA is indispensable for our experimental system because it contains the membrane binding sites, which are hypothesized to associate with lipid rafts. EGFP was inserted at a suitable position at the C-terminus of MA, where it is compatible with particle assembly (Muller et al., 2004). The other domains of Gag only indirectly contribute to membrane binding, via their multimerization capability, and were therefore substituted by artificial inducible dimerizing domains (Fig. 1B, scheme). These domains are derived from the FK506 binding protein (FKBP) and engineered to dimerize upon addition of the artificial ligand dimerizer (Clackson et al., 1998). The resulting fusion protein MA-EGFP-Fv3 is monomeric and can be induced to multimerize by the addition of dimerizer, which does not cause significant changes in osmolarity and is therefore compatible with model membranes. Furthermore, multimerized MA-EGFP-Fv3 forms planar, μm-sized patches, which can be comfortably studied by confocal microscopy (Fig. 1B), in contrast to full-length Gag and Gag-EGFP that co-assemble into sub-diffraction spots at the plasma membrane (Fig. 1A). Finally, myristoylated MA-EGFP-Fv3 can be purified in sufficient amounts and purity for biochemical studies (Fig. S1).

The Gag derivative MA-EGFP-Fv3 only binds to membranes when it is myristoylated, multimerized and when PI(4,5)P2 is present

To examine whether MA-EGFP-Fv3 requires myristoylation, multimerization and PI(4,5)P2 for membrane binding like native full-length Gag, 293T cells were transfected with MA-EGFP-Fv3 and incubated with dimerizer. Transfected cells showed large fluorescent membrane-associated patches upon the addition of dimerizer (Fig. 1B), while no plasma membrane binding of MA-EGFP-Fv3 was observed in the absence of dimerizer (Fig. 1E). To test the dependence on PI(4,5)P2, cells were depleted of this lipid by incubation with 4 mM DTT and 50 mM formaldehyde (Scott, 1976; Keller et al., 2009). A diffuse cytosolic distribution of MA-Fv3-EGFP was observed in this case, and no binding to the plasma membrane was detected when dimerizer was added (Fig. 1C). Likewise, a myristoylation-deficient MA-EGFP-Fv3 derivative, in which a glycine residue at position 2 was substituted by alanine (MAG2A-EGFP-Fv3), was diffusely distributed in the cytoplasm of intact cells in the absence or presence of dimerizer (Fig. 1D).

Similar results were obtained in vitro. Purified myristoylated MA-EGFP-Fv3 was incubated with GUVs in a binding assay. Non-phase-separated GUVs containing 56% DOPC, 25% cholesterol, 15% DOPS, 4% (18:0–20:4) PI(4,5)P2 and 0.01% fastDiI (Table 1 #1) were prepared by electroformation in a sucrose solution and sedimented in buffer containing physiological salt concentrations. For some GUVs, the fourfold negatively charged PI(4,5)P2 and parts of the DOPC were exchanged for an additional 16% of DOPS (single negative charge, Table 1 #2). These GUVs had the same surface charge as the former ones but lacked PI(4,5)P2. Different lipid dyes were used to distinguish the vesicles. The two types of GUVs were mixed and incubated at room temperature with MA-EGFP-Fv3 in the presence or absence of 500 nM dimerizer. Quantitative homogeneous MA-EGFP-Fv3 binding was observed for PI(4,5)P2-containing GUVs (Fig. 2A), while only few protein aggregates were detected on negatively charged vesicles lacking PI(4,5)P2 (Fig. 2B). No GUV binding of MA-EGFP-Fv3 was detected in the absence of dimerizer (Fig. 2C) or for MAG2A-EGFP-Fv3, which lacks the myristoylation site (Fig. 2D).

In summary, MA-EGFP-Fv3 behaved like Gag in all relevant aspects of membrane binding in the context of living cells and in vitro (Liang et al., 2003). Furthermore, the mode of membrane binding of Gag was confirmed in an independent in vitro system.

A model system for the inner leaflet of the plasma membrane

Gag association with lipid rafts via two saturated acyl chains, which are embedded into the cytosolic leaflet of the plasma membrane and serve as raft anchors, is an analogous concept to the well-understood lipid raft recruitment by cholera toxin on the extracellular leaflet of the plasma membrane. Therefore, the model membrane...
systems used in this study were designed based on phase-separated GUVs used in comparable studies, but extended to mimic the inner leaflet of the plasma membrane.

The two most common kinds of lipid mixtures showing L_d/Lo-type lipid phase separation were used as standard model systems. One contained sphingomyelin, cholesterol and an unsaturated phosphatidylcholine (PC) (see Table 1, #3). The other was a mixture of a saturated PC, an unsaturated PC and cholesterol (see Table 1, #4). For studying reversible phase transitions at physiologically relevant temperatures, a variant of the first mixture was adjusted to a miscibility transition temperature of 30–31°C (see Table 1, #6). To mimic the inner leaflet of the plasma membrane more closely, a more complex mixture was introduced with a phase separation based on saturated and unsaturated lipids (see Table 1, #5): here, much of the PC content was substituted by phosphatidylethanolamine and phosphatidylserine species because these lipids are major constituents of the inner leaflet (van Meer et al., 2008). For technical reasons, the PC content was still higher than it is assumed to be in the inner leaflet of the plasma membrane, according to available data from erythrocyte membranes (Daleke, 2008). PI(4,5)P_2 was added to all mixtures, because it is specifically bound by Gag and is a component of the inner leaflet of the plasma membrane. Furthermore, traces of the fluorescent dye fastDiI, which resembles unsaturated lipids, were added as marker of the L_d phase. Fluorescently labelled cholera toxin subunit B bound to traces of the glycosphingolipid GM1, which can be specifically bound by fluorescently labelled cholera toxin subunit B, a L_d phase marker. DOxx, dioleoyl phospholipids; DPxx, dipalmitoyl phospholipids; xxPC, phosphatidylcholines; xxPE, phosphatidylethanolamines; xxPS, phosphatidylserines; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate [in this case synthetic (18:0–20:4) PI(4,5)P_2]; SM, sphingomyelin (in this case natural extracts from bovine brain).

Table 1. Overview of lipid compositions of GUVs.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Phase separation</th>
<th>Phospholipid 1 (unsaturated)</th>
<th>Phospholipid 2</th>
<th>Cholesterol</th>
<th>PI(4,5)P_2</th>
<th>Other charged component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Homogeneous</td>
<td>None</td>
<td>56% DOPC</td>
<td>–</td>
<td>25%</td>
<td>4%</td>
<td>15% DOPS</td>
</tr>
<tr>
<td>2</td>
<td>Without PI(4,5)P_2</td>
<td>None</td>
<td>44% DOPC</td>
<td>–</td>
<td>25%</td>
<td>–</td>
<td>31% DOPS</td>
</tr>
<tr>
<td>3</td>
<td>SM-based</td>
<td>L_d/L_o</td>
<td>32.5% DOPC</td>
<td>40% SM</td>
<td>20%</td>
<td>7.5%</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Saturation-based</td>
<td>L_d/L_o</td>
<td>19.7% DOPC</td>
<td>46.3% DPPC</td>
<td>30%</td>
<td>7.5%</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>Complex mix</td>
<td>L_d/L_o</td>
<td>19.7% DOPC, DOPS, DOPE (41:36:23)</td>
<td>46.3% DPPC, DPPS, DPPE (41:36:23)</td>
<td>30%</td>
<td>7.5%</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>Low T_m</td>
<td>L_d/L_o</td>
<td>31.6% DOPC</td>
<td>30% SM</td>
<td>25%</td>
<td>7.25%</td>
<td>6% DOPS</td>
</tr>
</tbody>
</table>

Homogeneous (non-phase-separated) mixtures #1 and #2 were used for binding assays; phase-separated mixtures #3 to #6 were used for partitioning assays. Composition #6 was adjusted for a low miscibility transition temperature (T_m) of around 30–31°C. All lipid mixtures contained 0.1% of the fluorescent lipid dyes DiD-C18 (#2), fastDiI (#3 to #5) or bodipyTMR-PI(4,5)P_2 (#6) as L_d marker. Mixtures #3 to #6 additionally contained 0.02% of the glycosphingolipid GM1, which can be specifically bound by fluorescently labelled cholera toxin subunit B, a L_d phase marker. DOxx, dioleoyl phospholipids; DPxx, dipalmitoyl phospholipids; xxPC, phosphatidylcholines; xxPE, phosphatidylethanolamines; xxPS, phosphatidylserines; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate [in this case synthetic (18:0–20:4) PI(4,5)P_2]; SM, sphingomyelin (in this case natural extracts from bovine brain).

Fig. 2. Membrane binding determinants for MA-EGFP-Fv3 in vitro.
A. In the presence of 500 nM dimerizer, purified myristoylated MA-EGFP-Fv3 (250 nM) was incubated with PI(4,5)P_2-containing GUVs (composition #1 supplemented with 0.1% fastDiI, see Table 1). A colocalization of MA-EGFP-Fv3 and fluorescent GUVs was observed.
B. In the same sample chamber, no EGFP fluorescence was found to colocalize with GUVs, in which PI(4,5)P_2 was substituted for charge-compensating amounts of DOPS (composition #2), and the lipid dye fastDiI (red) was substituted for DiD-C18 (false-coloured in blue).
C. No colocalization of EGFP with GUV membranes was found either if dimerizer was omitted as compared to A or non-myristoylated MAG2A-EGFP-Fv3 (250 nM) was used (D). GUVs were imaged by laser scanning microscopy. In C and D the contrast in the EGFP channel was increased to show the diffuse background of unbound protein. Scale bars: 5 μm.
The Gag derivative MA-EGFP-Fv3 is excluded from the raft-like Lo phase

The partitioning of MA-EGFP-Fv3 on phase-separated GUVs was tested under conditions similar to the GUV binding assay above. GUVs of compositions #3 to #5 (Table 1) were incubated with 200 nM MA-EGFP-Fv3 in a physiological buffer resembling intracellular conditions (25 mM Hepes/KOH pH 7.4, 120 mM potassium acetate, 1 mM EDTA, 1 mM DTT). Upon addition of 500 nM dimerizer, EGFP fluorescence was observed on a section of the GUV perimeter, which was seen for all three tested lipid compositions (Fig. 3). The remaining surface area of the GUVs appeared dark, clearly indicating selective binding of the Gag model protein to specific subregions. Unexpectedly, however, MA-EGFP-Fv3 always colocalized with the Ld marker fastDiI (Fig. 3). This binding to the Ld phase was observed for the sphingomyelin/cholesterol-based lipid mixture #3 (Fig. 3A), the lipid mixture #4 with saturated and unsaturated PC species (Fig. 3B), and the complex lipid mixture #5 more closely resembling the inner leaflet of the plasma membrane (Fig. 3C). Thus, the partitioning was not dependent on the presence or absence of sphingomyelin or the complexity of the lipid mixture. The same result was obtained for several other lipid compositions with the same components, but different molar fractions of lipids and different miscibility transition temperatures in screening experiments. Counte rstaining with the Lo marker cholera toxin B microsopy.

The partition coefficient of MA-EGFP-Fv3, i.e. the fluorescence intensity in the Ld phase compared to the Lo phase, was very high (>10) and could not be determined accurately. For the sphingomyelin-based lipid mixture #3, no fluorescence signal above background could be detected in the Lo phase. For the simple saturation-based mixture #4, a variable hazy signal was measured in the Ld phase resulting in apparent partition coefficients ranging from 9 to 15. For the complex mixture #5, measurements were impeded by the small domain size and resulting fast diffusion of domains. The partition coefficients were obtained from experiments with at least three independently prepared GUV preparations.

Next, it was tested whether MA-EGFP-Fv3 is kinetically trapped in the Ld phase. This may happen if the initial membrane binding site of MA-EGFP-Fv3 is determined by the distribution of P(4,5)P2, which is expected to reside preferentially in the Ld phase. Then, the limited diffusion of extended cross-linked protein clusters could prevent a relocalization to the Lo phase. At room temperature, MA-EGFP-Fv3 was allowed to multimerize on GUVs produced from lipid mixture #6 (Fig. 4 upper panels), which show a miscibility transition temperature of about 30–31°C (Table 1). Also at this temperature very close to the miscibility transition temperature, MA-EGFP-Fv3 showed a highly selective binding to the Ld phase, which is characterized by the presence of the fluorescent lipid analogue bodipyTMR-Pi(4,5)P2 and absence of the Lo marker CtxB-Alexa 647. Besides serving as an Ld marker, bodipyTMR-Pi(4,5)P2 was used as an indicator for the distribution of P(4,5)P2. BodipyTMR-Pi(4,5)P2 was observed to be less strictly excluded from the Ld phase than MA-EGFP-Fv3 under these conditions. However, it has to be taken into account that the partitioning of fluorescent lipid analogues can be influenced by the presence of the fluorophore. Then the system was heated to 32°C, such that the lipid marker was distributed homogeneously. MA-EGFP-Fv3 was observed to spread over the whole surface of the GUVs at this temperature (Fig. 4 middle panels). The same homogeneous distribution was observed for the Lo marker CtxB-Alexa 647 if it was present in the same experiment. Thus, no component was found to be excluded from a certain area fraction at this temperature. Subsequently, the system was slowly cooled to ambient temperature, to induce phase separation again. MA-EGFP-Fv3 consistently colocalized with the Ld
marker and PI(4,5)P₂ after temperature-induced phase separation, and was excluded from areas with a high fluorescence intensity of the L₀ marker, as observed before (Fig. 4 lower panels).

In summary, MA-EGFP-Fv3 was found almost exclusively on the non-raft-like L₀ phase under all tested experimental conditions and there was no trapping of MA-EGFP-Fv3 in the L₀ phase because of higher initial concentrations of PI(4,5)P₂ in that phase.

**Discussion**

We designed a minimal system to mimic the presumable interaction of HIV Gag with lipid rafts or lipid raft precursors, a process that is supported by many indirect studies, but has not been directly proven so far. To this end, the artificial derivative of HIV Gag MA-EGFP-Fv3 was constructed. It contained only the MA domain of Gag, which is required for membrane binding. Additionally, the construct contained EGFP and artificial inducible multimerization domains, which functionally replace the Gag domains with multimerization capability. We showed that membrane binding of MA-EGFP-Fv3 in cells and *in vitro* is dependent on myristoylation, multimerization and interaction with PI(4,5)P₂. This confirms earlier results about the mode of membrane binding of HIV Gag in an independent *in vitro* system, and shows the validity of this experimental system (Lorizate and Krausslich, 2011).

Unexpectedly, multimerized MA-EGFP-Fv3 was found to reside almost exclusively in L₀ domains of phase-separated membranes, which resemble the non-raft fraction of the plasma membrane (Fig. 3). This challenges the initial hypothesis of this study that Gag associates with lipid rafts via multiple interlinked raft anchors. Model membrane systems with coexisting L₀ and L₃ phases, as used in this study, are established and mechanistically under-
stood for studying this kind of raft clustering or induction processes (Wolf et al., 1998; Hammond et al., 2005; Putzel and Schick, 2009). In particular, experiments and computer simulations have shown that physical linkage of raft anchors by protein scaffolds can induce lipid rafts. Raft anchors are lipids or proteins with a chemical affinity to other lipid raft components. One crucial difference to systems tested earlier is that in the case of Gag, the clustering process takes place on the inner leaflet of the plasma membrane and not on the outer leaflet. Because of the limited knowledge of the structure and composition of the inner leaflet of the plasma membrane, we tested several model membrane systems, covering established lipid mixtures and more complex variants. These covered both mechanisms of Lα/Ld-type lipid phase separation, demixing of saturated and unsaturated lipids, as well as separation of sphingomyelin and cholesterol from PC. The different model systems should thus detect raft association and raft clustering by any protein scaffold if it has membrane anchors with a higher affinity to other domain-forming membrane components, especially if the protein scaffolds are as big as in this case. Additionally to the shown model systems, cell-derived phase-separating model membrane systems like giant plasma membrane vesicles (Baumgart et al., 2007) and plasma membrane spheres (Lingwood et al., 2008) were tested earlier, but were found to be depleted of P(4,5)P2 and thus not to be suitable for studying the membrane binding of Gag (Keller et al., 2009). The possibility of kinetic trapping of the Gag model protein in the Ld phase of our GUVs could be excluded by showing a reversible and reproducible partitioning behaviour when we switched GUVs between the phase-separated and homogeneous state by heating and cooling.

Our experimental results suggest that the saturated acyl chains of Gag are not equivalent to raft anchors in the context of the tested phase-separating lipid systems. This is in agreement with a recent study by Hogue et al. (2011). They investigated the association of HIV Gag with lipid rafts and another type of membrane microdomains, so called tetranspanin-enriched microdomains (TEMs) in a cell-based assay system. Unexpectedly, they found that the association of Gag with lipid rafts or TEMs was not dependent on the MA domain. MA could be functionally replaced by several heterologous membrane binding domains with different binding modes, still achieving localization in membrane microdomains. This suggests that raft association of Gag does not exclusively depend on a specific raft anchor, or at least that it can be replaced by certain alternative anchors. Instead, the generation of curvature during assembly or subsequent events may be responsible for raft association, because a Gag mutant, which is deficient in inducing curvature and subsequent budding, associated less with lipid raft markers (Hogue et al., 2011). As we specifically tested for lipid raft association via interlinked raft anchors, our results do not argue against other possible modes of lipid raft association. In particular, the Gag derivative used in our minimal system was designed not to generate curvature to exclude effects, which were not part of our initial hypothesis. Furthermore, despite the mechanistic understanding of this model system for studying the lipid raft association of several proteins as discussed above, several other proteins, which were expected to reside in lipid rafts in the plasma membrane, were not found to partition preferentially into the Ld phase in vitro either (for example Kalvo dova et al., 2005; Nikolaus et al., 2010). This suggests that Lα/Ld-type model systems cannot detect every mechanism of lipid raft association or that lipid rafts are rather a class of several different but related membrane microdomains instead of one physicochemically defined compartment. These microdomains may colocalize partially or temporally or under certain conditions such as during extraction with cold detergents, a disruptive lipid raft localization assay.

Taken together, the association of HIV Gag with lipid rafts, as observed in many cell-based experiments, seems to be mechanistically different from raft association via lipid raft anchors on the outer leaflet of the plasma membrane, as exemplified by cholera toxin, which binds to the lipid GM1 as a raft anchor (Wolf et al., 1998). This is particularly unexpected as HIV Gag was shown to colocalize with cholera toxin in co-patching experiments (Ono and Freed, 2005). These observations raise the question whether lateral inhomogeneities of the inner leaflet of the plasma membrane generally follow the same organizing principle as microdomains of the outer leaflet. So far, most studies about raft-based processes on the inner leaflet reported unexpected sorting into Ld domains in vitro (Shogomori et al., 2005; Liu and Fletcher, 2006; Shaw et al., 2006; Sengupta et al., 2008; Tong et al., 2008), and this was also the case in the current study. One may speculate that microdomains on both leaflets could be fundamentally different, although being linked through trans-bilayer interactions and therefore often co-occurring locally. At least in the case of HIV Gag, it is conceivable that lipid raft recruitment according to the classical view, which relies on preferential association of specific lipids, is not the key mechanism on the inner leaflet. Instead, these membrane microdomains may be defined by physical or geometric properties such as curvature, stiffness, or a surrounding diffusion barrier like a bud neck or elements of the cytoskeleton (Roux et al., 2005; Parthasarathy et al., 2006; Gordon et al., 2008; Sorre et al., 2009). Nevertheless, raft anchors may still be necessary but not sufficient for proper HIV budding because an exchange of the myristate anchor of Gag by an unsaturated lipid impaired particle assembly (Lindwasser and Resh, 2002).
Along this line of thought, the Gag scaffold may alter general membrane properties locally, e.g. by inducing a large curvature bud, which subsequently, and possibly with a delay, leads to a recruitment of certain microdomains on the inner leaflet and lipid rafts on the outer membrane leaflet. This hypothesis could potentially be tested by extending the focus of this study from examining the influence of the cross-linked MA domains to analysing all physicochemical effects exerted by full-length Gag on the membrane model system as myristoylated full-length Gag was very recently purified in sufficient amounts and concentrations (Carlson and Hurley, 2012). The challenge would be to combine this extended model system with super resolution microscopy techniques to visualize the size scales involved.

Nevertheless, the mechanistic understanding of lipid raft recruitment by proteins acting on the inner leaflet of the plasma membrane, and in particular by HIV Gag, will require further intensive studies. Minimal in vitro systems, which model individual aspects of these processes with a rather well-defined set of parameters, will be invaluable to distinguish between various possible mechanisms.

**Experimental procedures**

**Reagents**

All general chemicals were purchased from Merck (Darmstadt, Germany). All lipids were from Avanti Polar Lipids (Alabaster, AL, USA). BodipyTM-R-Pl(4,5)P$_2$ was purchased from Echelon Biosciences (Salt Lake City, UT, USA). CtxB-Alexa 647, fastDiI, DiD-C18 and Dulbecco’s modified Eagle’s medium without sodium pyruvate (DMEM) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from PAA (Pasching, Austria) and Biochrom (Berlin, Germany). Penicillin/streptomycin solution was obtained from Biochrom, too. Sodium pyruvate (DMEM) were obtained from Invitrogen (Salt Lake City, UT, USA). CtxB-Alexa 647, fastDiI, DiD-C18 and Dulbecco’s modified Eagle’s medium without sodium pyruvate (DMEM) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from PAA (Pasching, Austria) and Biochrom (Berlin, Germany). Penicillin/streptomycin solution was obtained from Biochrom, too. Beta-casein, polyethylenimine and myristic acid were obtained from PAA (Pasching, Austria) and Biochrom (Berlin, Germany). All lipids were from Avanti Polar Lipids (Alabaster, AL, USA). CtxB-Alexa 647, fastDiI, DiD-C18 and Dulbecco’s modified Eagle’s medium without sodium pyruvate (DMEM) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from PAA (Pasching, Austria) and Biochrom (Berlin, Germany). Penicillin/streptomycin solution was obtained from Biochrom, too.

**Enzymes and buffers for molecular biology were obtained from New England Biolabs (Ipswich, MA, USA) with exception of PCR kits and Quikchange mutagenesis kits, which were purchased from Stratagene (La Jolla, CA, USA). Plasmid purification kits and Quikchange mutagenesis kits, which were purchased from Sigma-Aldrich (St Louis, MO, USA).**

**Plasmids**

The plasmid pSyngag containing the synthetic, codon-optimized HIV-1 gag gene was kindly provided by R. Wagner (Graf et al., 2000). pCMV1-BACE was kindly provided by D. Drechsel (Kalvodova et al., 2005). All following plasmids were generated according to standard procedures. The plasmids pC4-sMAAEGFP-Fv3-3c-FLAG (pHK72), pC4-sMAAEGFP-Fv3-3c-FLAG (pHK74), pC4-sMAAEGFP-Fv3-3c-FLAG (pHK80) and pC4-sMAAEGFP-Fv3-3c-FLAG (pHK81) were used for expression in human cell lines as described below and constructed in a modular fashion. All coding sequences of the final plasmids were flanked by unique restriction sites: frame-(EcoRI)-MA-(ClaI)-autofluorescent protein-(XbaI)-Fv domains-(SpeI)-tag-(BamHI)-frame. The frame originated from pC4-Fv1E, which was obtained from Ariad Pharmaceuticals (Clackson et al., 1998). It contains a single copy of the artificial inducible dimerization domain FKBP$_1$ (F$_1$, see Results section for more information), a cytomembrinions promoter and a polyadenylation signal. The sequence encoding for the MA domain was amplified by PCR from pSyngag and inserted via EcoRI/ClaI sites. Sequences encoding for EGFP as autofluorescent protein and three repeated FKBP$_1$ domains (Fv3), which originate from Ariad plasmids, were inserted via flanking restriction sites ClaI/XbaI and XbaI/SpeI. The coding sequence for the 3c-protease site in front of the FLAG tag and the whole TAP tag were amplified by PCR from the plasmid pCMV1-BACE and inserted via SpeI/BamHI sites. The TAP tag contained a zz-tag (2×z-domain of protein A), and a calmodulin binding domain, which allows for a second optional affinity purification step, which was not used in this case. The plasmid encoding for the myristoylation-deficient MA$_{A0A}$ mutant (pHK81) was created by site-directed mutagenesis. In plasmids pHK80 and pHK81, a R15K mutation was introduced in the MA domain. It matches the amino acid sequence of the HIV isolate NL4-3 and allows for the proteolytic generation of N-terminal peptides that are detectable in mass spectrometry.

**Live cell assembly assay**

293T cells were cultured in DMEM supplemented with 10% FBS. Cell cultures were maintained at 37°C and 8.5% CO$_2$. 293T cells were seeded on fibronectin-coated eight-well Lab-Tek imaging chambers at a density of 7×10$^4$ cells ml$^{-1}$ and transfected with Attractene 1 day later. 0.2 μl of Attractene and 80 ng of plasmid DNA (pHK72 and pHK74), which were pre-diluted in Opti-MEM reduced serum medium, were used for each well. Medium was exchanged after 4 h. One day post transfection, medium was exchanged for imaging buffer containing 150 mM NaCl, 20 mM Hepes pH 7.4, 20 mM trehalose, 15 mM glucose, 5.4 mM KCl, 0.9 mM MgSO$_4$ and 0.5 mM CaCl$_2$ (Ohrt et al., 2006). To induce the dimerization of FKBP$_1$ domains where indicated, cells were incubated for 1 h at 37°C with 50 nM of the artificial ligand termed dimerizer (Clackson et al., 1998). To deplete cells of free Pl(4,5)P$_2$ we used a protocol that finally leads to the formation of giant plasma membrane vesicles but causes immediate degra- dation of Pl(4,5)P$_2$ in the beginning (Keller et al., 2009). Cells were washed two times with Hepes buffer pH 7.4 containing 150 mM NaCl and 2 mM CaCl$_2$ and then incubated in a buffer additionally containing 2 mM DTT and 25 mM formaldehyde. Dimerizer was added 3 min later and observation was finished before cells started blebbing. Laser scanning microscopy was performed at room temperature on a LSM 510 from Zeiss (Jena, Germany) equipped with a C-Apochromat water immersion objective (40×, NA 1.2).

**Protein purification**

293T cells were expanded as described above with addition of 100 U ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin. Myristate-
supplemented medium was prepared by first saturating a 4% solution of delipidated BSA with 2.3 mg ml⁻¹ myristate. To this end, the myristate/BSA suspension was sonicated at 50 C and neutralized with NaOH while the myristate gradually dissolved. Then this solution was sterile filtered and added 1:100 to cell culture medium. 10⁶ cells were seeded on 15 cm dishes in myristate-supplemented medium. On the next day, the cells were transfected. Five hundred micrometers of plasmid DNA was diluted with 25 ml DMEM without supplements, 1.6 mg of pre-dissolved polyethyleneimine was added and the mixture was vortexed thoroughly. After 20 min, the solution was added to the cell culture dishes. The medium was exchanged after 6 h for myristate-supplemented and antibiotic-containing medium. After 48 h, cells were drained of medium and scraped in lysis buffer [20 mM Tris-HCl pH 7.9, 500 mM NaCl, 10 mM 2-mercaptoethanol, 0.05% (v/v) Tween-20, and protease inhibitors] at 4°C. All subsequent steps were carried out at 4°C unless indicated otherwise. Cell suspensions were homogenized with a tip sonicator (Sonifor 250 from Banson, Danbury, CT, USA) until the lysate appeared clear. The lysate was centrifuged at 10,000 g for 10 min. 1.5 ml of IgG beads were added to the supernatant and incubated for 1 h while shaking end-over-end. Subsequently, beads were washed with 50 ml of lysis buffer for 1 h, twice with 15 ml of lysis buffer containing 3 mM Mg²⁺ and 3 mM ATP for 10 min, and once with 15 ml of lysis buffer containing 3 mM EDTA. The beads were filled into a column, which had been blocked with 2.5 mg ml⁻¹ casein before. The column was washed with 8 ml of elution buffer (20 mM Tris-HCl pH 7.9, 150 mM NaCl, 5 mM 2-mercaptoethanol, protease inhibitors, 0.1 mg ml⁻¹ casein and 162 mM sucrose). Two hundred microliters of elution buffer containing four units of PreScission protease (approximately 4 µg) was added, the column was sealed and the resin was carefully resuspended and mixed. Proteolytic digestion was performed at 4°C overnight. The protein was eluted in 300 µl of fractions, which were pooled after analysis in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorometry. The eluate was concentrated using centrifuge concentrators (Vivaspin 2 with 30 kDa cut-off, Sartorius, Göttingen, Germany). The concentrate was supplemented with 1 mM DTT, frozen in liquid nitrogen and stored at –80°C. After thawing, the protein was eluted with an equal volume of assay buffer as used in the subsequent experiment supplemented with 5 mM fresh DTT. Then it was incubated for 1 h at 37°C to reduce all cysteines and prevent assembly without addition of dimerizer.

**GUV formation and binding assay**

Lipids in organic solvent mixtures were mixed, dried and re-dissolved at a concentration of 10 mg ml⁻¹ in a mixture of chloroform, methanol and water (20:6:1) to ensure complete dissolving of PI(4,5)P₂ at high concentrations. Indium tin oxide-coated glass slides were heated to 150°C for 30 min and cooled to 50°C. One microlitre of the lipid mixture was spread evenly on the glass slide and dried at 50°C for 5 min. Vacuum was applied for at least 2 h to remove residual organic solvents and water. An electroformation chamber was assembled by opposing the lipid-coated faces of two glass slides using a 2 mm thick Teflon ring as a spacer. The chamber was filled with a sucrose solution containing 1 mM EDTA. The concentration was adjusted to match the osmolarity of 1.5x assay buffer. An alternating current of 10 Hz and 1.5 V was applied for 1 h and the frequency was reduced to 2 Hz for additional 10 min. GUVs were harvested after cooling to room temperature. A 100 µl aliquot of GUV solution was mixed with 200 µl of 1.5x assay buffer (1x buffer: 25 mM Hepes/KOH pH 7.4, 120 mM potassium acetate, 1 mM EDTA, 1 mM DTT) by gentle inversion on a round bottom test tube. GUVs were allowed to sediment in a conical-bottom test tube containing an air bubble at the bottom to avoid collapsing of the GUVs upon adhesion to the plastic surface. After 10 min, 8 µl was recovered from the bottom by slow aspiration into a cut pipette tip.

In a casein blocked assay chamber, 1 µl of dimerizer solution in assay buffer, 3.5 µl of freshly reduced MA-EGFP-Fv3 or MA₂₀₀⁻EGFP-Fv3 solution and 6 µl of GUV suspension were carefully mixed to yield final concentrations of 200 nM MA-EGFP-Fv3 or MA₂₀₀⁻EGFP-Fv3 and 500 nM dimerizer. To allow for a direct comparison of binding to GUVs with and without PI(4,5)P₂, both kinds of GUVs with different fluorescent markers were mixed in the same assay chamber. Where indicated, dimerizer was omitted and only assay buffer was added instead. In control experiments, GUVs were mixed only with 90 nM CtxB-Alexa 647. The chamber was sealed and incubated at room temperature for at least 10 min. Images were acquired by laser scanning microscopy on a Nikon SP2 microscope equipped with a 4x/1.25 HCX PL APO oil objective. All experiments were conducted at least three times with independent GUV preparations. GUVs with a high content of charged lipids and/or phosphatidylethanolamine showed a compositional heterogeneity after electroformation and a significantly reduced stability during mixing resulting in disruption and inward or outward budding of Lₙ domains. Therefore, all GUVs that did not show phase separation and binding of MA-EGFP-Fv3 were excluded from analysis.

Assay chambers consisted of two glass coverslips with spacers made of double-stick tape. They were constructed to avoid evaporation and convection during microscopy. A 6 mm spot on the coverslip was activated by addition of a drop of 2 M NaOH in 50% ethanol and rinsing and drying after 30 min. This supports subsequent efficient blocking of the cleaned hydrophilic spot with a 20 mg ml⁻¹ solution of casein in assay buffer. After at least 20 min, the casein solution was removed and the blocked spot was rinsed with assay buffer without drying. Three layers of double-stick tape containing a hole with a diameter of 8 mm were attached to form a chamber with a height of 300 µm and a usable volume of 10 µl. The GUV suspension and other assay components were applied at the central hydrophilic spot to avoid spreading and direct contact to the tape. The chamber was sealed by a second coverslip, which had been treated the same way.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Purification of MA-EGFP-Fv3.

A. Coomassie-stained PAGE shows purity of MA-EGFP-Fv3 and the myristoylation-deficient mutant MA<sub>Δ</sub>-EGFP-Fv3 (arrow). The asterisk indicates the added carrier protein casein.

B. Mass spectrometry trace of the myristoylated N-terminal peptide of MA-EGFP-Fv3.

**Fig. S2.** The markers for L<sub>d</sub> and L<sub>d</sub> phases are complementary. LSM section through GUVs of complex lipid mixture (see Table 1, #5) containing 0.1% fastDiI (red) and 0.02% GM1. 90 mM CtxB-Alexa 647 was added as L<sub>d</sub> phase marker (false-coloured in blue). FastDiI and CtxB are very well established markers for L<sub>d</sub> and L<sub>d</sub> phases respectively. This control shows that their distribution is not affected by the complex lipid mixture.