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DEPLETION OF CELLULAR CHOLESTEROL INHIBITS MEMBRANE BINDING AND HIGHER-ORDER MULTIMERIZATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 GAG

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Abstract

Recent studies have suggested that the plasma membrane contains cholesterol-enriched microdomains known as lipid rafts. HIV-1 Gag binds raft-rich regions of the plasma membrane, and cholesterol depletion impairs HIV-1 particle production. In this study, we sought to define the block imposed by cholesterol depletion. We observed that membrane binding and higher-order multimerization of Gag were markedly reduced upon cholesterol depletion. Fusing to Gag a highly efficient, heterologous membrane binding sequence reversed the defects in Gag-membrane binding and multimerization caused by cholesterol depletion, indicating that the impact of reducing the membrane cholesterol content on Gag-membrane binding and multimerization can be circumvented by increasing the affinity of Gag for membrane. Virus release efficiency of this Gag derivative was minimally affected by cholesterol depletion. Altogether, these results are consistent with the hypothesis that cholesterol-enriched membrane microdomains promote HIV-1 particle production by facilitating both Gag-membrane binding and Gag multimerization.

Keywords

HIV-1; Gag; retrovirus assembly; cholesterol; lipid raft; myristyl switch; membrane binding

Introduction

Human immunodeficiency virus type 1 (HIV-1), like many other retroviruses, assembles into virus particles in association with cellular membrane (Freed, 1998;Swanstrom and Wills, 1997). Retrovirus particle production, driven by the viral Gag protein, is a multistep process that includes: 1) Gag transport to the site of virus assembly, 2) Gag binding to a lipid bilayer, 3) Gag multimerization, and 4) budding and pinching-off of nascent virus particles from the host cell membrane. In the case of HIV-1, depending on the cell type in which Gag is expressed, virus particle formation takes place either at the plasma membrane (PM) or in late endosomes/multivesicular bodies (MVBs)(Grigorov et al., 2006;Nydegger et al., 2003;Ono and Freed,

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2004;Pelchen-Matthews et al., 2003;Perlman and Resh, 2006;Raposo et al., 2002;Rudner et al., 2005;Sherer et al., 2003). HIV-1 Gag is synthesized as a precursor polyprotein, $Pr55^{Gag}$, that consists of four major structural domains: matrix (MA), capsid (CA), nucleocapsid (NC), and p6 and two spacer peptides, SP1 and SP2. Upon virus particle release, these domains and spacer peptides are cleaved by the viral protease (PR) to generate p17MA, p24CA, p7NC, p6, SP1, and SP2, respectively.

The MA domain contains determinants for Gag binding to the lipid bilayer and Gag targeting to the PM. The former function is mediated in part by the N-terminal myristate moiety (Freed, 1998;Swanstrom and Wills, 1997). The myristyl chain is thought to adopt either of two conformational states: exposed or sequestered within the globular head of MA. According to the myristyl switch model (Zhou and Resh, 1996), Gag binds lipid bilayers efficiently when the myristate is exposed but poorly when it is sequestered. Consistent with this model, amino acid substitutions near the N-terminus of MA or deletions in the MA globular head modulate Gag-membrane binding (Ono and Freed, 1999;Paillart and Gottlinger, 1999;Perez-Caballero et al., 2004). In the WT context, Gag multimerization likely promotes the exposure of the myristate moiety (Hermida-Matsumoto and Resh, 1999;Perez-Caballero et al., 2004;Sandefur et al., 2000;Sandefur et al., 1998;Spearman et al., 1997;Tang et al., 2004;Zhou and Resh, 1996). In addition to the myristyl chain, interactions between acidic phospholipids and a highly basic region of MA (located between MA amino acids 17 and 32) are implicated in Gag-membrane binding (Zhou et al., 1994). This highly basic sequence is also a determinant of Gag localization to the PM; amino acid substitutions in this region cause retargeting of Gag to the late endosome/MVB even in cell types in which WT Gag is targeted to the PM (Freed et al., 1994;Hermida-Matsumoto and Resh, 2000;Ono and Freed, 2004;Ono et al., 2000b;Yuan et al., 1993). Gag multimerization is mediated by a region spanning the C-terminal domain of CA, SP1, and the N-terminal portion of NC. The CA C-terminal domain likely mediates Gag dimerization while NC promotes higher-order Gag multimerization by binding to RNA molecules that may serve as assembly scaffolds (Adamson and Jones, 2004). p6 facilitates pinching-off of nascent virus particles from the PM by recruiting cellular machinery that usually functions in the budding of intraluminal vesicles into late endosomes (Demirov and Freed, 2004;Morita and Sundquist, 2004).

It is now well established that the PM is not a homogenous lipid bilayer but rather is composed of various microdomains containing unique sets of proteins and lipids. Among these, cholesterol- and saturated-lipid-enriched microdomains known as lipid rafts have been the subject of much interest and debate across several disciplines of biology. Cholesterol likely promotes formation of a liquid-ordered structure through interactions with saturated acyl chains in lipid rafts (Brown and London, 2000;Simons and Toomre, 2000;Simons and Vaz, 2004). Lipid rafts can be isolated biochemically based on their insolubility in a number of nonionic detergents (e.g., Triton X-100) at low temperature (Brown and Rose, 1992). Although detergent-resistant membrane (DRM) is unlikely to represent intact rafts present in undisrupted cells (Edidin, 2003;Heerklotz, 2002;Munro, 2003), and a population of raft-associated molecules may be lost from DRM during isolation procedures (Harder et al., 1998), DRM binding can serve as a useful criterion for raft association. To corroborate results obtained by such biochemical methods, molecules of interest are often examined for their copatching or colocalization with raft marker proteins and lipids. As cholesterol plays an important role in maintaining lipid raft integrity (Brown and London, 2000;Simons and Toomre, 2000), the functional significance of rafts in cellular processes has been assessed by using cholesterol depletion approaches. Based on results obtained by a combination of biochemical, microscopic, and pharmacological methods, rafts have been implicated in a large number of cellular functions such as signal transduction and protein trafficking (Brown and London, 2000;Simons and Toomre, 2000). In such cellular pathways, rafts are postulated to serve as concentration platforms that facilitate interactions among raft-associated molecules. Alternatively or in

addition, rafts may function as transport vehicles that allow specific proteins and lipids to target a specific subcellular location.

Over the past several years, lipid rafts have been implicated in the replication of an increasing number of viruses (Ono and Freed, 2005). In particular, members of several families of enveloped viruses have been reported to utilize rafts during the assembly and release phase of their replication cycles (for reviews, see references Briggs et al., 2003;Ono and Freed, 2005;Suomalainen, 2002). In these reports, viral glycoproteins and/or inner structural proteins of the orthomyxoviruses, paramyxoviruses, filoviruses, and retroviruses were observed to interact with rafts based either on their association with DRM, their colocalization with raft markers, or both. Genetic studies of viral mutants that are defective in DRM association suggested correlations between the extent of DRM association of these viral structural proteins and the efficiency of virus particle production (e.g., Panchal et al., 2003;Takeda et al., 2003). Because mutations in viral structural proteins often have pleiotropic effects on virus assembly and release, however, the physiological significance of the reported interactions between viral proteins and lipid rafts remains to be determined.

Multiple lines of evidence suggest that lipid rafts play a role in HIV-1 assembly and release. 1) The virion lipid bilayer is enriched in cholesterol and sphingolipids relative to the host cell plasma membrane (Aloia 93, Brugger 06). 2) The Gag proteins of HIV-1 and other retroviruses have been shown to associate with rafts in DRM binding assays (Ding et al., 2003;Feng et al., 2003;Halwani et al., 2003;Holm et al., 2003;Lindwasser and Resh, 2001;Nguyen and Hildreth, 2000;Ono and Freed, 2001;Pickl et al., 2001;Yang and Ratner, 2002). Although the association of Gag with DRM has been the subject of debate (Ding et al., 2003;Holm et al., 2003), technical differences between studies likely explain the differences in interpretation (Ono et al., 2005). 3) Gag proteins colocalize or copatch with raft markers (Holm et al., 2003;Nguyen and Hildreth, 2000;Ono and Freed, 2005). 4) Virus particle production is inhibited by cholesterol depletion (Ono and Freed, 2001;Pickl et al., 2001) and by substitution of myristate with unsaturated analogs, which inhibits Gag association with liquid-ordered microdomains (Lindwasser and Resh, 2002). Altogether, these results suggest that lipid rafts play an important role in HIV-1 particle production. In this report, we sought to define the role(s) of cholesterol-enriched membrane microdomains in HIV-1 assembly and release. To this end, we examined the impact of cellular cholesterol depletion on each step of the virus particle production process. The results demonstrate that PM cholesterol is important for efficient binding of Gag to membrane and for higher-order Gag multimerization, but is not required for proper subcellular Gag targeting. We suggest that the primary function of rafts in HIV-1 assembly is to provide a platform for stable binding of Gag to membrane and for efficient Gag multimerization.

Results

Cholesterol depletion does not affect targeting of Gag to the PM

It was reported that disruption of HIV-1 Gag-raft association with unsaturated myristate analogs caused a mislocalization of Gag to intracellular compartments (Lindwasser and Resh, 2002). To examine whether Gag targeting to the PM is affected by cholesterol depletion, HeLa cells were transfected with WT pNL4-3, treated with either methyl-β-cyclodextrin (MβCD), which acutely extracts PM cholesterol, or simvastatin, which inhibits cholesterol biosynthesis, and immunostained with a mixture of anti-p17MA and anti-p24CA antibodies. In cholesterol-depleted cultures, Gag still localized to the PM and not to intracellular compartments, although a number of cells showed an increase in hazy cytoplasmic localization relative to cell-surface punctate signal (data not shown). These results indicate that cholesterol depletion does not cause a mistargeting of Gag to intracellular compartments, but suggest that cholesterol removal may reduce binding of Gag to membrane.

Depletion of cellular membrane cholesterol reduces Gag-membrane binding

We have shown previously that Gag binding to total cellular membrane precedes DRM association (Ono and Freed, 2001). These data suggest that initial Gag-membrane binding does not require the presence of intact lipid rafts. Nonetheless, it is possible that Gag interaction with the lipid bilayer may be stabilized or enhanced by the presence of lipid rafts in the membrane. To address this possibility, we sought to determine whether cholesterol depletion from virus-producing cells affects the binding of Gag to membrane. HeLa cells were infected with vesicular stomatitis virus G (VSV-G)-pseudotyped virus expressing NL4-3/PR^{-}, which lacks a functional viral protease (PR). Infected cells were either left untreated or were depleted of cholesterol with MβCD or simvastatin. In the conditions used in these experiments, we typically observe an approximately 3-fold reduction in plasma membrane cholesterol (Ono and Freed, 2001). To monitor global effects on membrane that might be induced by cholesterol depletion, HeLa cells expressing FynGFP were mixed with Gag-expressing cells prior to cholesterol depletion. FynGFP contains the myristylated and palmitoylated Fyn N-terminus linked to GFP (van't Hof and Resh, 1997). Cells were pulse-labeled for 5 min with [^{35}S]Met/Cys and chased for 15 min. Post-nuclear supernatants (PNSs) of labeled cell homogenates were subjected to equilibrium flotation centrifugation in sucrose gradients. Gag proteins in top (membrane-bound) and bottom (non-membrane-bound) fractions were recovered by immunoprecipitation with HIV immunoglobulin (HIV-Ig) and the efficiency of Gag-membrane binding was compared between untreated and cholesterol-depleted cells. Approximately half of the total Gag was detected in membrane fractions in untreated cells whereas, in cholesterol-depleted cells, only 25-30% of total Gag was membrane-bound (Fig. 1A and B). Similar results were obtained when pNL4-3/PR^{-} was introduced into cells by transfection (see below). In contrast, membrane binding of FynGFP was unaffected (Fig. 1A and C) indicating that cholesterol depletion does not globally disrupt cellular membrane. These results demonstrate that Gag-membrane binding is facilitated by PM cholesterol.

The efficiency of higher-order Gag multimerization is reduced by cholesterol depletion

As we reported recently, membrane-bound Gag undergoes higher-order multimerization that masks Gag epitopes and impairs immunoprecipitation of Pr55^{Gag} (Ono et al., 2005). For this reason, in the experiments described in Fig. 1A-C, Gag-containing fractions were denatured prior to immunoprecipitation so that higher-order Gag multimers were dissociated and fully recognized by anti-Gag antibodies. By comparing the efficiency of Gag immunoprecipitation from denatured and non-denatured samples, we can assess the level of higher-order Gag multimerization (Ono et al., 2005). Using this approach, we examined the impact of cholesterol depletion on higher-order Gag multimerization. In the untreated cultures, only 25% of membrane-bound Gag was recovered without denaturation versus with denaturation (Fig. 1D), suggesting that a majority of membrane-bound Gag is in higher-order multimers. In contrast, in cultures treated with cholesterol-depleting agents, nearly half of the total membrane-bound Gag was readily immunoprecipitated without prior denaturation (Fig. 1D). These results indicate that PM cholesterol enhances higher-order Gag multimerization in addition to Gag-membrane binding.

A heterologous membrane-binding signal reverses the impact of cholesterol depletion on Gag-membrane binding and higher-order multimerization

As described above, membrane binding of FynGFP, unlike that of Gag, was insensitive to cholesterol depletion. We hypothesized that Gag-membrane binding mediated by the native Gag N terminus requires intact lipid rafts whereas membrane binding mediated by the Fyn N terminus, which is myristylated and dually-palmitoylated, does not require raft structures. To analyze directly the involvement of the Gag N-terminus in the sensitivity of Gag-membrane binding to cholesterol depletion, we constructed a Gag derivative in which the Fyn N-terminus

is fused to the N-terminus of Gag [Fig. 2A, Fyn(10)fullMA]. We first examined whether Fyn (10)fullMA Gag forms virus particles at the plasma membrane like WT Gag. We transfected HeLa cells with either HIV-1 molecular clones encoding WT or Fyn(10)fullMA Gag or the plasmid encoding FynGFP (Fig. 2B). To detect Gag associated with assembling particles, the anti-p17 monoclonal antibody was used for immunostaining. As observed with WT Gag and FynGFP, a majority of the Fyn(10)fullMA Gag signal localized to the PM, indicating that Fyn (10)fullMA Gag, like WT Gag, forms virus particles at the PM.

To examine the involvement of N-terminal sequences in the cholesterol dependence of Gag binding to membrane, we then transfected HeLa cells with pNL4-3/PR$^-$ or pNL4-3/Fyn(10) fullMA/PR$^-$. Cells were metabolically pulse-labeled for 5 min and chased for 15 min, and PNSs were subjected to equilibrium flotation centrifugation. To examine simultaneously the impact of MβCD treatment on higher-order Gag multimerization, immunoprecipitation of labeled Gag in membrane and non-membrane fractions was performed with or without prior denaturation (Fig. 3). In this series of experiments, ~30% of WT Gag was associated with membrane whereas ~80% of Fyn(10)fullMA Gag was in membrane fractions within the 15-min chase period. As observed in Fig. 1A and B, partitioning of WT Gag into membrane fractions was reduced approximately 50% by cholesterol depletion (Fig. 3A). In contrast, membrane binding of Fyn(10)fullMA Gag was insensitive to cholesterol depletion. Interestingly, unlike WT Gag, higher-order multimerization of Fyn(10)fullMA Gag was also resistant to cholesterol depletion (Fig. 3B). These results show that the effect of cholesterol depletion on Gag-membrane binding and higher-order Gag multimerization can be reversed by increasing the ability of Gag to bind membrane.

The Fyn N-terminus largely reverses the impact of cholesterol depletion on virus particle production

To examine whether the effects of cholesterol depletion can be reversed by the Fyn membrane binding signal, we transfected HeLa cells with molecular clones expressing either WT Gag or Fyn(10)fullMA Gag and metabolically labeled with [^{35}S]Met/Cys following cholesterol depletion with MβCD. Labeled Gag proteins were immunoprecipitated from cell and virus lysates with HIV-Ig and virus release efficiency was determined by measuring the amount of released, virion-associated p24 normalized for total labeled Gag (Fig. 3C). As we reported previously (Ono and Freed, 2001), virus particle production from cells expressing WT Gag was reduced approximately five-fold by cholesterol-depleting agents. By comparison, virus release efficiency from cells expressing Fyn(10)fullMA Gag was significantly less sensitive to MβCD treatment. These results indicate that the N terminus of Fyn confers upon Gag resistance to cholesterol depletion. Similar results were obtained when we examined the effect of MβCD on the release of a Gag chimera containing the Fyn membrane targeting signal in the context of a mutant Gag containing deletions in both MA and NC. We observed that release of VLPs generated by this construct, Fyn(10)ΔMAdelNC (Ono et al., 2004), was minimally affected, with a reduction of only ~10% relative to the WT. These results support the conclusion that linking Gag to a potent, heterologous membrane targeting signal reverses the impact of cholesterol depletion on virus release.

Discussion

Depletion of cellular cholesterol, which is essential for the integrity of lipid raft microdomains, markedly inhibits HIV-1 particle production. However, which step(s) in the virus particle assembly and release pathway requires membrane cholesterol and/or lipid rafts is unknown. To address this question, in this study we examined the impact of cholesterol depletion on each step of virus particle production using microscopic and biochemical methods. Immunofluorescence and EM experiments suggest that cholesterol depletion has no major

impact on Gag localization to the plasma membrane, or on the pinching-off of budding particles (unpublished data). In biochemical experiments, however, we found that depletion of cellular cholesterol markedly reduced the binding of Gag to membrane (Fig. 1). Importantly, membrane binding of the lipid raft marker FynGFP, as well as Fyn(10)fullMA Gag, was insensitive to cholesterol depletion (Fig. 1 and 3) even though, like WT Gag, both FynGFP and Fyn(10) fullMA Gag associate with the PM (Fig. 2). These results indicate that the decrease in Gag-membrane binding is not due to a global disruption of cellular membrane by cholesterol depletion. Together with the previous reports supporting the notion that Gag associates with lipid rafts (Ding et al., 2003;Feng et al., 2003;Guo et al., 2005;Halwani et al., 2003;Holm et al., 2003;Jolly and Sattentau, 2005;Lindwasser and Resh, 2001;Lindwasser and Resh, 2002;Nguyen and Hildreth, 2000;Ono and Freed, 2001;Ono et al., 2005;Pickl et al., 2001;Yang and Ratner, 2002), our data suggest that membrane binding of Gag is enhanced by the presence of cholesterol-enriched microdomains in the plasma membrane.

In addition to Gag-membrane binding, higher-order Gag multimerization was also reduced by cholesterol depletion (Fig. 1). Interestingly, upon addition of Fyn-derived sequences to the Gag N-terminus, not only Gag-membrane binding but also higher-order Gag multimerization was rendered insensitive to cholesterol depletion (Fig. 3). It is possible that the high-affinity membrane binding mediated by the Fyn N-terminus substantially increased the number of Gag molecules present on the membrane and thereby diminished the role of intact rafts in promoting efficient Gag multimerization. In other words, enhancement of Gag multimerization by rafts is observable only when a limited number (i.e., the WT level) of Gag molecules is present on the membrane. As a two-fold decrease in the efficiency of Gag-membrane binding results in a significant inhibition of virus particle production (Ono and Freed, 1999), the cumulative defects in Gag-membrane binding and higher-order Gag multimerization likely account for the virus release defects caused by cholesterol depletion. Indeed, the relative efficiency of virus release after cholesterol depletion was largely restored upon addition of the Fyn N-terminal sequence, which reversed defects in both Gag membrane binding and multimerization (Fig. 3C). Infectivity of virus particles formed by Fyn(10)fullMA Gag was reduced more than 50 fold (AW and EF, unpublished observation).

How would PM cholesterol facilitate Gag-membrane binding? It is possible that the observed effect of cholesterol depletion on Gag-membrane binding is due to disruption of a cholesterol-mediated function unrelated to rafts. In addition to disruption of raft microdomains, cholesterol depletion has been shown to inhibit clathrin-mediated endocytosis (Rodal et al., 1999;Subtil et al., 1999). However, we did not observe any major reduction in virus particle production when clathrin-mediated endocytosis was blocked by expression of a dominant-negative version of a regulatory protein (Ono et al., 2004). Cholesterol depletion has also been reported to reduce levels of the plasma membrane phospholipid, phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P_2] (Kwik et al., 2003). Since PI(4,5)P_2 depletion severely impairs virus production (Ono et al., 2004), and Gag appears to interact directly with this lipid (Saad et al., 2006;Shkriabai et al., 2006), it is possible that cholesterol depletion might indirectly affect Gag-membrane binding through PI(4,5)P_2 reduction. However, reductions in the levels of plasma membrane PI(4,5)P_2 caused relocalization of Gag to late endosomes/MVBs (Ono et al., 2004); this effect on Gag targeting was not observed upon cholesterol depletion (unpublished data). Therefore, these reportedly raft-independent cholesterol functions are unlikely to be involved in the disruption of Gag-membrane binding observed upon cholesterol depletion.

We postulate a model in which lipid rafts stabilize Gag-membrane interaction by facilitating the exposure of the N-terminal myristate moiety. A recent NMR analysis of myristylated MA in solution showed that the exposed and sequestered states of the myristate moiety are in equilibrium (Tang et al., 2004). Importantly, Gag-Gag interaction substantially shifted the equilibrium towards myristate exposure (Tang et al., 2004). If rafts enhance Gag-Gag

interactions, exposure of the myristate moiety will likely be increased upon raft association. Alternatively, raft binding may stabilize the exposed conformation of the N-terminal myristyl chain. The NMR study suggested that the exposure of myristate is coupled with MA trimerization (Tang et al., 2004) and that, at least in solution, MA trimerization is driven by interactions between exposed myristyl groups (Tang et al., 2004). In the presence of membrane lipids, hydrophobic interactions between the myristate moiety and lipid acyl chains would likely replace the interactions between myristates observed in solution (Tang et al., 2004). Lipid rafts, which are enriched in saturated lipids, are proposed to contain tightly packed liquid-ordered structures (Brown and London, 2000;Simons and Toomre, 2000;Simons and Vaz, 2004). The myristyl moiety attached to Gag may thus form hydrophobic interactions with acyl chains of membrane lipids more efficiently and thereby support more stable Gag-membrane interaction in rafts than in non-ordered membrane microdomains.

Even though palmitoylation is well known to promote raft association, membrane binding of Fyn(10)fullMA Gag was unaffected by cholesterol depletion (Fig. 3). In this case, probably due to the high affinity of the 16-carbon palmitate moiety for membrane, the presence of liquid-ordered membrane microdomains did not enhance further the membrane binding of Fyn(10) fullMA Gag. There are, however, two other reported cases in which rafts are not required for HIV-1 Gag-membrane binding (Guo et al., 2005;Lindwasser and Resh, 2002). In one study, the N-terminal myristate moiety was substituted with a kinked acyl chain (Lindwasser and Resh, 2002). In the other study, Gag-membrane binding was artificially enhanced by a MA mutation (Guo et al., 2005). According to the model described above, in these cases, Gag-membrane binding might not have been regulated by the equilibrium between the exposure and sequestration of the myristate moiety, and as a result, membrane binding became raft-independent. It would be interesting to analyze effects of cholesterol depletion on membrane binding of retroviral Gag proteins that naturally do not require myristylation for Gag-membrane interaction (e.g., Gag proteins of equine infectious anemia virus and Rous sarcoma virus). In this regard, it is noteworthy that Rous sarcoma virus MA showed no preference for raft-like membranes in *in vitro* liposome binding assays (Dalton et al., 2005).

In summary, the results reported here demonstrate that plasma membrane cholesterol facilitates virus particle production by promoting Gag-membrane binding and higher-order Gag multimerization presumably as a component of liquid-ordered membrane microdomains. To our knowledge, this is the first report showing that membrane cholesterol facilitates the interaction of a myristylated protein with the cytoplasmic leaflet of the plasma membrane. An increasing number of enveloped and non-enveloped viruses are proposed to associate with lipid rafts during assembly (Briggs et al., 2003;Ono and Freed, 2005;Suomalainen, 2002). Analysis of the relationships between cholesterol-enriched lipid rafts and particle production mediated by these viruses will likely elucidate common aspects of membrane microdomains that are exploited by diverse viruses.

Materials and Methods

Plasmids

Molecular clones encoding Gag derivatives, pNL4-3/Fyn(10)fullMA and pNL4-3/Fyn(10) ΔMA/delNC (Ono et al., 2004), or an inactive PR [pNL4-3/PR$^-$, (Huang et al., 1995)] were described previously. A PR$^-$ version of pNL4-3/Fyn(10)fullMA [pNL4-3/Fyn(10)fullMA / PR$^-$] was constructed by introducing the SphI-to-EcoRI fragment (pNL4-3 nt 1443-5743) from pNL4-3/PR$^-$ into pNL4-3/Fyn(10)fullMA. Construction of pCMVNLGagPolRRE using pCMVGagPolRRE [a kind gift from D. Rekosh, University of Virginia (Srinivasakumar et al., 1997)] was described previously (Ono and Freed, 2001). The VSV-G expression vector pHCMV-G (Yee et al., 1994) was generously provided by J. Burns (University of California,

San Diego). An expression plasmid encoding FynGFP (van't Hof and Resh, 1997) was kindly provided by M. Resh (Memorial Sloan-Kettering Cancer Center).

Cells, transfections, and infections

HeLa cells were cultured as previously described (Freed and Martin, 1994). Gag was expressed either by transfecting cells with molecular clones or by infecting with high-titer vector virus stocks. Transfection of HeLa cells was performed by the calcium phosphate method as previously described (Freed and Martin, 1994) or by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Infection of HeLa cells with virus stocks pseudotyped with VSV-G was performed as described previously (Ono et al., 2005). VSV-G-pseudotyped virus stocks were prepared by transfecting HeLa cells with pCMVNLGagPolRRE, pHCMV-G, and the molecular clones pNL4-3/PR$^-$.

Cholesterol depletion, metabolic labeling, and immunoprecipitation

Depletion of cholesterol and metabolic labeling were performed as described previously (Ono and Freed, 2001). Briefly, HeLa cells were cultured in Met$^-$/Cys$^-$ RPMI-1640 supplemented with 2% cholesterol-depleted serum (CDS) in the presence of 10 mM MβCD for 30 min. Subsequently, cells were metabolically labeled with [^{35}S]Met/Cys in Met$^-$/Cys$^-$ RPMI-1640 supplemented with 2% CDS for 2 h for the analyses of virus release efficiency. Preparation of cell lysates, pelleting of virions in the ultracentrifuge, and immunoprecipitation of cell- and virion-associated proteins with HIV-Ig (obtained from the NIH AIDS Research and Reference Reagent Program) was detailed previously (Freed and Martin, 1994). Quantification of immunoprecipitated proteins was performed by phosphorimager analysis. Virus release efficiency was calculated as the amount of virion-associated Gag as a fraction of total (cell plus virion) Gag synthesized during a 2-h labeling period. For membrane binding analyses, MβCD-treated cells were pulse labeled with [^{35}S]Met/Cys in Met$^-$/Cys$^-$ RPMI-1640 supplemented with 2% CDS for 5 min and chased in DMEM containing 5% CDS for 15 min. Alternatively, cells were cultured for 2 days in DMEM containing 5% CDS in the presence of 2 M simvastatin and 500 M mevalonate. After starving in RPMI-Met$^-$/Cys$^-$ medium containing 2 M simvastatin and 500 M mevalonate for 30 min, cells were labeled in the same medium with [^{35}S]Met/Cys for 5 min followed by a 15-min chase period.

Membrane binding analyses and denaturation of Gag

Analyses of Gag-membrane binding were performed as previously described (Ono et al., 2000a;Ono and Freed, 1999;Ono and Freed, 2001;Ono et al., 2005). Denaturation of Gag was performed as described previously (Ono et al., 2005).

Immunofluorescence microscopy

Fixation, permeabilization, and immunostaining of transfected HeLa cells were performed as previously described (Ono et al., 2004;Ono and Freed, 2004;Ono et al., 2000b). Gag proteins were detected using monoclonal anti-p17 antibody or a mixture of monoclonal anti-p17 and anti-p24 antibodies (Advance Biotechnologies). After staining, cells were mounted with Fluoromount G (Electron Microscopy Sciences, Fort Washington, PA) and examined with a Nikon TE2000 microscope equipped with CoolSnap ES CCD camera (Photometrics). Images were deconvolved using Huygens software (Scientific Volume Imaging).

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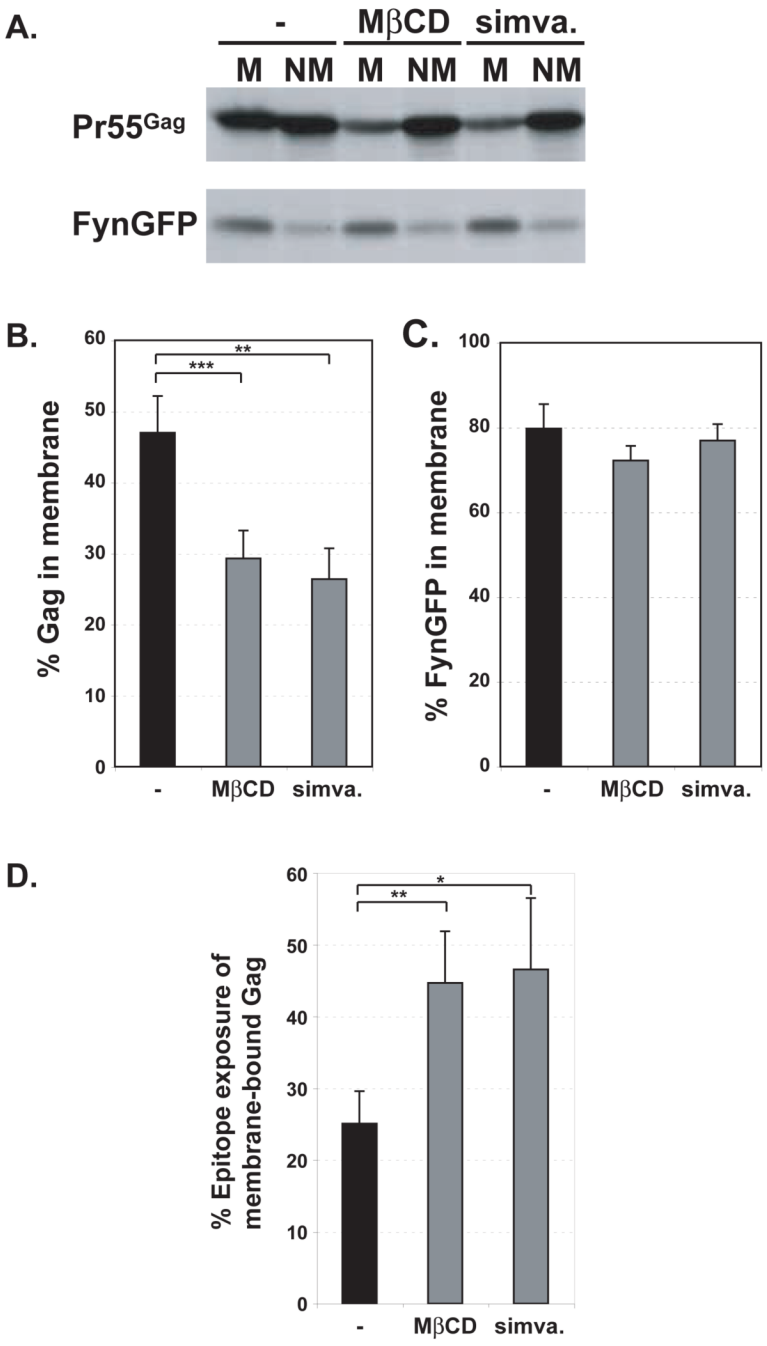

Fig 1.

Depletion of cellular cholesterol disrupts membrane binding and higher-order multimerization of HIV-1 Gag. HeLa cells were either infected with VSV-G-pseudotyped virus transducing NL4-3/PR^{-} or transfected with an expression plasmid encoding FynGFP. Cells were pooled, and cellular cholesterol was depleted using 10 mM MβCD (MβCD) or 2 M simvastatin (Simva.) as described in Materials and Methods. Cells were pulse-labeled with [^{35}S]Met/Cys for 5 min and were chased for 15 min. PNSs of cell homogenates were fractionated by membrane flotation. Fractions were treated with RIPA buffer and membrane (M) and non-membrane (NM) fractions were pooled. Labeled Pr55^{Gag} and FynGFP in each pooled fraction were recovered by immunoprecipitation using HIV-Ig and rabbit anti-GFP antibody, respectively.

Prior to immunoprecipitation with HIV-Ig, the fractions were denatured to expose epitopes masked by Gag multimerization. Immunoprecipitated material was analyzed by SDS-PAGE followed by autoradiography (A) and signal intensity of $Pr55^{Gag}$ (B) and FynGFP (C) was quantified by phosphorimager analysis. Data from six independent experiments are shown as means +/− standard error of the means. P values were determined by the Student's t-test (*** $p < 0.001$; ** $p < 0.01$). In (D), labeled $Pr55^{Gag}$ in pooled membrane fractions was recovered by immunoprecipitation either without or with prior denaturation. Signal intensity was quantified by phosphorimager analysis and epitope exposure of Gag in non-denatured samples was calculated relative to denatured samples. Data from six independent experiments are shown as means +/− standard error of the means. P values were determined by the Student's t-test (** $p < 0.005$, * $p < 0.05$).

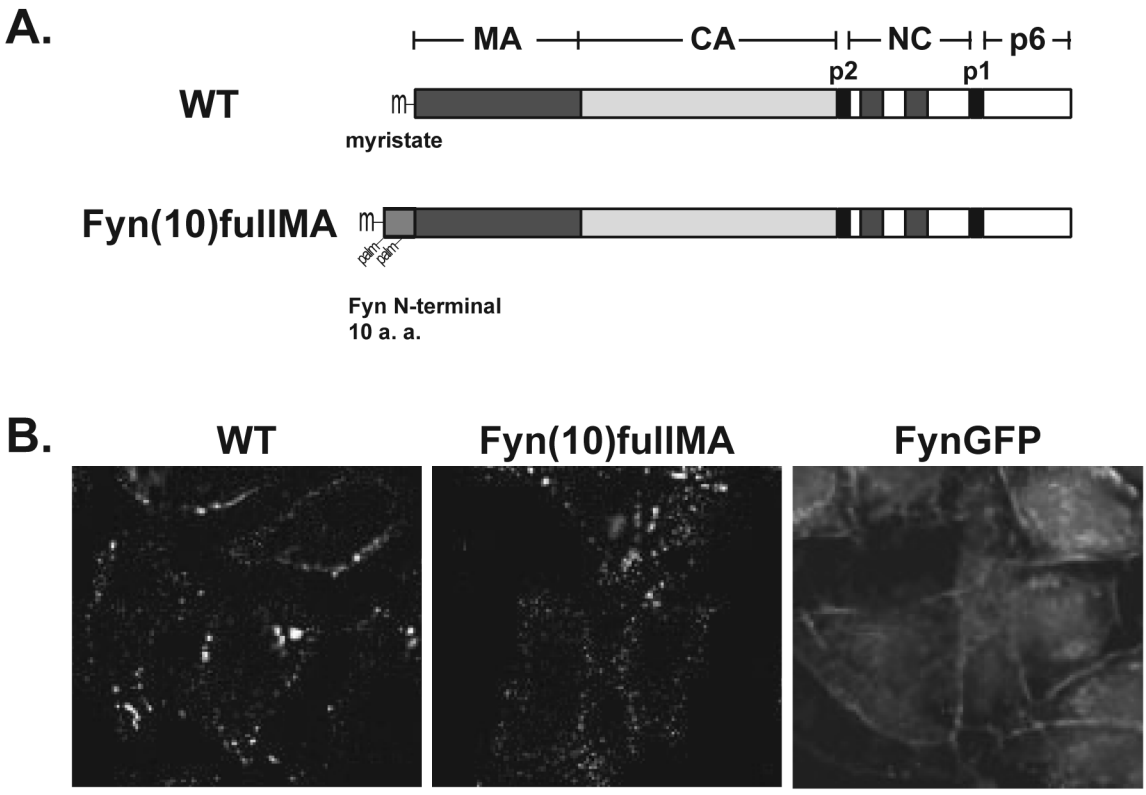

Fig 2.

Addition of the Fyn membrane-binding signal to Gag N terminus does not alter the site of virus assembly. (A) Schematic representation of Gag mutants. Positions of N-terminal myristate (m) and palmitate (palm) moieties are shown. (B) HeLa cells were transfected with either pNL4-3 derivatives encoding WT or Fyn(10)fullMA Gag or a plasmid expressing FynGFP. Gag was detected by immunostaining using an anti-p17 monoclonal antibody and anti-mouse IgG conjugated with AlexaFluor 488. Images were acquired by a Nikon TE2000 microscope equipped with a CCD camera and deconvolved with the Huygens software.

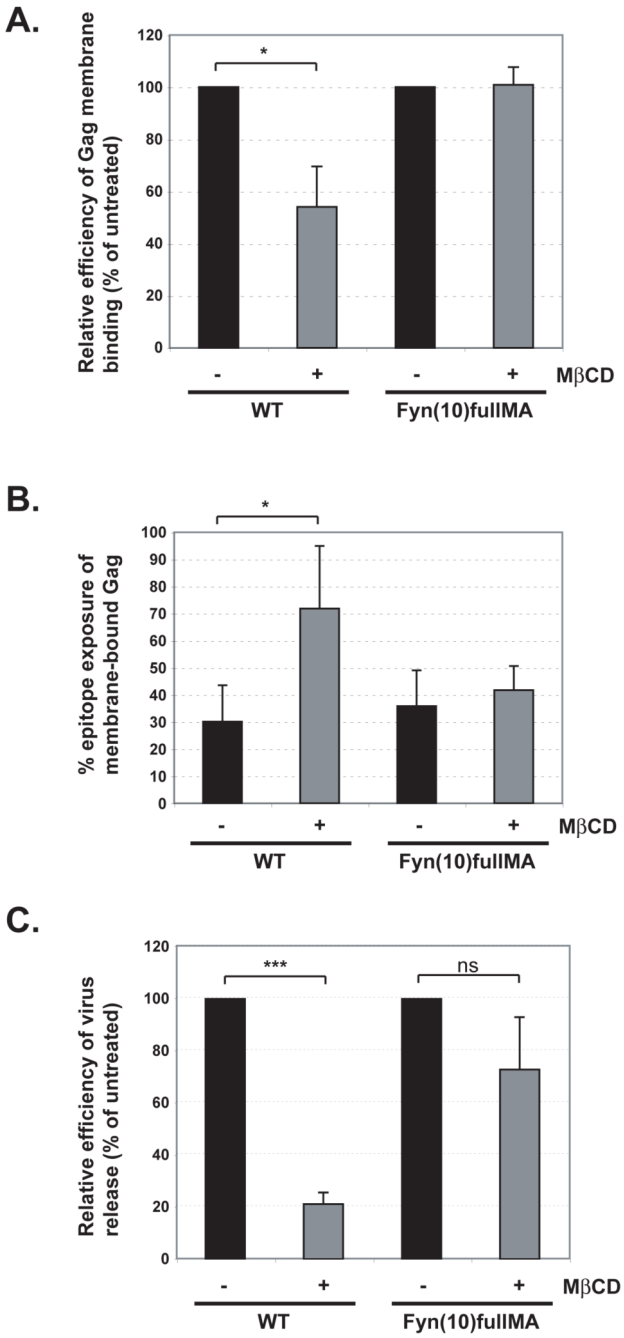

Fig 3.

Defects in Gag membrane binding, higher-order Gag multimerization, and VLP release in cholesterol-depleted cells are restored upon addition of the Fyn membrane-binding signal to the Gag N terminus. HeLa cells were transfected with pNL4-3/PR$^-$ or the pNL4-3/PR$^-$ derivative encoding Fyn(10)fullMA Gag. Cells were treated (+) or not (−) with MβCD and membrane binding (A) and epitope exposure (B) of Gag were analyzed as in Fig. 1. Data from three independent experiments were quantified by phosphorimager analysis and are shown as means +/− standard deviations. P values were determined by the Student's t-test (* $p < 0.05$). In (C), HeLa cells transfected with pNL4-3 or its derivative encoding Fyn(10)fullMA Gag were treated (+) or not (−) with MβCD and labeled metabolically with [^{35}S]Met/Cys for 2 h.

Labeled Gag proteins in cell and virus lysates were immunoprecipitated and quantified by phosphorimager analyses. Virus release efficiency was calculated as described in Materials and Methods and normalized to untreated cultures. P values were determined by the Student's t-test (*** $p < 0.0001$; ns, not significant). The actual average virus release efficiency of untreated cultures expressing WT Gag is 11 % and that of Fyn(10)fullMA Gag is 33%.