Gag Induces the Coalescence of Clustered Lipid Rafts and Tetraspanin-Enriched Microdomains at HIV-1 Assembly Sites on the Plasma Membrane[▽]

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The HIV-1 structural protein Gag associates with two types of plasma membrane microdomains, lipid rafts and tetraspanin-enriched microdomains (TEMs), both of which have been proposed to be platforms for HIV-1 assembly. However, a variety of studies have demonstrated that lipid rafts and TEMs are distinct microdomains in the absence of HIV-1 infection. To measure the impact of Gag on microdomain behaviors, we took advantage of two assays: an antibody-mediated copatching assay and a Förster resonance energy transfer (FRET) assay that measures the clustering of microdomain markers in live cells without antibody-mediated patching. We found that lipid rafts and TEMs copatched and clustered to a greater extent in the presence of membrane-bound Gag in both assays, suggesting that Gag induces the coalescence of lipid rafts and TEMs. Substitutions in membrane binding motifs of Gag revealed that, while Gag membrane binding is necessary to induce coalescence of lipid rafts and TEMs, either acylation of Gag or binding of phosphatidylinositol-(4,5)-bisphosphate is sufficient. Finally, a Gag derivative that is defective in inducing membrane curvature appeared less able to induce lipid raft and TEM coalescence. A higher-resolution analysis of assembly sites by correlative fluorescence and scanning electron microscopy showed that coalescence of clustered lipid rafts and TEMs occurs predominately at completed cell surface virus-like particles, whereas a transmembrane raft marker protein appeared to associate with punctate Gag fluorescence even in the absence of cell surface particles. Together, these results suggest that different membrane microdomain components are recruited in a stepwise manner during assembly.

The plasma membrane (PM) is heterogeneous, consisting of diverse microdomains. This partitioning of membrane components, which compartmentalizes cellular processes, is regulated by lipid-lipid, protein-protein, and protein-lipid interactions (27, 87). Human immunodeficiency virus type 1 (HIV-1) assembly, which occurs on the cytoplasmic leaflet of the PM (68), is thought to preferentially associate with particular microdomains, lipid rafts and tetraspanin-enriched microdomains (TEMs), during assembly (22, 102, 131, 137).

HIV-1 assembly is driven by the structural polyprotein Gag, which is necessary and sufficient for the formation of virus-like particles (VLPs). Gag binding to the PM is mediated by its N-terminal matrix (MA) domain, which is myristoylated and contains basic residues that bind the PM phospholipid phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂] (12, 23, 28, 46, 56, 118, 125, 145). Prior to membrane binding, the myristoyl moiety is sequestered in a hydrophobic patch on the MA domain (129), and its exposure may be regulated by PI(4,5)P₂ binding (118) and multimerization of Gag molecules (129, 146). Gag multimerization is primarily driven by its capsid (CA) and nucleocapsid (NC) domains, but membrane binding

also enhances Gag multimerization (1). The CA domain forms an interface that mediates Gag homodimerization (29, 40, 58, 67, 107, 136). The NC domain binds RNA, which is thought to serve as a scaffold promoting Gag multimerization (13, 14, 25, 73, 95, 107). Similarly, the ability of Gag to bind membrane seems to allow Gag to use the PM as a scaffold for multimerization (58, 83). In particular, multimerization may be facilitated by Gag molecules binding to and concentrating within specific membrane microdomains. Two types of PM microdomains, lipid rafts and tetraspanin-enriched microdomains, are currently proposed to be platforms for HIV-1 assembly (for reviews, see references 22, 102, 131, and 137).

Lipid rafts are dynamic, submicroscopic domains enriched in sterols, sphingolipids, glycosylphosphatidylinositol-anchored (GPI-anchored) proteins, and proteins modified with saturated acyl chains (27, 87). Proteomics, lipidomics, and biochemical studies have shown that the HIV-1 envelope is enriched in lipids and proteins that are also markers for lipid rafts (3, 11, 16, 21, 48, 96, 109, 119), and envelope lipids appear ordered like those in rafts (90). Immunofluorescence microscopy studies have revealed that Gag colocalizes/copatches with lipid raft markers in cells (59, 98, 105) and cofractionates with lipid raft markers in detergent-resistant membranes (DRMs) (9, 31, 32, 53, 59, 84, 85, 98, 104, 107) although qualitative differences between canonical DRMs and Gag-containing DRMs have been noted (31, 59, 84). Depletion of cellular cholesterol, which disrupts lipid rafts, reduces virus particle production and disrupts the behavior of Gag in cells as measured by a variety of assays (43, 104, 106, 113). Importantly, one study loaded

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cells with an unsaturated myristate analogue which blocked Gag fractionation into DRMs and reduced virus production, suggesting that N-terminal saturated acylation is a necessary molecular determinant of lipid raft association and assembly of Gag (85). An additional mechanism that may promote Gag-raft association was suggested by a nuclear magnetic resonance (NMR) study showing that, upon PI(4,5)P₂ binding and myristoyl exposure, MA sequesters the typically unsaturated 2' acyl chain of PI(4,5)P₂ in a hydrophobic cleft in its globular head domain. If this unique mode of PI(4,5)P₂ binding occurs on cellular membranes, it is predicted to facilitate Gag association with lipid rafts as Gag could associate with membranes using two saturated acyl chains, the myristoyl moiety and the saturated 1' acyl chain of PI(4,5)P₂ (118). However, chimeric Gag constructs that bind membrane via nonacylated, phosphoinositide-binding domains (e.g., pleckstrin homology [PH] domains), which do not sequester the 2' acyl chain of phosphoinositides, can still produce VLPs (68, 121, 133). Although the lipid raft association of these Gag chimeras has not yet been examined, the ability of these to assemble VLPs suggests that neither acylation nor sequestration of PI(4,5)P₂ acyl chains plays a necessary role in assembly. Overall, the precise determinants of lipid raft association during assembly remain to be elucidated.

TEMs are membrane microdomains organized by homo- and hetero-oligomerization of tetraspanins, a family of homologous proteins with four transmembrane domains (18, 131, 140). A variety of studies have suggested roles for tetraspanins and TEMs during entry, but research into whether TEMs facilitate virus assembly and release remains contradictory (131). Tetraspanins, including CD9, CD63, and CD81, are incorporated into virus particles (21, 42, 74, 93, 94, 97, 100, 108, 112, 120), coimmunoprecipitate with Gag (51), and strongly colocalize/copatch with Gag by immunofluorescence and electron microscopy assays (10, 30, 41, 50, 58, 64, 101, 132, 138). Tetraspanins CD63 and CD81 have been shown to associate with phosphatidylinositol 4-kinase, a critical enzyme in creating PI(4,5)P₂ (8). Thus, PI(4,5)P₂ might recruit Gag to TEMs; however, this hypothesis has not been tested. The molecular determinants of Gag association with TEMs are currently unknown.

Lipid rafts and TEMs can be biochemically distinguished as they have different detergent sensitivities, different sensitivities to cholesterol depletion, and distinct protein constituents (4, 17, 19, 20, 26, 81, 141). Furthermore, many microscopy-based studies have observed segregation or distinct behaviors of lipid raft and TEM markers (7, 34, 101). Yet, as described above, Gag appears to associate with both lipid rafts and TEMs, raising the question of whether lipid rafts and TEMs are coalesced by Gag. Individual microdomains, especially lipid rafts that may be as small as 10 nm (27, 87), are too small to serve as a preformed platform for the assembly of a ~150-nm virion. Thus, it is plausible that Gag brings together multiple microdomains, possibly coalescing disparate classes of microdomains during assembly.

In this study, we aimed to answer two questions: (i) does Gag reorganize cellular microdomains, inducing the coalescence of lipid rafts and TEMs, and (ii) what are the molecular determinants or mechanisms of such Gag-induced microdomain reorganization? Using an antibody-mediated copatching

assay and an assay of fluorescent protein clustering based on Förster resonance energy transfer (FRET), we observed that Gag does induce the coalescence of lipid rafts and TEMs. Gag membrane binding was necessary to induce coalescence of these microdomains; however, the particular mode of Gag membrane binding did not affect microdomain association, as Gag derivatives with alternative membrane binding motifs could still induce microdomain coalescence. Finally, a Gag derivative that is defective in inducing membrane curvature appeared less able to induce coalescence of lipid rafts and TEMs. In a higher-resolution analysis of this Gag derivative and wild-type (WT) Gag by correlative fluorescence and scanning electron microscopy (SEM), we found that a canonical lipid raft marker appeared to associate with punctate Gag fluorescence even in the absence of cell surface VLPs, whereas a TEM marker appeared primarily where cell surface VLPs existed, suggesting a stepwise model of microdomain coalescence during assembly.

MATERIALS AND METHODS

Plasmids. Derivatives of the HIV-1 molecular clone pNL4-3 encoding Gag with a C-terminal monomeric Venus (mVenus) yellow fluorescent protein (YFP) fusion were described previously (23, 58). These chimeric derivatives do not express *pol*, *vif*, or *vpr*. A derivative expressing Gag that is completely defective in membrane binding was constructed by combining two previously described mutations, 1GA and 6A2T. This construct contains amino acid substitutions within the MA domain, replacing eight basic residues that bind acidic phospholipids and abolishing the N-terminal myristoylation signal (24, 39). Derivatives in which the MA start codon or the entire MA sequence was replaced with the sequence encoding the 10-residue myristoylation and dual-palmitoylation signal of Fyn kinase [Fyn(10)fullMA or Fyn(10)ΔMA, respectively] were described previously (23). Another derivative, PH_{PLC81}delMA, was created by replacing the sequence encoding the MA globular head (codons 1 to 114) with the sequence encoding the first 170 amino acids, containing the PH domain of phospholipase C81, isoform 2 (PH_{PLC81}). The junctions between pNL4-3 and PH_{PLC81} DNA sequences are as follows (bold type represents pNL4-3 sequence, italic type represents PH_{PLC81} sequence, and the start codon is underlined): upstream junction, **GAGGCTAGAAGGAGAGAGATGGACTCGGGCCGGGAC**; downstream junction, **GAGTGCAGAACTTCCTGAAGGCACAGCAA** **GCAGCA**.

The cloned PH_{PLC81} sequence was a kind gift from T. Balla and J. Swanson (70, 135). PH_{PLC81}delMA Gag derivatives did not express well, but normal expression levels were rescued by cotransfecting pCMV-Rev (a kind gift from S. Venkatesan, National Institutes of Health) and pcDNA3.1-HIVtat101 (a kind gift from D. Markovitz, University of Michigan; originally from E. Verdin [110]), expressing HIV-1 *rev* and *tat* genes, respectively (data not shown).

The mutation P99A in the Gag CA N-terminal domain was created by PCR mutagenesis. It has been previously reported that this mutation reduces particle production and disrupts core formation in mature particles (37, 75), and a 12-residue insertion at an analogous position in murine leukemia virus Gag is reported to cause production of planar multimers (6).

A vector that expresses HIV-1 Gag derived from NL4-3 in an HIV-1 Rev-dependent manner was previously described (pCMVNLGagPolRRE) (104). Previously described mutations abolishing N-terminal myristoylation of Gag and protease activity (39, 61) were introduced to express nonmyristoylated 1GA Gag. Alternatively, Gag fused to monomeric red fluorescent protein (Gag-RFP), was transferred from previously described pNL4-3/Gag-mRFP (23) into this expression vector.

Expression vectors encoding an mVenus YFP and monomeric Cerulean (mCerulean) cyan fluorescent protein (CFP) were described previously (58). mNonFP, a nonfluorescent protein derivative of mCitrine (60), containing a mutation (Y67C) that abolishes fluorophore formation (76), was a kind gift from S. Straight at the University of Michigan Center for Live-Cell Imaging. All fluorescent protein constructs contain the mutation A206K to suppress fluorescent protein dimerization (142).

We generated constructs expressing GPI-anchored mCerulean (CFP-GPI) or mNonFP (NonFP-GPI) based on a previously described GPI-anchored fluorescent protein construct (a kind gift from J. Silver, National Institutes of Health;

originally from K. Simons [71]). We generated constructs expressing mNonFP fused to the C-terminal 72 amino acid residues of influenza virus hemagglutinin (HA) including the transmembrane and cytoplasmic domains (NonFP-HA-TMD) based on previously described constructs, a kind gift from A. Herrmann (122).

A plasmid encoding CD81 with mVenus (YFP) inserted into its large extracellular loop (CD81-EC2-iYFP) was created using CD81 coding sequence from CD81-GFP (a kind gift from F. Sánchez-Madrid) (45). In this construct, mVenus replaced helix D (residues 181 to 186, IISNLF) of the CD81 large extracellular loop (123). The junctions between CD81 sequence and fluorescent protein sequence are as follows (CD81 sequence is in bold type, and fluorescent protein sequence is in italic type): upstream junction, **KNNLCPSGSGNPPVATMVSKGEELFT**; downstream junction, **ITLGMDELYKGGKEDCHQKIDD**.

Cells and transfection. HeLa cells were cultured as described previously (38). For the membrane flotation assay, 5.6×10^5 cells were seeded in each well of a six-well tissue culture plate (Corning). Cells were grown for 24 h and then transfected with 2 μ g/well of pNL4-3-based plasmid using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. For confocal microscopy, 4.2×10^4 cells were seeded in each well of eight-well chamber slides (Lab-Tek; Thermo Fisher). Cells were grown for 24 h and transfected with 0.6 μ g/well of pNL4-3-based plasmid as above. When the pNL4-3-based plasmid was coexpressed with any other plasmids, cells were transfected with 0.6 μ g/well of pNL4-3-based plasmid and 0.06 μ g/well (each) of the other plasmid(s), as above. For correlative fluorescence and SEM (see below), 2×10^5 cells were seeded on top of 25-mm diameter photoetched gridded coverslips (Bellco Glass) in each well of a six-well plate, grown overnight, and transfected with 2 μ g/well of pNL4-3-based plasmid, as above. When a pNL4-3-based plasmid was coexpressed with any other plasmids, cells were transfected using 2 μ g/well of pNL4-3-based plasmid and 0.2 μ g/well (each) of the other plasmid(s), as above.

Membrane flotation assay. HeLa cells were harvested at 16 h posttransfection. Cells were prepared for membrane flotation centrifugation on sucrose gradients as previously described (103). Gag constructs were detected by immunoblotting using HIV-Ig antiserum (NABI and NHLBI, AIDS Research and Reference Reagent Program, NIH) and Alexa Fluor 488-conjugated anti-human IgG (Invitrogen) as a secondary antibody. Fluorescent secondary antibody was quantified using a Typhoon Trio imager (GE Healthcare), and percent membrane binding was calculated by the sum of Gag in membrane fractions 1 and 2 divided by the sum total Gag in all five fractions.

Copatching assay and confocal fluorescence microscopy. At 16 to 18 h posttransfection, chamber slides were brought to room temperature for 10 min and then incubated for 10 min with primary antibodies detecting CD46 (mouse monoclonal IgG2a; BD Biosciences), CD59 (mouse monoclonal IgG2a; BD Biosciences), CD81 (mouse monoclonal IgG1; BD Biosciences), CD9 (mouse monoclonal IgG2b; AbD Serotec), or GFP derivative NonFP (rabbit polyclonal; Clontech), diluted 1:100 in cell culture medium. Slides were then rinsed three times in phosphate-buffered saline (PBS) and incubated for 10 min with isotype-specific fluorescent secondary antibodies (Invitrogen) diluted 1:200 in cell culture medium. Slides were again rinsed three times in PBS and immediately fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences) in PBS at 4°C overnight, rinsed in PBS once more, and mounted in Fluoromount-G (Southern Biotech). Slides were imaged at the University of Michigan Microscopy and Image-Analysis Laboratory using a Leica TCS SP5 X laser scanning confocal microscope equipped with a multiline argon laser and a broad-spectrum (i.e., white-light) laser. The confocal pinhole was set to 1 Airy unit, and the focal plane was selected to image the dorsal surface of each cell. We used a 100 $\times$ oil immersion (1.40 numerical aperture) PL APO objective with 6 $\times$ scanning zoom, producing a 1,024- by 1024-pixel image at a resolution of 25 by 25 nm per pixel. Note that the inherent resolution limit of light microscopy is $\sim$ 200 nm; thus, these imaging parameters are oversampling the optical signal. Excitation and emission ranges were set for each fluorophore as follows: YFP, 514 nm excitation and 525 to 575 nm emission; Alexa Fluor 594 or monomeric RFP, 590 nm excitation and 600 to 640 nm emission; and Alexa Fluor 647, 650 nm excitation and 660 to 750 nm emission. Samples were imaged with each excitation wavelength sequentially, and excitation and emission wavelengths were chosen to minimize cross talk between fluorescence channels.

Image analysis, quantitation, and statistics. The degree of copatching between microdomain markers was quantified using ImageJ (version 1.43o; NIH [http://rsb.info.nih.gov/ij/]). Images were first cropped, with the dorsal surface where the PM is nearly parallel to the focal plane retained. To avoid spuriously colocalized signals, the lateral surface, where the PM is not parallel to the focal plane and largely out of focus, was excluded.

The R^2 coefficient of determination between microdomain marker fluorescence channels was calculated using the JACoP plug-in of ImageJ and Microsoft

Excel. This measure of the strength of correlation ranges from 0 to 1, where 1 represents perfect colocalization and 0 represents random distributions of fluorescence intensities. Statistical significance was assessed using a nonparametric Mann-Whitney U test (implemented in Microsoft Excel). Fluorescence images were prepared for publication by auto-scaling brightness and contrast in ImageJ.

To ensure that differences in the amount of Gag at the dorsal plasma membrane (e.g., due to differences in expression or membrane binding of Gag derivatives) were not systematically biasing calculation of the R^2 coefficient between microdomain markers, data were additionally analyzed as follows. First, images were cropped, and the R^2 coefficient was calculated, as above. Next, fluorescence background was subtracted from Gag-YFP images using a 40-pixel median filter in ImageJ. A threshold was then set in ImageJ to select Gag-YFP-positive pixels greater than 10 relative fluorescence units (on a 0 to 255, 8-bit scale) above the calculated background. The fraction of above-threshold Gag-YFP-positive pixels per total number of pixels was then calculated in ImageJ. This process was repeated creating a set of R^2 coefficients and fractions of Gag-YFP-positive pixels for each cell. These values were plotted, and Pearson's correlation was calculated to determine if there is a relationship between these measures that might bias the calculation of the R^2 coefficient between microdomain markers.

Förster resonance energy transfer by FLIM. HeLa cells were seeded in four-well chamber coverslips (Lab-Tek; Thermo Fisher) and transfected with a Gag expression plasmid (pCMVNLgagpol/1GA/PR-RRE or pCMVNLgag-mRFP-RRE), an HIV-1 *rev* expression plasmid (pCMV-Rev), and plasmids expressing CFP-GPI and CD81-EC2-iYFP, as described above. At 18 h posttransfection, cell culture medium was replaced with Dulbecco's modified Eagle medium (DMEM) with HEPES buffer, without phenol red (Invitrogen), and supplemented with 5% fetal bovine serum. Cells were imaged live, immersed in this medium, at room temperature. Fluorescence lifetime imaging microscopy (FLIM) was performed using the time-correlated single-photon counting (TCSPC) method. Data were acquired using the Leica TCS SP5 X confocal microscope described above, with TimeHarp (Picoquant) lifetime imaging hardware, a pulsed infrared laser for multiphoton excitation, and LAS AF (Leica) and SymPhoTime (Picoquant) software. The confocal pinhole was set completely open, and the focal plane was selected at the mid-plane of each cell. We used the 100 $\times$ objective described above with 3 $\times$ scanning zoom and 512 by 512 pixels per image. For lifetime imaging, CFP was excited at 820 nm, and CFP emission was acquired at 455 to 495 nm. CFP fluorescence signal was accumulated in lifetime imaging mode for approximately 3 to 5 min, until at least one pixel had accumulated 600 photons. Subsequently, a YFP image of the same field was acquired using 514-nm excitation and 530- to 566-nm emission, and an RFP image was recorded using 584-nm excitation and 595- to 644-nm emission. Regions of interest (ROIs) were manually selected to include the plasma membrane at the periphery of each cell but to exclude fluorescence from the cytosol or intracellular membranes. For each region of interest, the variable τ , which describes the distribution of CFP fluorescence lifetimes, was calculated by fitting a monoexponential (for donor only) or a biexponential (for donor and acceptor) function to the TCSPC histogram using the SymPhoTime software. For this fitting, the experimentally determined instrument response function (kindly provided by Haridas Pudavar, Life Science Division, Leica Microsystems) was taken into account. For biexponential fits, τ was calculated as the weighted average of individual exponential components. The average τ of CFP in the absence of acceptor was averaged over six cells on each day the experiment was repeated. FRET efficiency (E) was calculated as follows: $E = 100\% \times [1 - (\tau_{\text{FRET}}/\tau_{\text{donor}})]$. To determine whether FRET is highly dependent on YFP concentration, indicating no specific clustering of microdomain markers, or whether FRET is less dependent on YFP concentration, indicating clustering of markers, we used a previously described method of analysis (33, 72, 122, 142). YFP fluorescence intensity was measured for each cell using ImageJ by taking the average pixel intensity within the ROIs determined as described above. FRET efficiency (E) was plotted against YFP intensity (F). Data points were fitted according to the hyperbolic function $E = (E_{\text{max}} \times F)/(F + K_d)$, a simple saturable ligand binding (i.e., Michaelis-Menten) model, where K_d is a variable analogous to a dissociation constant (33, 122).

Transmission electron microscopy. Transfected HeLa cells were processed and examined on a Hitachi H7000 transmission electron microscope at the Electron Microscopy Laboratory, SAIC-Frederick, NCI Frederick, as previously described (44).

Correlative fluorescence/SEM. HeLa cells were cultured, transfected, treated with antibodies to patch microdomain markers, and fixed in 4% PFA in PBS, as described above. Cells were then rinsed in PBS, and the remaining PFA was quenched by incubating the cells in 0.1 M glycine (Sigma) in PBS for 10 min; cells were then blocked by incubation in 3% bovine serum albumin (BSA; Sigma) in PBS (3% BSA-PBS) for 30 min. Cells were then stained with biotinylated anti-

CD71 antibody (mouse monoclonal; BD Biosciences) diluted 1:10 in 3% BSA-PBS for 30 min and then incubated with streptavidin-coated 0.5- μ m silica microspheres (Bangs Laboratories) diluted 1:100 in 3% BSA-PBS. These silica microspheres serve as landmarks for the registration of SEM and light microscopy images, as described below. Cells were then washed in PBS and imaged by confocal fluorescence microscopy as described above, except that cells remained immersed in PBS instead of being mounted in Fluoromount-G and were imaged with a 40 $\times$ PL APO objective with 20 $\times$ scanning zoom. Additionally, silica microspheres and cell structural features were detected by differential interference contrast. After fluorescence imaging, cells were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences) in PBS at 4°C overnight. Cells were then rinsed with Sorenson's buffer (0.1 M sodium phosphate buffer, pH 7.4), stained with 1% OsO₄ in Sorenson's buffer for 30 min, rinsed again in Sorenson's buffer, and then dehydrated in a series of 10-min ethanol washes at 30%, 50%, 70%, 90%, 100%, and 100% (vol/vol in water). Cells were then rinsed twice for 15 min each in hexamethyldisilazane (Electron Microscopy Sciences) and allowed to dry overnight. After dehydration, coverslips were affixed to specimen mounts and sputter coated with gold for 45 s (Polaron). Cells were identified by their location on the gridded coverslip and imaged on an Amray 1910FEG scanning electron microscope at 10 kV. Digital SEM images were collected with an X-Stream Digital Image Capture System (SEMTECH Solutions). Fluorescence and SEM images were roughly brought into registration by scaling, rotating, and translating images in Adobe Photoshop, similarly to other correlative fluorescence/SEM studies (80). Landmarks used for registration included silica beads and cell edges. Virus particles at the cell surface served as additional landmarks in cells expressing WT Gag-YFP, where surface particles matching to fluorescent Gag puncta are readily detectable. Due to scarcity of surface particles, registration of light and electron microscopy images for P99A Gag-YFP were performed based on positions of beads and cell edges. Final high-resolution image registration was performed by matching landmarks visible in both image sets and calculating an elastic transformation using the bUnwarpJ plug-in (5) in ImageJ.

Correlative fluorescence and SEM data were quantified by categorizing fluorescent Gag puncta according to the presence of each microdomain marker and presence of cell surface VLPs in the SEM image. First, individual fluorescent Gag puncta were automatically identified using the GranFilter plug-in in ImageJ, as previously described (58). This plug-in selects a circular ROI (radius, 10 pixels) around Gag puncta. To compensate for the variations in the fluorescence background across the cell, background was subtracted from fluorescent microdomain marker images using a 40-pixel median filter in ImageJ. ROIs containing Gag fluorescent puncta were categorized as positive for a microdomain if the average microdomain marker fluorescence intensity within that region was greater than 10 relative fluorescence units (on a 0 to 255, 8-bit scale) above the calculated background. Cell surface structures visible in SEM were manually classified as VLPs if they appeared spherical and near 150 nm in diameter. To perform statistical tests on these data, the above process of averaging microdomain marker intensity within circular ROIs was repeated across the entire cell surface, and the fraction of cell surface positive for the microdomain marker was calculated by thresholding, as above. The significance of association between Gag puncta and microdomain marker was then assessed using a binomial test (implemented in Microsoft Excel).

RESULTS

An antibody-mediated copatching assay distinguishes lipid rafts and TEMs. Without antibody-mediated copatching, association between microdomain markers was often inconsistent and obscured by diffuse surface and intracellular fluorescence signals (data not shown). To overcome these limitations, we took advantage of an antibody-mediated copatching assay to observe the relationships between Gag, lipid raft markers, and TEM markers. It has long been appreciated that binding of exogenous multivalent proteins, such as antibodies or lipid-binding toxins, can induce the clustering of membrane components into microscopically visible patches on the cell surface (15, 91, 114, 128, 130). When two microdomain markers are independently clustered using specific antibodies, these markers can colocalize within the same patch, or "copatch," which is thought to indicate shared microdomain affinity. Inversely, markers independently clustered into patches can segregate,

TABLE 1. Microdomain markers used in this study

Marker type and name	Expression type	Membrane association	Detection method
Raft markers ^a			
CD59	Endogenous	GPI anchor	Antibody patching
NonFP-GPI	Transgene	GPI anchor	Antibody patching
NonFP-HA-TMD	Transgene	Transmembrane	Antibody patching
CFP-GPI	Transgene	GPI anchor	Intrinsic fluorescence
TEM markers			
CD81	Endogenous	Transmembrane	Antibody patching
CD9	Endogenous	Transmembrane	Antibody patching
CD81-EC2-iYFP	Transgene	Transmembrane	Intrinsic fluorescence
Non-TEM/nonraft control			
CD46	Endogenous	Transmembrane	Antibody patching

^a NonFP, nonfluorescent GFP derivative.

indicating a lack of shared affinity (55, 91). It is thought that this shared affinity (or lack thereof) during antibody copatching is representative of the markers' native submicroscopic microdomain affinities in the absence of experimental manipulation as a number of biophysical and biochemical studies have corroborated copatching data regarding microdomain association of membrane proteins (49, 55, 62, 86, 92, 126).

To validate the ability of this assay to distinguish different classes of microdomains in the absence of Gag, we performed a copatching assay detecting markers within or between classes of microdomains. Microdomain markers used in these experiments are summarized in Table 1. Consistent with previous observations (101), independently patched TEM markers CD81 and CD9 colocalized strongly (Fig. 1A, top row). Also in agreement with previous copatching studies (55), different lipid raft markers, CD59, NonFP-GPI, and NonFP-HA-TMD, copatched on a punctum-by-punctum basis (Fig. 1A, rows 2 and 3). We also confirmed previous reports showing that lipid rafts and TEMs are distinct microdomains (see introduction): lipid raft markers CD59 and NonFP-HA-TMD segregated from the TEM marker CD81 in this copatching assay (Fig. 1A, rows 4 and 5). CD81, NonFP-GPI, and NonFP-HA-TMD all segregated from the control non-TEM/nonraft marker CD46 (Fig. 1B). These results confirm that this copatching assay is able to distinguish different classes of microdomain markers.

We calculated the R^2 coefficient of determination, a measure of the strength of correlation, between the fluorescence intensities of each microdomain marker, over 5 to 20 cells. Pairs of microdomain markers within the same class (i.e., TEM markers copatching with TEM markers or raft markers copatching with raft markers) produced stronger correlations than markers between classes (Fig. 1C).

WT Gag, but not a non-membrane-bound Gag derivative, appears to coalesce lipid rafts and TEMs. As described in the introduction, studies using a wide variety of methods have shown that Gag associates with lipid raft markers and that Gag associates with TEM markers. However, it is well established that lipid rafts and TEMs are distinct from each other in cells not expressing Gag. Therefore, to examine whether Gag associates with lipid rafts and TEMs simultaneously, and thereby induces coalescence of these two types of microdomains, we next tested whether lipid raft and TEM markers can be copatched in HIV-1-expressing cells. Importantly, other mem-

FIG. 1. Antibody-mediated copatching assay distinguishes lipid rafts and TEMs. (A) Cells were treated with antibodies to independently induce patching of microdomain markers, and the dorsal surfaces of cells were imaged. Copatching between two TEM markers (row 1), between two lipid raft markers (rows 2 and 3), and between lipid raft and TEM markers (rows 4 and 5) was examined. (B) Copatching of TEM and lipid raft markers with a negative-control non-TEM/nonraft marker, CD46, was examined. Fields shown in panels A and B are each 12 μ m by 12 μ m. (C) The R^2 strength of correlation between fluorescence intensities of pairs of microdomain markers was calculated and averaged from 5 to 20 cells per condition. Error bars represent ± 1 standard error of the mean.

brane-associated HIV-1 proteins, including Env, Nef, Vpu, and Tat, may interact with membrane microdomains. In particular, Nef has been proposed to alter lipid rafts and their constituent lipids and proteins (2, 99, 127, 143, 144). To control for the effects of other HIV-1 proteins on microdomains, we compared the copatching of microdomain markers in cells transfected with a WT HIV-1 molecular clone or cells transfected with a molecular clone containing multiple MA mutations (1GA/6A2T). In this 1GA/6A2T mutant, both Gag myristoylation and basic residues that bind PI(4,5)P₂ were disrupted to ensure that this derivative cannot interact with membrane by either mechanism. This Gag derivative was diffuse in the cytosol and did not form PM puncta although confocal microscopy

focusing on the dorsal surface of cells showed that the fluorescence signal had a mottled appearance likely due to surface irregularities like ruffles (Fig. 2A). In the presence of non-membrane-bound Gag, antibody-patched lipid raft markers (CD59, NonFP-GPI, and NonFP-HA-TMD) and a TEM marker (CD81) remained largely segregated (Fig. 2A). These conditions were also repeated to measure copatching with the TEM marker CD9, with similar results (data not shown). These findings indicate that neither non-membrane-bound Gag nor other HIV-1 proteins are sufficient to induce coalescence of lipid rafts and TEMs although these data do not preclude other HIV-1 proteins playing a supportive role. In contrast, in the presence of Gag with WT membrane binding

FIG. 2. Lipid rafts and TEMs appear coalesced in the presence of membrane-bound Gag but not non-membrane-bound Gag. Cells expressing Gag-YFP derivatives, lipid raft markers, and TEM markers were treated with antibodies to independently induce patching of the microdomain markers, and the dorsal surfaces of cells were imaged. Copatching was examined in cells expressing 1GA/6A2T Gag-YFP (A) or WT Gag-YFP (B). Copatching was also examined between a TEM marker and a non-TEM/nonraft marker, CD46 (C). Fields shown in panels A, B, and C are each 12 μ m by 12 μ m. (D) The R^2 strength of correlation between fluorescence intensities of pairs of markers was calculated and averaged from 10 to 28 cells per condition. Error bars represent ± 1 standard error of the mean. *, significantly different ($P < 0.01$); n.s., not significantly different ($P > 0.01$).

motifs (WT Gag-YFP), antibody-patched lipid raft and TEM markers were strongly coalesced at Gag puncta (Fig. 2B). Importantly, this coalescence of microdomain markers by WT Gag-YFP is specific to lipid raft and TEM markers because

WT Gag-YFP did not coalesce TEMs with a negative-control nonraft/non-TEM marker, CD46 (Fig. 2C).

To quantify this copatching, we calculated the R^2 strength of correlation between lipid raft and TEM markers, averaged

FIG. 2—Continued.

over 10 to 28 cells for each experimental condition. In all combinations of lipid raft and TEM markers tested, the correlation between markers was significantly greater in the presence of WT Gag-YFP than non-membrane-bound 1GA/6A2T Gag-YFP (Fig. 2D). However, there was no significant difference in correlation between TEM marker CD81 and negative-control marker CD46 in the presence of either 1GA/6A2T or WT Gag-YFP (Fig. 2D). Altogether, these results indicate that lipid rafts and TEMs are coalesced in the presence of WT Gag, that Gag membrane binding is necessary for this coalescence, and that other HIV-1 proteins are not sufficient for coalescence in the absence of membrane-bound Gag.

A FRET assay reveals that WT Gag increases coclustering between lipid raft and TEM markers. Despite the usefulness of antibody-mediated copatching assays, there remains the possibility that antibody cross-linked microdomain markers do not accurately reflect the association of markers in the absence of experimental manipulation. In addition, due to the limits of light microscopy resolution, it is possible that lipid raft and TEM markers remain segregated on a submicroscopic scale despite apparent copatching on a microscopic scale. To address these limitations, we took advantage of a FRET-based assay that detects submicroscopic clustering of membrane proteins in live cells.

To measure FRET between microdomain markers on the

extracellular leaflet of the PM, we coexpressed GPI-anchored CFP (CFP-GPI), a raft marker, and a derivative of TEM marker CD81 with YFP inserted into its large extracellular loop (CD81-EC2-iYFP) (Fig. 3A). In a copatching assay, CD81-EC2-iYFP copatched with endogenous CD9 similarly to endogenous CD81 (compare Fig. 3B to the top row of Fig. 1), indicating that CD81-EC2-iYFP behaves as a TEM marker despite modification with YFP. To determine the effect of assembling Gag on microdomain marker clustering, we additionally coexpressed WT Gag-RFP or a negative-control Gag derivative with a mutation that abolishes its myristoylation signal and disrupts membrane binding (1GA Gag).

In this assay, energy transfer (FRET) between donor and acceptor fluorescent proteins (CFP and YFP, respectively) in nanometer-scale proximity is detectable as shortened average fluorescence lifetime of donor molecules in fluorescence lifetime imaging microscopy (FLIM). The schematic presented in Fig. 3C illustrates the relationship between FRET efficiency and YFP intensity with and without fluorescent protein clustering. If microdomain markers are not specifically clustered, FRET will occur by random molecular collisions dependent on YFP concentration. On the other hand, if markers are submicroscopically clustered, FRET will be less dependent on YFP concentration (33, 122, 142).

In the presence of FRET acceptor CD81-EC2-iYFP, we

FIG. 3. Assembling Gag increases submicroscopic coclustering of a lipid raft marker and a TEM marker. (A) CFP-GPI, a raft marker and FRET donor, is coexpressed with CD81-EC2-iYFP, a TEM marker and FRET acceptor, in the presence or absence of assembly-competent Gag-RFP. In nanometer-scale proximity due to clustering in a microdomain, CFP transfers energy to YFP (FRET). (B) To ensure that CD81-EC2-iYFP behaves as a TEM marker despite modification with YFP, copatching between CD81-EC2-iYFP and endogenous tetraspanin CD9 was examined. CD81-EC2-iYFP copatches with CD9 similarly to endogenous CD81 (compare to Fig. 1, top row). The fields

detected a shorter average fluorescent lifetime of FRET donor CFP-GPI, which is indicative of FRET (Fig. 3D). When we analyzed the relationship between FRET efficiency and YFP intensity as described above, FRET efficiency in the presence of WT Gag-RFP appeared largely independent of YFP intensity, representing submicroscopic clustering of lipid raft and TEM markers (Fig. 3E). In contrast, when cells were transfected with negative-control 1GA Gag construct, FRET efficiency appeared dependent on YFP intensity, representing little or no clustering of markers (Fig. 3E). These results indicate that Gag-dependent clustering of lipid raft and TEM markers occurs in live cells, on a submicroscopic scale, and is not an artifact of antibody-mediated patching, fixation, or post-fixation mobility of markers.

Replacing the MA domain with heterologous membrane binding motifs has no effect on association with, and coalescence of, lipid rafts and TEMs. It has been suggested that Gag acylation or a potential unique mode of PI(4,5)P₂ binding may determine Gag association with lipid rafts. It is also conceivable that the presence of the phosphoinositide-producing enzyme phosphatidylinositol 4-kinase in TEMs may govern Gag association with TEMs (see introduction). To test whether acylation, PI(4,5)P₂ binding, or other functions within the MA domain are molecular determinants of microdomain interaction, we generated a panel of Gag derivatives containing functional substitutions of MA membrane binding motifs (Fig. 4).

Fyn(10)fullMA Gag-YFP is acylated but retains PI(4,5)P₂-binding MA basic residues. This construct is less dependent on PI(4,5)P₂ for membrane binding than WT Gag (23). Fyn(10)ΔMA Gag-YFP is also acylated but has its entire MA domain deleted. This construct does not have any detectable affinity for PI(4,5)P₂ (23). We have previously reported that these Fyn(10) derivatives bind cellular membranes more efficiently than WT Gag (23, 58). PH_{PLC81}delMA Gag-YFP contains a PI(4,5)P₂-binding PH domain in place of the MA globular domain. This construct contains no acylation signals. Although these constructs have very different modes of membrane binding, all are capable of forming VLPs (23, 68, 121, 133).

Under each experimental condition tested, Gag-YFP with membrane binding motif substitutions induced the copatching of lipid raft and TEM markers (Fig. 4A), similarly to WT Gag-YFP (Fig. 2B). Correlations between microdomain marker fluorescence intensities were measured (Fig. 4B) and compared to WT Gag-YFP and negative-control 1GA/6A2T Gag-YFP values from the experiments shown in Fig. 2D. Because coexpressing Tat and Rev *in trans* was necessary to res-

shown are each 12 μm by 12 μm. (C) Schematic of clustering analysis. Randomly distributed fluorescent proteins produce FRET due to random collisions dependent on acceptor YFP density (left). Clustering of fluorescent proteins produces high FRET, even at low YFP density (right). (D) In FLIM, FRET between CFP-GPI and CD81-EC2-iYFP causes a shortened average fluorescence lifetime of CFP (right field) compared to that of the CFP-GPI donor only (left field). (E) FRET efficiency calculated from FLIM data is plotted against YFP intensity and fit to a saturable ligand binding model. Each data point represents the average value of one cell.

FIG. 4. Gag membrane binding motif substitutions have no effect on interaction with microdomains. Cells expressing Gag-YFP derivatives containing membrane binding motif substitutions, Fyn(10)fullMA, Fyn(10) Δ MA, and PH_{PLC δ 1}delMA, were treated with antibodies to independently induce patching of microdomain markers, CD59 and CD81. (A) Images of dorsal cell surfaces are shown. Fields are each 12 μ m by 12 μ m. (B) The R^2 strength of correlation between CD59 and CD81 fluorescence intensities was calculated and averaged from 13 to 33 cells per condition. The values for 1GA/6A2T and WT Gag-YFP from the experiments shown in Fig. 2D are included here to facilitate comparison. Error bars represent ± 1 standard error of the mean. *, significantly greater than 1GA/6A2T ($P < 0.01$).

cue PH_{PLC δ 1}delMA Gag-YFP expression (see Materials and Methods), we controlled for this difference by also coexpressing WT Gag-YFP with Tat and Rev. Coexpressing Tat and Rev had no effect on microdomain marker correlations (Fig. 4B). Each Gag derivative with membrane binding motif substitutions coalesced lipid rafts and TEMs similarly to WT Gag-YFP

and significantly more than non-membrane-binding 1GA/6A2T Gag-YFP (Fig. 4B). These results indicate that while Gag membrane binding is necessary (Fig. 2 and 3), the particular mode of membrane binding has no detectable effect on interaction with, and coalescence of, lipid rafts and TEMs: either acylation or PI(4,5)P₂ binding is sufficient. Moreover,

these results show that other functions within the MA domain, such as acyl chain sequestration or unknown interactions with other host factors, do not play an irreplaceable role in microdomain coalescence, as the entire MA sequence is dispensable.

A Gag mutation that reduces membrane curvature and particle budding also reduces lipid raft and TEM marker coalescence. Upon Gag membrane binding and multimerization, the assembling virus particle induces membrane curvature to bud a nascent enveloped VLP. A derivative of Gag-YFP, P99A, containing a mutation in its CA N-terminal domain, produced electron-dense patches on the PM, like WT Gag-YFP (Fig. 5A, black arrowheads), as shown by transmission electron microscopy. However, P99A Gag-YFP multimers appear to be arrested at a prebudding or early budding step and largely fail to progress to a completely budded VLP (Fig. 5A, compare P99A Gag-YFP to WT GFP-YFP, open arrows) although completed particles are rarely observed (Fig. 5A, inset).

To determine whether microdomain reorganization at Gag assembly sites depends solely on the presence of Gag multimers or on additional steps after Gag multimerization (e.g., membrane curvature and budding), we performed copatching between microdomain markers in the presence of P99A Gag-YFP. Under each experimental condition tested, P99A Gag-YFP produced a low correlation between lipid rafts and TEMs (Fig. 5B and C), which is significantly less than that of WT Gag-YFP and not significantly different from that of non-membrane-bound 1GA/6A2T Gag-YFP (compare images in Fig. 5B to those in 2A and B). 1GA/6A2T and WT Gag-YFP correlation values from Fig. 2D are included in Fig. 5C to facilitate comparison. These results are consistent with Gag-dependent microdomain coalescence occurring late during particle assembly, i.e., during membrane budding and completion of a spherical particle.

Using the same microscopy data, we also calculated the R^2 strength of correlation between P99A Gag-YFP and each microdomain marker. TEM marker CD81 and lipid raft markers CD59 and NonFP-GPI each did not copatch with P99A Gag-YFP. In contrast, raft marker NonFP-HA-TMD copatched with P99A Gag-YFP to a significantly greater degree (Fig. 5D). The difference in behaviors among canonical lipid raft markers in the presence of Gag suggests that these different markers may represent distinct subsets of lipid rafts with different affinities for Gag multimers. These results further suggest that Gag-mediated coalescence of disparate microdomains may occur in a stepwise manner. For example, HA-TMD-containing microdomains may be recruited earlier than TEMs and GPI anchor-containing microdomains.

Microdomain copatching results are not explained by differences in Gag membrane binding. It is possible that differences in membrane binding between Gag derivatives may influence the microdomain copatching observations presented above. For example, if more Gag molecules binding to the plasma membrane cause more microdomain copatching and higher R^2 values, a Gag derivative that is intrinsically less able to cause coalescence of microdomain markers might still produce high R^2 coefficients by virtue of more efficient membrane binding. Inversely, reduced copatching by a Gag derivative (e.g., P99A) might be due to inefficient membrane binding.

To compare the membrane binding efficiency of Gag derivatives used in this study, we performed a membrane flotation assay quantifying the amount of Gag-YFP derivatives that fractionate with cellular membranes. As expected based on previous studies (23, 58), mutations that disrupt Gag membrane binding motifs (1GA/6A2T) almost completely reduced membrane binding, and Gag derivatives containing the Fyn(10) acylation motif had enhanced membrane association relative to WT Gag-YFP (Fig. 6A). Like the Fyn(10) derivatives, PH_{PLC81}delMA Gag-YFP had enhanced membrane association (Fig. 6A). However, P99A Gag-YFP bound cellular membranes at levels equal to those of WT Gag-YFP (Fig. 6A). Therefore, the inability of P99A Gag-YFP to induce microdomain copatching (Fig. 5) is not due to a reduction in membrane binding.

Since the Fyn(10) and PH_{PLC81} Gag-YFP derivatives have enhanced membrane association, we reanalyzed the microscopy data presented in Fig. 2D and 4B to determine whether this increased membrane binding could be systematically affecting the measured R^2 correlations between microdomain markers. If increased amounts of Gag at the plasma membrane generally cause higher measured R^2 correlations, this relationship should be apparent on a cell-by-cell basis. To determine whether such a trend exists, we calculated the R^2 coefficient between microdomain markers for each image as before and determined the fraction of each image that is positive for the Gag-YFP derivative. Plotting these measures revealed no apparent positive relationship, suggesting that a greater amount of Gag-YFP at the plasma membrane does not cause greater R^2 coefficients, at least within the ranges naturally present in our data set. Thus, increased membrane binding of Fyn(10) and PH_{PLC81} Gag-YFP derivatives is not likely compensating for any decreased ability to induce copatching of microdomain markers.

Altogether, these results and reanalyses show that the inability of P99A Gag-YFP to induce microdomain coalescence is not due to a lack of membrane binding and suggest that,

FIG. 5. A Gag mutation that reduces membrane curvature and particle budding also reduces coalescence of lipid raft and TEM markers. (A) Transmission electron micrographs show that WT Gag-YFP produces both completed immature VLPs (open arrows) and electron-dense prebudding Gag multimers (filled arrowheads). P99A Gag-YFP produces primarily prebudding multimers, but VLPs are rarely observed (inset). Scale bar, 1 μ m. (B) Cells expressing Gag-YFP derivatives and microdomain markers were treated with antibodies to independently induce patching of the microdomain markers, and the dorsal surfaces of cells were imaged. Fields are each 12 μ m by 12 μ m. (C) The R^2 strength of correlation between fluorescence intensities of pairs of markers was calculated and averaged from 15 to 37 cells per condition. The values for 1GA/6A2T and WT Gag-YFP from the experiments shown in Fig. 2D are included to facilitate comparison. (D) Using the same microscopy data used in panel C, the R^2 between fluorescence intensities of each marker and P99A Gag-YFP was calculated. Error bars represent ± 1 standard error of the mean. *, significantly different ($P < 0.01$); n.s., not significantly different ($P > 0.01$).

FIG. 6. Differential copatching levels among Gag derivatives are not explained by differences in membrane binding. (A) HeLa cells expressing Gag derivatives were disrupted and subjected to membrane flotation centrifugation and divided into five fractions, and Gag derivatives were detected by immunoblotting. Fractions 1 and 2 contain membrane-associated material (memb.), and fractions 4 and 5 contain non-membrane-associated material (non-memb.). The percentage of membrane binding was calculated by the ratio of membrane-associated signal to total signal. (B) Data presented in Fig. 2D and 4B were reanalyzed, and percent membrane area occupied by Gag (x axis) and R^2 strength of correlation between CD59 and CD81 (y axis) in individual cells were plotted. There is little or no linear relationship apparent between these measures ($R^2 \leq 0.05$).

while membrane binding is necessary (Fig. 2A), the differences in Gag membrane binding between Gag derivatives are not great enough to have an apparent effect on copatching.

Lipid raft marker HA-TMD is associated with Gag puncta even in the absence of cell surface VLPs, whereas TEM marker CD81 appears predominately in association with VLPs. To obtain a higher-resolution view of microdomain coalescence phenotypes, we performed antibody-mediated copatching of lipid raft marker NonFP-HA-TMD and TEM marker CD81 in the presence of WT or P99A Gag-YFP as above and subsequently imaged the same cells by SEM to detect cell surface particles. In cells expressing WT Gag-YFP, we observed many spherical cell surface particles near 150 nm in diameter, approximately the size of an authentic HIV-1 VLP (Fig. 7A and B and pseudocolored red in C). Aligning fluorescence and SEM images (see Materials and Methods), we observed a

strong correspondence between WT Gag-YFP fluorescent puncta and particles (Fig. 7D), indicating that these particles are likely Gag-YFP-derived VLPs. In contrast, in cells expressing P99A Gag-YFP, there were few particulate structures and little or no correspondence between these cell surface structures (Fig. 7G and H and pseudocolored red in I) and P99A Gag-YFP fluorescence signals (Fig. 7J). Thus, the few particulate structures in P99A Gag-YFP-expressing cells are likely not Gag-YFP-derived VLPs. In light of the transmission electron microscopy data showing that P99A Gag-YFP produces electron-dense patches on the PM (Fig. 5A), fluorescent puncta without cell surface particles likely represent P99A Gag-YFP multimers that are arrested prior to membrane budding and particle morphogenesis.

To determine the relationship between particle production and microdomain coalescence, we quantified 170 fluorescent puncta from three cells expressing WT Gag-YFP and 179 fluorescent puncta from four cells expressing P99A Gag-YFP (Table 2). Gag-YFP fluorescent puncta were automatically selected in an unbiased manner using an image-processing algorithm (58; see also Materials and Methods). WT Gag-YFP fluorescent puncta were then classified according to their colocalizations with cell surface particles, as illustrated in Fig. 7. For cells expressing P99A Gag-YFP, very few particulate structures were found in areas displaying fluorescent Gag puncta. Gag-YFP fluorescent puncta were then scored for the presence or absence of each microdomain marker (Table 2).

In the case of WT Gag-YFP, 91% of particle-positive fluorescent puncta were double positive for NonFP-HA-TMD and CD81. In each individual cell measured, particle-positive fluorescent puncta were significantly more frequently positive for NonFP-HA-TMD ($P < 0.01$) and CD81 ($P < 0.01$) than by chance.

In contrast, in both WT and P99A Gag-YFP-expressing cells, fluorescent puncta that are not associated with particles were mostly positive for NonFP-HA-TMD but mostly negative for CD81 (Table 2). In individual cells expressing WT Gag-YFP, there were too few particle-negative fluorescent puncta to perform statistical tests, but in each cell expressing P99A Gag-YFP, fluorescent puncta were significantly more frequently positive for NonFP-HA-TMD than by chance ($P < 0.01$). However, the association of P99A Gag-YFP fluorescent puncta with CD81 was not significant in three of the four cells ($P = 0.26$ and higher). In the fourth cell, although CD81 association with P99A Gag-YFP Gag puncta was observed more frequently than by chance ($P < 0.01$), the number of Gag puncta with this tetraspanin was still lower than the number with the raft marker NonFP-HA-TMD (25 CD81-positive puncta versus 36 NonFP-HA-TMD-positive puncta out of 41 total fluorescent puncta).

These higher-resolution analyses revealed the following: (i) as opposed to the larger aggregate structures that have been observed in cells producing human T-cell leukemia virus type 1 (HTLV-1) (111), the coalescence phenomenon observed in this study occurs in association with individual VLPs; (ii) NonFP-HA-TMD appears to be recruited to Gag-YFP fluorescent puncta with or without cell surface particles, consistent with a model in which this lipid raft marker is recruited to Gag multimers early in assembly, prior to membrane budding; (iii) the TEM marker CD81 appears predominately in association

FIG. 7. Correlative fluorescence and scanning electron microscopy. Cells expressing WT Gag-YFP or P99A Gag-YFP were imaged by SEM to detect cell surface structures and by fluorescence microscopy to detect Gag (green), raft marker NonFP-HA-TMD, and TEM marker CD81 (red). Cells were treated with antibodies to induce patching of microdomain markers prior to fixation. (A and G) SEM images of a cell expressing WT Gag-YFP or P99A Gag-YFP. Panels B to F and H to L represent a $\times 10$ magnification of the boxed areas in panels A and G, respectively. (B and H) SEM image. (C and I) SEM images with pseudocolored ~ 150 -nm spherical cell surface structures (red). (D and J) Fluorescence image of Gag (green) with pseudocolored cell surface structures superimposed (red) from panel C or I, respectively. (E and K) Fluorescence image of Gag (green) and NonFP-HA-TMD (red). (F and L) Fluorescence image of Gag (green) and CD81 (red). White numbered arrowheads indicate examples of Gag-YFP punctum classifications: 1, WT Gag-YFP fluorescent puncta that are particle positive are mostly double positive for microdomain markers; 2, WT and P99A Gag-YFP fluorescent puncta that are not particle positive are mostly NonFP-HA-TMD positive and CD81 negative (Table 2). Scale bars, 10 μm (A and G) and 1 μm (B to F and H to L).

TABLE 2. Quantification of correlative fluorescence and SEM data

Gag-YFP punctum type (<i>n</i>) ^a	Association with patched microdomain marker(s)	No. (%) of puncta
WT puncta (170)		
Particle positive (130)	NonFP-HA-TMD positive	126 (97)
	CD81 positive	121 (93)
	Double positive	118 (91)
Particle negative (40)	NonFP-HA-TMD positive	30 (75)
	CD81 positive	17 (43)
	Double positive	15 (38)
P99A puncta (179)	NonFP-HA-TMD positive	121 (68)
	CD81 positive	54 (30)
	Double positive	45 (25)

^a *n*, number of puncta in group.

with cell surface particles, consistent with recruitment of TEMs late in assembly, during membrane budding and VLP production. Altogether, these results suggest a stepwise model of microdomain coalescence during HIV-1 assembly.

DISCUSSION

As viruses rely on host cell structures and functions for most steps of their life cycle, it would not be surprising for viruses to take advantage of preexisting host membrane microdomains to compartmentalize their own viral processes. Additionally or alternatively, viruses might hijack membrane organization, restructuring microdomains to facilitate viral processes or modulate cellular processes for their benefit. Using a wide variety of methods, previous studies have demonstrated that Gag associates with lipid rafts and/or TEMs, both of which have been proposed to be platforms for HIV-1 assembly. However, it is well established that lipid rafts and TEMs are distinct microdomains in the absence of HIV-1. Two possible, but not mutually exclusive, models can explain this contradiction, as described below.

First, it is possible that individual Gag multimers interact with distinct microdomains bimodally or quantally—interacting with one or another, but not both simultaneously. This “quantal” model is supported by a study that coexpressed HIV-1 Gag, HIV-1 Env glycoprotein, and Ebola glycoprotein (82). The two viral glycoproteins were observed to segregate into distinct microscopic-scale domains in the presence or absence of Gag, and individual VLPs produced by Gag were found to have incorporated either HIV-1 Env glycoprotein or Ebola glycoprotein but not a mixture of both.

Alternatively, our data support a “coalescence” model in which Gag actively reorganizes the membrane by inducing the coalescence of otherwise distinct microdomain constituents, lipid raft markers and tetraspanin proteins. Thus, in this model, Gag does not associate with preexisting virus-sized microdomains organized by cellular factors. Rather, Gag itself appears to function as a microdomain-organizing factor to create a novel virus-induced microdomain in a stepwise manner during the course of assembly. Consistent with this model, a kinetic analysis of Gag binding to membranes previously showed that association of Gag with DRMs is delayed relative to Gag binding to total membrane (104).

While the manuscript was in preparation, Kremontsov et al.

showed that a GPI-anchored lipid raft marker and tetraspanin CD9 both accumulate at Gag puncta, that CD9 becomes trapped and unable to freely diffuse at Gag puncta, and that this accumulation and confinement correlate with the Gag expression level and ability to multimerize (77). Their results are largely in agreement with ours, and both support the idea that Gag reorganizes microdomains during assembly.

Fang et al. previously showed that membrane binding plus higher-order multimerization are sufficient for proteins to be targeted to specialized endosome-like microdomains of the PM and become exosome cargo (35). Exosomes are small membranous vesicles either produced by budding directly through these endosome-like microdomains or initially generated as intraluminal vesicles of late endosomes/multivesicular bodies (LE/MVB) and then released into the extracellular space by fusion of the LE/MVB with the PM. In light of their findings, Gould et al. proposed a model according to which Gag, as a membrane binding and multimerizing protein, is cargo for the preexisting exosome pathway and virions are exosomes containing infectious viral components (35, 47). A critical question raised by this “Trojan exosome” model is to what degree viral components actively hijack and alter cellular machinery for viral purposes instead of merely masquerading as substrates of preexisting host processes. Though it does not preclude an active role for Gag, this Trojan exosome model does not require any more complex role for Gag in VLP assembly than as a passive substrate. In contrast to this notion, we observed differences in membrane microdomain organization in the presence and absence of Gag. Thus, we favor a more active role for Gag interacting with and altering host membrane systems. It remains to be seen whether the cellular exosome machinery induces similar microdomain reorganization in response to passive exosome cargo or if this reorganization is unique to active viral assembly.

To date, the biochemical or biophysical underpinnings that govern the association of Gag with lipid rafts and TEMs and the Gag-induced coalescence of these microdomains remain to be determined. Lipid raft formation is thought to be at least partially driven by preferential associations between planar cholesterol molecules and saturated acyl chains (87). Thus, it has been suggested that Gag N-terminal myristoylation (a saturated acylation) causes partitioning of Gag into lipid rafts. Additionally, it has been suggested that sequestration of unsaturated 1' acyl chain of MA-bound PI(4,5)P₂ into the MA globular domain may drive lipid raft association of Gag (118). However, our observation that PH_{PLC81}delMA Gag copatches lipid rafts and TEMs as efficiently as WT Gag suggests that neither myristoylation of Gag nor acyl chain sequestration by Gag plays an irreplaceable role in association with and coalescence of these microdomains. PI(4,5)P₂ binding may be responsible for association of WT and PH_{PLC81}delMA Gag with phosphatidylinositol 4-kinase-containing TEMs. However, our results show that Fyn(10)ΔMA Gag, which contains no known PI(4,5)P₂-binding motifs, copatches lipid rafts and TEMs as well as WT Gag, indicating that PI(4,5)P₂ binding is also not a prerequisite for association with and coalescence of these microdomains.

How can Gag derivatives using very different molecular interactions with the membrane all cause similar lipid raft/TEM coalescence? It is intriguing to speculate that a more

general membrane phenomenon underlies this coalescence of microdomains—a property or function of the membrane that Gag derivatives can affect regardless of membrane binding mode. It has been proposed that cellular membranes exist in a metastable state: affinities between membrane components provide a latent propensity or potential for membrane components to partition or compartmentalize (87). Thus, relatively small effects on local membrane dynamics by microdomain-organizing proteins may trigger this underlying predisposition for partitioning, amplifying small protein-based signals into larger-scale membrane reorganization. Our data suggest that membrane curvature may be one such mechanism by which Gag derivatives with diverse modes of membrane binding may influence host membrane dynamics since Gag multimers that have not induced or are unable to induce membrane budding are also unable to cause the copatching of lipid raft and TEM markers. Indeed, membrane curvature has been shown to stabilize microdomains (134). In this case, we might predict that many unrelated viruses that assemble on and bud through the PM induce similar microdomain reorganization. Consistent with this possibility, a canonical lipid raft-associated virus, influenza A virus, was shown to incorporate tetraspanins CD9 and CD81 in addition to raft markers HA and CD59 (124).

The ability of Gag to reorganize host membranes may have many important implications for viral fitness. (i) If small Gag multimers induce specialized membrane microdomains that attract subsequent Gag monomers, this cooperativity could promote Gag multimerization and virion assembly. (ii) Since biophysical studies of artificial membranes have shown relationships between lipid composition and membrane curvature (116), it is conceivable that changes in microdomain composition induced by Gag may contribute to membrane curvature during virus budding. (iii) Gag interaction with membrane microdomains may modulate recruitment of HIV-1 envelope glycoproteins and may underlie the phenomenon of viral pseudotyping by recruiting heterologous viral envelope glycoproteins (66, 113; for recent reviews, see references 63 and 137). (iv) By altering host microdomains, Gag may influence the incorporation of host factors that may directly or indirectly affect viral fitness. For example, it has been shown that tetraspanin proteins modulate virus infectivity (51, 78, 120) and formation of syncytia (139). Also, lipid rafts are proposed to mediate targeting of the antiviral restriction factor tetherin to assembling virions, thereby preventing their release (36, 52, 54, 57, 69, 79, 89, 115). (v) Finally, microdomains appear to be involved in the formation of cell synapses that mediate direct cell-to-cell virus transmission, which is thought to be the primary mode of virus spread within an infected host (64, 65, 117). Our recent data suggest that the rearward flow of PM microdomains may be responsible for the localization of virus assembly to the uropod of polarized T cells, which is the site of frequent cell-cell contact (88). In light of these potentially critical roles for microdomains in HIV-1 replication, further investigation is necessary to better define cellular microdomains, further clarify the molecular determinants by which Gag reorganizes these microdomains, and elucidate what impacts these membrane phenomena have on the virus replication cycle.

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