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Differential Membrane Binding of the Human Immunodeficiency Virus Type 1 Matrix Protein

WENJUN ZHOU AND MARILYN D. RESH*

Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, Cornell University Graduate School of Medical Sciences, New York, New York 10021

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The human immunodeficiency virus type 1 matrix protein (p17MA) plays a central role at both the early and late stages of the virus life cycle. During viral assembly, the p17MA domain of Pr55^{gag} promotes membrane association, which is essential for the formation of viral particles. When viral infection occurs, the mature p17MA dissociates from the plasma membrane and participates in the nuclear targeting process. Thus, p17MA contains a reversible membrane binding signal to govern its differential subcellular localization and biological functions. We previously identified a membrane binding signal within the amino-terminal 31 amino acids of the matrix domain of human immunodeficiency virus type 1 Gag, consisting of myristate and a highly basic region (W. Zhou, L. J. Parent, J. W. Wills, and M. D. Resh, J. Virol. 68:2556–2569, 1994). Here we show that exposure of this membrane binding signal is regulated in different Gag protein contexts. Within full-length Pr55^{gag}, the membrane targeting signal is exposed and can direct Pr55^{gag} as well as heterologous proteins to the plasma membrane. However, in the context of p17MA alone, this signal is hidden and unable to confer plasma membrane binding. To investigate the molecular mechanism for regulation of membrane binding, a series of deletions within p17MA was generated by sequentially removing α-helical regions defined by the nuclear magnetic resonance structure. Removal of the last α helix (amino acids 97 to 109) of p17MA was associated with enhancement of binding to biological membranes in vitro and in vivo. Liposome binding experiments indicated that the C-terminal region of p17MA exerts a negative effect on the N-terminal MA membrane targeting domain by sequestering the myristate signal. We propose that mature p17MA adopts a conformation different from that of the p17MA domain within Pr55^{gag} and present evidence to support this hypothesis. It is likely that such a conformational change results in an N-terminal myristyl switch which governs differential membrane binding.

Gag proteins, the major structural components of human immunodeficiency virus type 1 (HIV-1), play a central role in viral replication at both the early and late stages of the virus life cycle (43). HIV-1 Gag is initially synthesized as the polyprotein precursors Pr55^{gag} and Pr160$^{gag\text{-}pol}$ (a ribosomal frameshift product of Pr55^{gag}) on free ribosomes in the cytoplasm (43). The 14-carbon fatty acid myristate is cotranslationally attached to the N terminus of HIV-1 Gag proteins (4, 15). Pr55^{gag} is then targeted to the plasma membrane, where viral budding occurs. During budding, the Gag precursor is cleaved into several smaller proteins by a viral protease. The cleavage products of Pr55^{gag} include matrix protein (p17MA), capsid protein (CA), nucleocapsid protein (NC), and a proline-rich protein (p6) (25). In the mature virion, the matrix protein (p17MA) lines the inner face of the viral membrane, while the other Gag cleavage products (CA, NC, and p6) localize in the core and the internal space within the core (12).

Plasma membrane targeting of HIV-1 Gag is an essential step for the viral assembly and budding process. The N-terminal p17MA domain of the Gag precursor plays a major role in this step. Myristylation of the N terminus of the p17MA domain is crucial for plasma membrane association of HIV-1 Gag and assembly of infectious viral particles (4, 13, 15). However, myristate alone is insufficient for membrane association, since myristate is also found attached to cytosolic as well as membrane proteins (39). Recently, we and others established that a basic region within the N terminus of p17MA (amino acids 15 to 31) is also required for membrane association of HIV-1 Gag (27, 46, 47). Myristate promotes hydrophobic interactions with the interior of the membrane lipid bilayer, and the basic region forms a positively charged platform to promote electrostatic interactions with negatively charged phospholipids in the inner leaflet of the membrane (23, 24, 28, 47). Together, myristate and the basic region within the N terminus of p17MA domain form a bipartite membrane targeting signal to direct HIV-1 Gag to the plasma membrane (47).

In addition to its crucial role in the viral assembly process, the mature p17MA protein performs important functions at the early stage of viral infection. In contrast to oncoretroviruses, which depend on the breakdown of the nuclear envelope at mitosis for productive viral infection, lentiviruses, including HIV-1, can infect both dividing and nondividing cells (21). The ability of HIV-1 to infect nondividing cells is attributed to two viral proteins, p17MA and Vpr, which promote active nuclear import of the preintegration complex in a partially redundant manner (5–7, 9, 17, 22, 41). The N-terminal basic region of p17MA closely resembles the nuclear localization signal of simian virus 40 large T antigen and can direct a heterologous protein to the nucleus (6, 18, 26).

Thus, the p17MA domain of Pr55^{gag} and its corresponding cleavage product, p17MA, exhibit different subcellular localizations and functions at different stages of the virus life cycle. While the p17MA domain of Pr55^{gag} promotes the plasma membrane binding during the assembly process, the mature p17MA must dissociate from the plasma membrane in order to participate in the nuclear targeting process during a new round of infection. p17MA must therefore contain a reversible mem-

* Corresponding author. Mailing address: Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., Box 143, New York, NY 10021. Phone: (212) 639-2514. Fax: (212) 717-3317. Electronic mail address: m-resh@ski.mskcc.org.

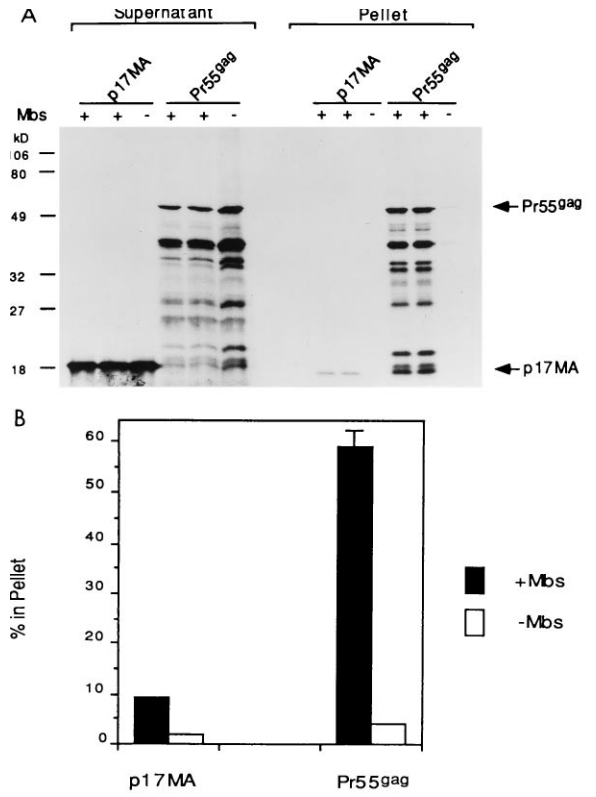

FIG. 1. Membrane association of in vitro-translated p17MA and Pr55^{gag}. In vitro-translated, [^{35}S]methionine-labeled p17MA and Pr55^{gag} were incubated with (+Mbs) or without (−Mbs) plasma membrane-enriched fractions (P100) from COS-1 cells for 30 min at 20°C. Following ultracentrifugation at 100,000 × *g*, the pellet (membrane-bound) and supernatant (unbound) fractions were analyzed by gel electrophoresis, autoradiography, and scintillation counting. (A) Typical autoradiogram. p17MA and Pr55^{gag} products are indicated by the arrows. The lower-molecular-weight bands under Pr55^{gag} are proteolysis products. (B) Graphic representation of the results obtained from duplicate experiments.

brane binding signal whose effects are tightly regulated. In this report, we present evidence that p17MA utilizes a myristyl switch mechanism to govern the accessibility of myristate and membrane binding affinity, thereby regulating Gag subcellular localization and biological functions.

MATERIALS AND METHODS

Antibodies. Mouse anti-β-galactosidase (β-gal) monoclonal antibody was purchased from Promega. Rabbit anti-green fluorescent protein (GFP) polyclonal antiserum was purchased from Clontech Laboratories, Inc. Mouse anti-HIV-1 p17 monoclonal antibody was purchased from Advanced Biotechnologies Inc. Human HIV-1 immunoglobulin (30) was obtained from the NIH AIDS Research and Reference Reagent Program.

Construction of plasmids for use in in vitro transcription and translation. pGEM-p17MA was made by cloning the coding sequences of p17MA from pHXB2.ΔBal.D25S (a kind gift from John W. Wills) (2, 44) into the pGEM3Z vector. pGEM-Pr55^{gag} (a kind gift from John W. Wills) was constructed by cloning the *Sac*I-*Bal*I fragment (nucleotides 680 to 2618) from pHXB2.ΔBal.D25S into pGEM7Z vector. pGEM-Gag-dihydrofolate reductase (DHFR) chimeras were made as previously described (47) (see Fig. 3A).

Construction of plasmids for expression in COS-1 cells. pCMV5-p17MA was constructed by cloning the coding sequences of p17MA from pB17M1234 (a kind gift from George N. Pavlakis, National Cancer Institute, Frederick, Md.) (33) into the COS-1 cell expression vector pCMV5. p65-VALO (a kind gift from Didier Picard), which utilizes the α-1 globulin promoter to express wild-type β-Gal, was used to construct the p65–Gag–β-Gal chimeras. The Gag coding sequences synthesized by PCR were fused in frame onto the N terminus of β-Gal. p65-Gag-GFP chimeras were made by replacing β-Gal with GFP (improved version) coding sequences from pRSET.GFP.S65T (a kind gift from Roger Y. Tsien) (16) with two additional amino acids (Ser and Arg) introduced at the fusion point. All constructs were verified by DNA sequencing.

In vitro membrane binding assays. mRNA synthesized by in vitro transcription of pGEM clones was translated in rabbit reticulocyte lysates as previously described (8) in the presence of [^{35}S]methionine. The P100 membrane fraction was prepared as before (32) except that the membranes were derived from COS-1 cells and subjected to a 10,000 × *g* spin to rid the fraction of mitochondria before the final 100,000 × *g* spin. The binding assay was performed as previously described (31, 35). Briefly, after protein synthesis in reticulocyte lysates, Triton X-100 was added to a final concentration of 0.05%. A 20-μl aliquot of the translation mixture, which had been subjected to a 110,000 × *g* spin for 15 min to remove aggregated protein, was incubated with 30 μl of NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 7.4]) or with 30 μl of a plasma membrane-enriched fraction (P100) in NTE (protein concentration, 0.9 mg/ml) for 30 min at 20°C. Following ultracentrifugation at 100,000 × *g*, the pellet and supernatant fractions were analyzed by gel electrophoresis and autoradiography. The amount of labeled protein in each fraction was quantitated by excising the corresponding bands from the dried gels and analyzing radioactivity by liquid scintillation counting.

Liposome preparation. Liposomes were prepared by a previously described procedure (1, 34), with modifications. Lipids were purchased from Avanti Polar-Lipids, Inc. Phospholipids (phosphatidylcholine) in chloroform were stripped of solvent by rotary evaporation. The dry lipids were then suspended in NTE buffer. Lipid dispersions were sonicated (Heat Systems Sonicator, W-385, standard tapered microtip, output setting at 3) intermittently at 0°C for 4 to 5 min and then cooled for 5 min, for a maximum sonication time of 15 min. Following sonication, the vesicle dispersion was centrifuged for 10 min at 10,000 × *g* to remove any probe particles as well as undispersed phospholipid. The supernatant was then centrifuged for 20 min at 70,000 × *g*. The pellet, which contains large liposomes, was resuspended in NTE buffer.

Liposome binding assay. Proteins were made by in vitro translation as described above. Following translation, the mixture was diluted 1:5 by addition of NTE buffer, and Triton X-100 was added to a final concentration of 0.05%. After a 20-min spin at 180,000 × *g* to remove aggregates, a 100-μl aliquot of the translation mixture was incubated with 100 μl of 1 mM liposomes at room temperature for 20 min. The binding mixture was then centrifuged at 150,000 × *g* for 20 min. The amount of material which pelleted (liposome bound) and stayed in the supernatant (free) was quantitated as described for the in vitro membrane binding assay.

Immunofluorescence. COS-1 cells transfected with expression plasmids were grown on 25-mm-diameter glass coverslips. All subsequent steps were performed

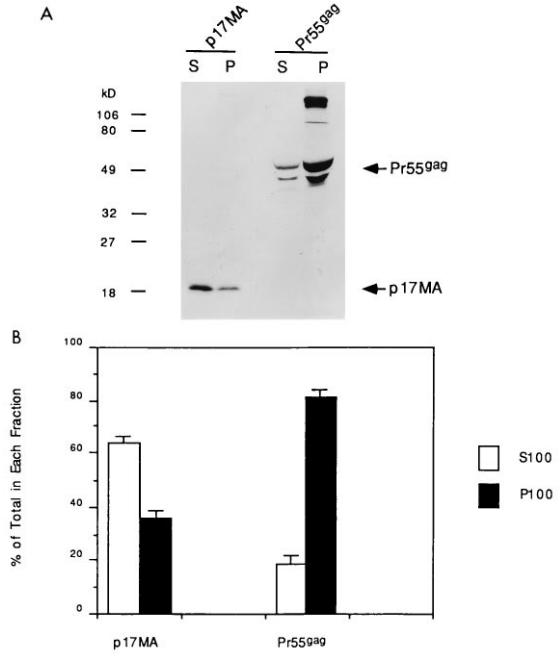

FIG. 2. Subcellular localization of p17MA and Pr55^{gag}. COS-1 cells expressing p17MA or Pr55^{gag} were labeled with [^{35}S]cysteine, lysed, and fractionated by differential centrifugation into P1, P100, and S100 fractions. P100 and S100 fractions were immunoprecipitated with human HIV-1 immunoglobulin and subjected to SDS-PAGE, autoradiography, and scintillation counting. (A) Typical autoradiogram. p17MA and Pr55^{gag} products are indicated by arrows. (B) Graphic representation of the results obtained from duplicate experiments.

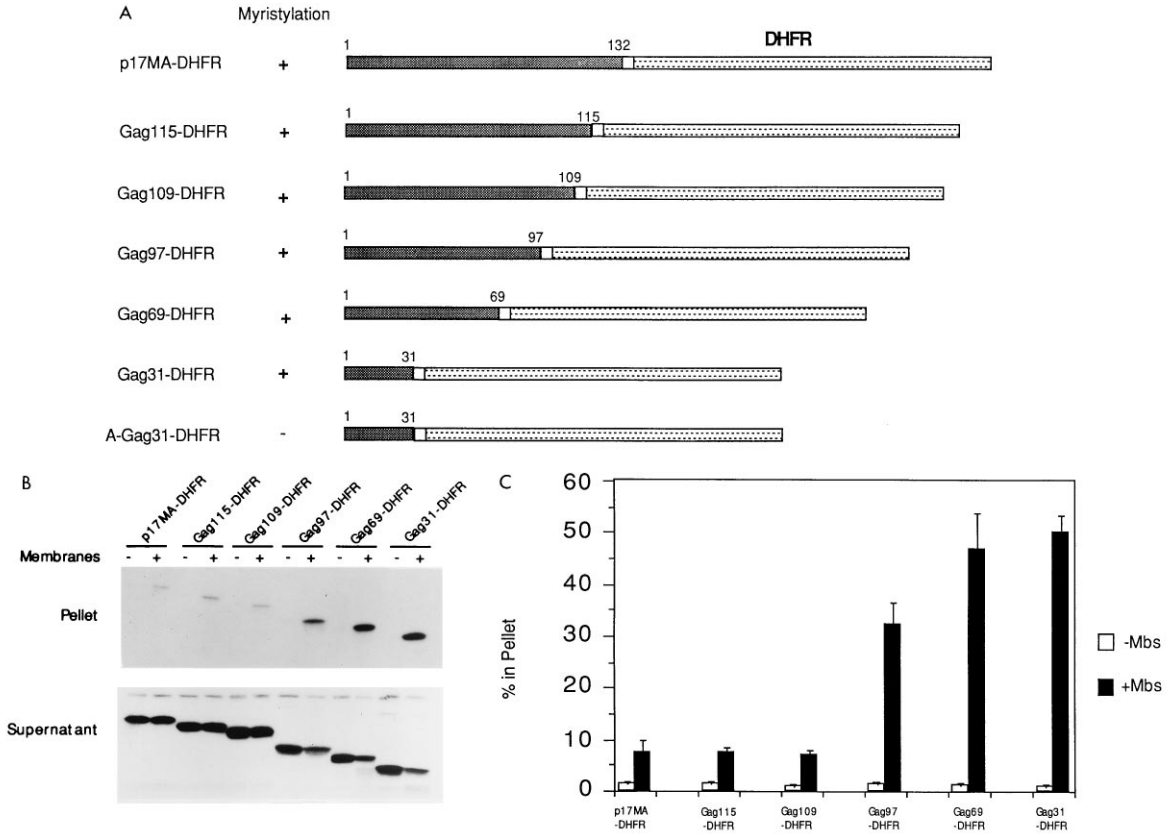

FIG. 3. Membrane association of in vitro-translated Gag-DHFR chimeras. (A) Schematic representation of Gag-DHFR constructs analyzed in this experiment. (B) In vitro-translated, [^{35}S]methionine-labeled Gag-DHFR chimeras were incubated with or without plasma membrane-enriched fractions (P100) from COS-1 cells for 30 min at 20°C. Following ultracentrifugation at 100,000 × *g*, the pellet (membrane-bound) and supernatant (unbound) fractions were analyzed by gel electrophoresis, autoradiography, and scintillation counting. A typical autoradiogram is illustrated. (C) Graph representing the results obtained from three experiments. Mbs, plasma membrane-enriched fractions.

at room temperature (32). The cells were rinsed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS for 15 min. After being washed with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and washed with PBS. Reaction with the primary antibody was in PBS containing 10% calf serum and a 1:2,000 dilution of mouse monoclonal anti-β-Gal antibody (Promega) for 45 min, using 125 μl per coverslip. The coverslips were washed three times with PBS and then incubated with PBS containing 10% calf serum and a 1:75 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim Biochemicals), 300 μl per coverslip, for 30 min. The coverslips were washed three times with PBS, mounted in 90% glycerol–0.1% phenylenediamine in PBS (pH 9.0) on microscope slides (25 by 75 mm), and sealed with nail polish. Cells were observed with a 63× oil immersion objective on a Zeiss Axiophot microscope and photographed with Kodak TMAX 400 film.

Cell fractionation and immunoblotting. The subcellular fractionation of proteins expressed in vivo has been previously described (32). Briefly, cells were lysed by Dounce homogenization in hypotonic buffer and fractionated by differential centrifugation into a 1,000 × *g* pellet (P1), a 100,000 × *g* pellet (P100), and supernatant (S100). Each fraction was adjusted to the same volume and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting as previously described (19, 20). The corresponding bands on the film were quantified with a densitometer (Millipore).

Metabolic labeling and immunoprecipitation. Two days after transfection, COS-1 cells were labeled for 3 h with 100 μCi of L-[^{35}S]cysteine (NEN Research Products) or 100 μCi of [^{3}H]myristate (NEN Research Products) per ml. After the labeling period, cells were washed twice with cold STE buffer (100 mM NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA) and lysed on ice with cold radioimmunoprecipitation assay buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA). After clarification, the lysates were immunoprecipitated and the products were resolved by SDS-PAGE (12.5% gels) and fluorography.

RESULTS

Differential membrane binding of p17MA and Pr55^{gag}. We recently identified a membrane binding motif within the N-terminal MA domain of HIV-1 Gag. This motif, consisting of the first 31 amino acids of Gag, can direct heterologous proteins to the membrane. We next wished to assess the function of the membrane binding signal within the context of authentic HIV-1 Gag molecules. p17MA and Pr55^{gag} were produced by in vitro transcription and translation in rabbit reticulocyte lysates. Membrane binding activity was assayed and quantitated by incubating the radiolabeled proteins with plasma membrane-enriched fractions from COS-1 cells. Although both proteins were efficiently expressed (Fig. 1A) and N myristylated (not shown), their membrane binding properties differed dramatically. Nearly 60% of the total Pr55^{gag} became bound to membranes, whereas less than 10% of p17MA was membrane bound under the in vitro assay conditions (Fig. 1). Since the N-terminal sequences of Pr55^{gag} and p17MA are identical, these data imply that the membrane binding signal functions well in the context of Pr55^{gag} and poorly in the context of p17MA.

To test whether the membrane binding measured in vitro reflects the behavior of the Gag proteins in vivo, COS-1 cells expressing p17MA and Pr55^{gag} were subjected to subcellular fractionation (Fig. 2). Pr55^{gag} was mostly localized to the membrane fraction (81% was associated with the particulate fraction), while the majority of p17MA (64%) was present in the cytosolic fraction. A similar cytosolic distribution for p17MA has been observed by others (36). Taken together, the results from both the in vitro and in vivo membrane binding studies indicate that p17MA exhibits a weaker interaction with the

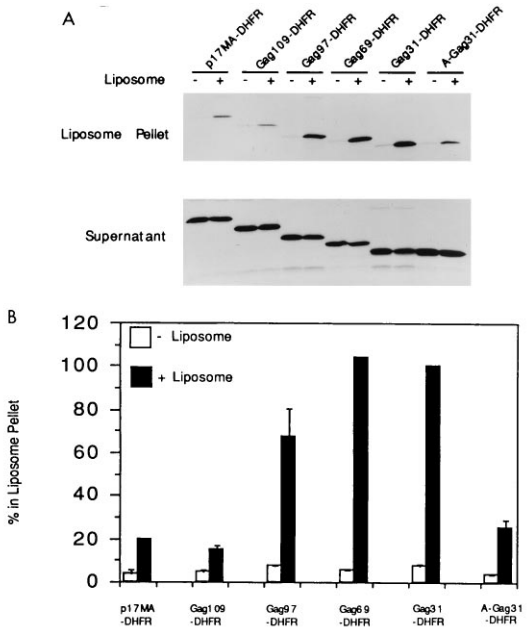

FIG. 4. Liposome binding of Gag-DHFR chimeras. In vitro-translated, radiolabeled Gag-DHFR chimeras depicted in Fig. 3A were incubated with large liposomes containing the neutral lipid phosphatidylcholine (see Materials and Methods). After ultracentrifugation, the amount of material in the pellet (liposome-bound protein) and supernatant (free protein) fractions was quantitated as described for Fig. 3. (A) Autoradiogram from a typical experiment. To facilitate visualization for liposome-bound proteins, four times more pellet fractions than supernatant fractions were loaded on the gel. (B) Graphic representation of results obtained from two independent experiments. The amount of Gag31-DHFR bound to liposomes was normalized to 100%.

membrane than does Pr55^{gag}. It is therefore possible that the Gag membrane binding signal is somehow altered in the context of p17MA.

The last α helix of p17MA exerts a negative effect on the N-terminal membrane binding signal. To elucidate the molecular basis for the decreased membrane binding of p17MA, a series of deletion mutants within p17MA was constructed by sequentially removing structural fragments on the basis of the nuclear magnetic resonance (NMR) structure (23, 24). DHFR, a small cytosolic protein, was fused to the C terminus of each construct as a carrier (47) (Fig. 3A). The membrane binding affinity of each construct was then assessed in vitro. As depicted in Fig. 3B and C, p17MA-DHFR behaved similarly to p17MA and exhibited very low membrane binding. Partial or complete removal of the C-terminal tail of p17MA (Gag115-DHFR and Gag109-DHFR) did not affect the extent of membrane binding. However, after removal of the last α helix (amino acids 97 to 109), the membrane binding affinity increased over threefold (Gag97-DHFR). Further removal of C-terminal sequences (Gag69-DHFR and Gag31-DHFR) resulted in only a slight increase in membrane binding levels. All of the constructs were N myristylated to equivalent extents (data not shown). These results demonstrate that within the context of p17MA, the last α helix exerts a negative effect on its membrane binding, perhaps by altering the exposure of the N-terminal membrane binding signal. The negative effect can be eliminated by removing the last α helix.

Sequestering of the N-terminal myristate by the last α helix of p17MA may be responsible for the loss of membrane binding. The N-terminal membrane binding signal of HIV-1 Gag consists of two components, myristate and a cluster of basic amino acids. Both the hydrophobic interaction contributed by myristate and the electrostatic interactions provided by the basic domain are required for efficient membrane binding. In the NMR solution structure of p17MA, the basic domain forms an amphipathic β sheet exposed on the surface of the protein. The disposition of the myristate moiety is not known, as only the structure of nonmyristylated p17MA is available and the extreme N terminus of p17MA is disordered in the published NMR structures (23, 24). To account for the reduced membrane binding capacity of p17MA, we hypothesized that myristate is sequestered within the molecule and therefore not available to participate in membrane binding. To assess the accessibility of the myristate moiety, binding of Gag-DHFR constructs to liposomes containing the neutral phospholipid phosphatidylcholine was monitored. Under these conditions, only hydrophobic interactions contribute to the binding energy. As depicted in Fig. 4, p17MA bound poorly to phosphatidylcholine liposomes, indicating that the myristyl group of p17 was not accessible for hydrophobic insertion into the lipid bilayer. Removal of the last α helix significantly increased the binding to liposomes (Gag97-DHFR). Further deletions led to only moderate increases in liposome binding (Gag69-DHFR and Gag31-DHFR). This pattern is nearly identical to that observed for binding to biological membranes (Fig. 3 and 4). To confirm that the myristate moiety was mainly responsible for the observed liposome binding, a nonmyristylated Gag protein (A-Gag31-DHFR) was constructed. Liposome binding was fourfold less than for the equivalent myristylated Gag31-DHFR protein (Fig. 4). Taken together, these results strongly suggest that the reduced membrane binding of p17MA is due to sequestration of the myristyl group, either directly or indirectly by the last α helix of p17MA.

The last α helix of p17MA exerts a negative effect on membrane binding in vivo. To verify that the membrane binding results obtained in vitro reflect the behavior of p17MA dele-

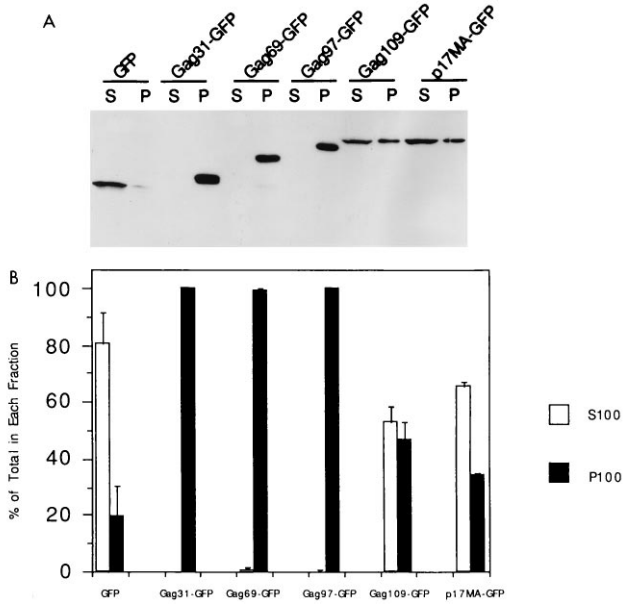

FIG. 5. Subcellular localization of Gag-GFP chimeras. COS-1 cells expressing wild-type GFP and Gag-GFP chimeras were lysed and fractionated by differential centrifugation into P1, P100, and S100 fractions. S100 and P100 fractions were subjected to SDS-PAGE, immunoblotting (with anti-GFP polyclonal antibodies), and densitometry quantitation. Each bar represents the average of two independent experiments.

tion mutants in vivo, the coding sequences of various p17MA constructs were fused to the N terminus of GFP (16), with two additional amino acids (Ser and Arg) introduced at the fusion point (analogous to the construction of Gag-DHFR chimeras depict in Fig. 3A). The chimeric proteins were expressed in COS-1 cells, and the subcellular localization of Gag-GFP proteins was determined by subcellular fractionation studies (Fig. 5).

Wild-type GFP was present mainly in the cytosolic fraction. The addition of the first 31, 69, and 97 amino acids of p17MA (Gag31-GFP, Gag69-GFP, and Gag97-GFP) directed GFP chimeras exclusively to the membrane fractions. However, constructs containing the last α helix of p17MA (Gag109-GFP and p17-GFP) were distributed in both membrane and cytosolic fractions. These results indicate that the presence of the last α helix of p17MA decreases its membrane binding affinity in vivo.

To further support the conclusions obtained with Gag-GFP chimeras, a second non-membrane-bound protein, β-Gal, was chosen as a carrier. Gag–β-Gal chimeras were constructed by fusing the coding sequences of p17MA mutants to the N terminus of β-Gal (analogous to the construction of Gag-DHFR chimeras depicted in Fig. 3A). All chimeras were efficiently expressed in COS-1 cells. To verify that the Gag chimeras were modified by myristate, COS-1 cells were metabolically labeled with [^{3}H]myristate. After immunoprecipitation with a monoclonal antibody against β-Gal, the proteins were analyzed by SDS-PAGE and fluorography (Fig. 6A). Except for the parent β-Gal, all chimeras were myristylated to equivalent extents.

The subcellular localization of each protein was evaluated by subcellular fractionation studies (Fig. 6B and C) and indirect immunofluorescence microscopy (Fig. 7). As expected, wild-type β-Gal was mostly localized to the soluble fraction (Fig. 6B and C) and exhibited diffuse cytoplasmic staining (Fig. 7A), confirming that β-Gal is a cytosolic, non-membrane-bound protein. Gag31–β-Gal, Gag69–β-Gal, and Gag97–β-Gal, which have the N-terminal membrane binding signal but not the last α helix of p17MA, all exhibited association with the membrane fractions (Fig. 6B and C). These results were generally consistent with those obtained for Gag-GFP and Gag-DHFR constructs. The decreased extent of membrane binding for Gag31–β-Gal compared with Gag31-GFP and Gag31-DHFR may be due to the extremely large size of the β-Gal carrier protein and its ability to form a tetramer. However, immunofluorescence microscopy revealed that Gag31–, Gag69–, and Gag97–β-Gal proteins all exhibited distinct plasma membrane staining (Fig. 7B to D).

In contrast, Gag109–β-Gal and p17MA–β-Gal, which have the N-terminal membrane binding signal as well as the last α helix, exhibited reduced association with the membrane fractions compared with Gag97 and diffuse cytoplasmic staining similar to that of wild-type β-Gal (Fig. 7A, E, and F). Limited staining was seen on the edge of the cells expressing Gag109–β-Gal (Fig. 7E). Staining in the perinuclear region of the cell, most likely representing binding of the overexpressed constructs to intracellular membranes, was also observed.

p17 epitopes are exposed differently in p17MA compared with Pr55^{gag}. We next investigated why the membrane binding signal is altered within the context of p17MA but maintained functional within the context of Pr55^{gag}. We speculated that the p17MA protein adopts a new conformation, different from that of the p17MA domain within Pr55^{gag}. Such a conformational change could alter the N-terminal membrane binding signal and result in loss of membrane binding for p17MA. To investigate this possibility, the ability of a mouse monoclonal anti-p17MA antibody to immunoprecipitate p17MA and

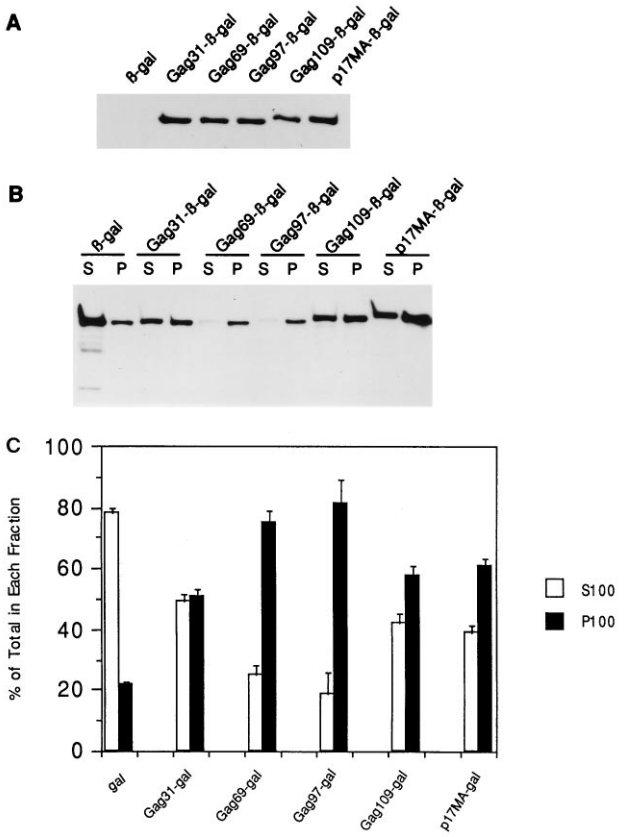

FIG. 6. (A) Myristylation of Gag–β-Gal chimeras were labeled with [^{3}H]myristate. Following cell lysis, proteins were immunoprecipitated by an anti-β-Gal monoclonal antibody and analyzed by SDS-PAGE and fluorography. Differences in molecular weight among the various Gag constructs were not readily apparent because of the large size of the β-Gal carrier protein (116 kDa). (B and C) Subcellular localization of Gag–β-Gal chimeras. COS-1 cells expressing wild-type β-Gal or Gag–β-Gal chimeras were lysed and fractionated by differential centrifugation into P1, a P100, and S100 fractions. S100 and P100 fractions were subjected to SDS-PAGE, immunoblotting (with anti-β-Gal monoclonal antibodies), and densitometry quantitation. Each bar represents the average of two independent experiments.

Pr55^{gag} was assessed. As shown in Fig. 8, under nondenaturing conditions, the anti-p17MA antibody immunoprecipitated p17MA but not Pr55^{gag}, even though both proteins contain the same coding sequences for p17MA. As a control, human HIV-1 immunoglobulin (30) immunoprecipitated both proteins under the same conditions. These results strongly suggest that the epitope recognized by this anti-p17MA monoclonal antibody is exposed in the context of p17MA but not in the context of Pr55^{gag}, indicating that p17MA has a conformation different from that of its counterpart in the context of Pr55^{gag}.

DISCUSSION

We have previously identified a membrane binding signal within the p17MA domain of Pr55^{gag} (47). In the present study, we show that this membrane binding signal functions distinctly in different Gag contexts and confers differential membrane binding to p17MA and the p17MA domain of Pr55^{gag}. We also provide evidence that a myristyl switch mechanism is likely accountable for the differential membrane binding.

The last α helix of p17MA regulates membrane binding. Pr55^{gag} and its cleavage product, p17MA, possess the same N-terminal sequence. Because this sequence functions as a mem-

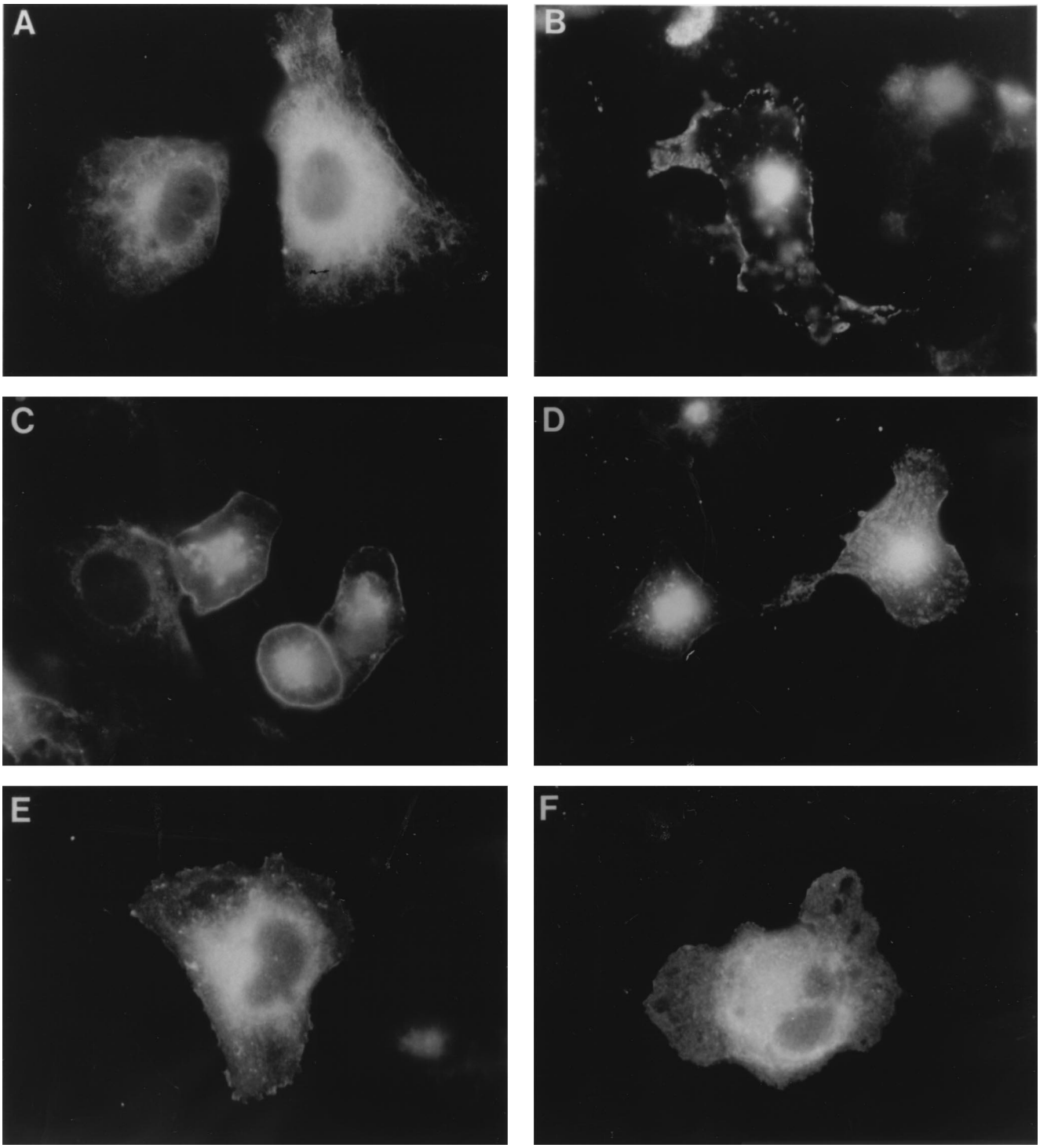

FIG. 7. Subcellular localization of Gag–β-Gal chimeras by immunocytochemistry. β-Gal and Gag–β-Gal chimeras expressed in COS-1 cells were detected by indirect immunofluorescence microscopy with anti-β-Gal monoclonal antibodies. (A) β-Gal; (B) Gag31–β-Gal; (C) Gag69–β-Gal; (D) Gag97–β-Gal; (E) Gag109–β-Gal; (F) p17MA–β-Gal.

brane binding signal in Pr55^{gag}, one would predict that p17MA would also be mostly membrane bound. Instead, we observed that p17MA does not bind to the membrane as well as Pr55^{gag}, as illustrated in Fig. 1 and 2. Using deletion mutants of p17MA, we found that the presence of the last α helix of p17MA is responsible for the reduced membrane binding, both in vitro and in vivo (Fig. 3, 5, 6, and 7). The recently published NMR structures of p17MA reveal that the last α helix is separated from the globular core domain of the protein and projects into solution. It is therefore possible that the C-terminal helix can "flip" and alter the exposure of the N-terminal membrane binding signal.

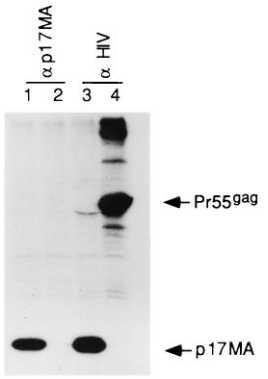

FIG. 8. Immunoprecipitation of p17MA and Pr55^{gag}. After labeling with [^{35}S]cysteine, COS-1 cells expressing p17MA (lanes 1 and 3) or Pr55^{gag} (lanes 2 and 4) were lysed and immunoprecipitated with a mouse anti-p17MA monoclonal antibody (Advanced Biotechnologies; see Materials and Methods) (lanes 1 and 2), or human HIV-1 immunoglobulin (lanes 3 and 4). The samples were then subjected to SDS-PAGE and fluorography.

The myristate moiety is sequestered in the context of p17MA. The data presented in Fig. 4 indicate that the presence of the last α helix of p17MA reduces liposome binding, supporting the notion that the myristate moiety is no longer accessible for hydrophobic interaction with the lipid bilayer. We propose that cleavage of Pr55^{gag} triggers a myristyl switch which sequesters myristate within the MA molecule. This myristyl switch mechanism has already been reported for at least two other proteins, recoverin and ADP-ribosylation factor. In each case, the accessibility of the myristate moiety is regulated, resulting in reversible membrane binding (3, 37).

At present, it is not clear how the last α helix of p17MA affects the disposition of the N-terminal myristate. This region of the protein may directly interact with the N terminus, thereby trapping myristate inside the protein. Alternatively, the last α helix may control the accessibility of myristate by indirectly influencing the N-terminal conformation. Because the published three-dimensional structures of p17MA were determined for nonmyristylated protein and do not reveal the location of the N-terminal myristate, we cannot distinguish between these two possibilities.

Recently, it has been demonstrated that the presence of myristate is the key to controlling subcellular localization of HIV-1 Gag. Myristate acts as a dominant membrane targeting signal, preventing the adjacent nuclear localization signal from functioning in virus-producing cells (10). In the absence of myristate, the nuclear localization signal is revealed, directing a nonmyristylated mutant p17MA to the nucleus (10). Our study provides the first evidence that the accessibility of myristate is regulated in wild-type HIV-1 Gag. We therefore propose a model in which p17MA plays a dual role at different stages of the virus life cycle (Fig. 9). In the context of Pr55^{gag}, both myristate and the basic domain are exposed and together form a dominant plasma membrane targeting signal. This allows Pr55^{gag} to be directed to the plasma membrane during viral assembly. After viral budding and cleavage, mature p17MA adopts a new conformation, in which myristate is sequestered and membrane binding is disrupted. During a new round of viral infection, p17MA dissociates from the membrane, allowing the N-terminal basic sequence to serve as a nuclear targeting signal.

Cleavage of Pr55^{gag} triggers a conformational change within p17MA. The data depicted in Fig. 8 show that a mouse anti-p17MA monoclonal antibody can immunoprecipitate p17MA but not Pr55^{gag}, indicating that the epitope recognized by this antibody is exposed in p17MA but not in Pr55^{gag}. Although it is not known where the epitope is located, the data suggest a conformational difference between p17MA and the p17MA domain in the context of Pr55^{gag}. Such a conformational change may result in alteration of the N-terminal membrane

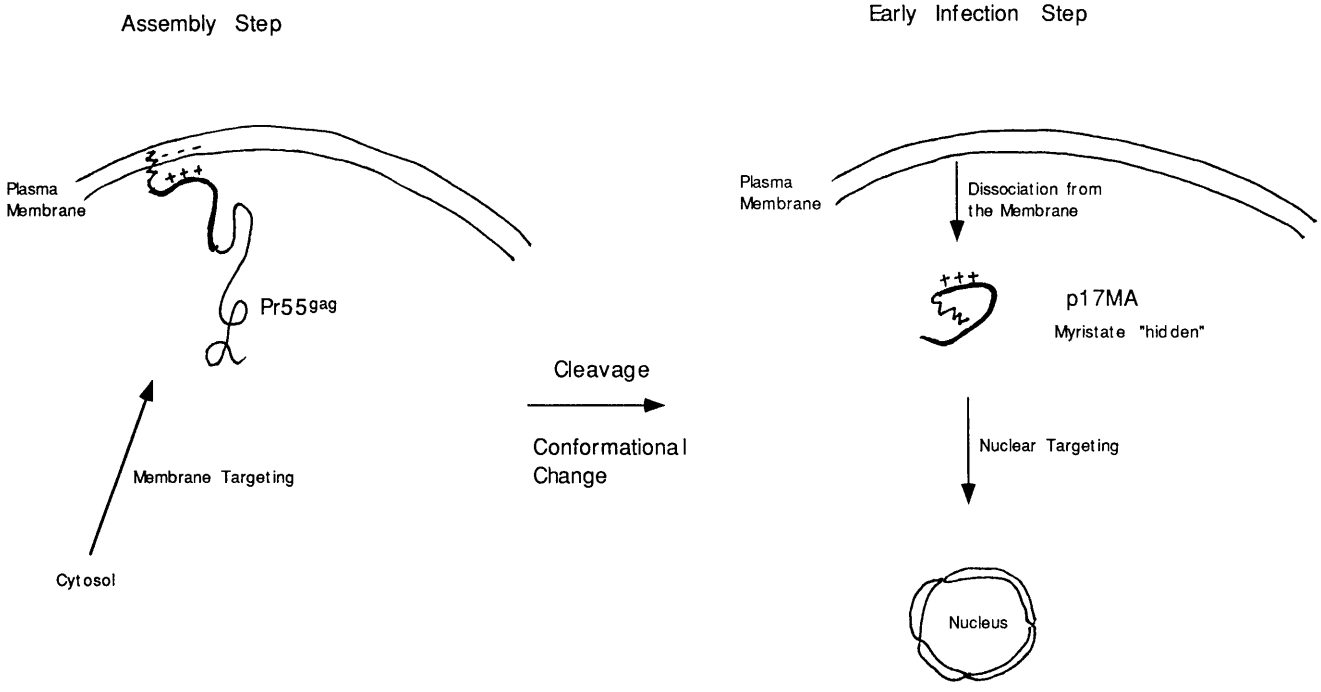

FIG. 9. Schematic representation of our model for differential membrane binding of HIV-1 Gag MA.

binding signal, as proposed in our model (Fig. 9). Alternatively, it is possible that this monoclonal antibody recognizes the C-terminal end of p17, in which case the cleavage of $Pr55^{gag}$, rather than a conformational change, would account for the differential antibody reactivity. Recently, others (40) have inferred from cryoelectron microscopy studies that the structure of the p17MA domain could be different in immature particles than in mature particles, suggesting that a conformation change occurs upon proteolytic maturation. Ultimately, a three-dimensional crystal structure of $Pr55^{gag}$ will be required to determine the MA domain conformation.

Effect of downstream sequences of $Pr55^{gag}$ on membrane binding. Other studies have suggested that downstream sequences of $Pr55^{gag}$ are required for particle formation (14) and membrane binding (29). However, our data indicate that the N-terminal membrane binding signal (amino acids 1 to 31) can independently direct DHFR, GFP, and β-Gal to the membrane in the absence of any downstream sequences of $Pr55^{gag}$. It is likely that the distal sequences of $Pr55^{gag}$ do not directly mediate membrane binding but rather contribute to maintaining the N-terminal Gag amino acids in a membrane binding-competent conformation.

It is also important to consider the cooperativity of Gag interactions. The NC regions of $Pr55^{gag}$ interact with each other (via RNA) to form large oligomeric complexes. Gag-Gag interactions mediated by NC as well as CA may also account for the higher membrane binding affinity of $Pr55^{gag}$ than of p17MA.

The N-terminal Gag sequence is a plasma membrane targeting signal. The eukaryotic cell contains multiple intracellular membranes as well as the plasma membrane. Gag molecules, however, are targeted exclusively to the plasma membrane. Our gain-of-function approach using chimeric Gag proteins illustrates that the N-terminal membrane binding domain of HIV-1 Gag is also a plasma membrane targeting motif. Immunofluorescence studies with Gag31–, Gag69–, and Gag97–β-Gal chimeras revealed distinct plasma membrane staining at the surface of the cell. (Similar studies with Gag-GFP chimeras were difficult to evaluate because of a high background of nuclear fluorescence from the parent GFP molecule). The plasma membrane targeting ability of the N-terminal Gag sequence most likely promotes viral assembly at the plasma membrane.

Effect of phosphorylation on the membrane binding of the HIV-1 Gag. Recently, the effects of phosphorylation on subcellular localization of the HIV-1 Gag have been studied by other groups. One study showed that phorbol myristate acetate-stimulated phosphorylation at Ser-111 of p17MA by protein kinase C increased membrane binding of p17MA (45). A second study demonstrated that C-terminal tyrosine phosphorylation of p17MA enables p17MA to translocate to the nucleus by stimulating binding of p17MA to the integrase (10, 11).

Binding of several other myristylated proteins, such as MARCKS and $pp60^{c\text{-}src}$, to membranes is regulated by phosphorylation (38, 42). It is conceivable that by introducing negative charges in p17MA, phosphorylation would disrupt electrostatic interactions between the N-terminal basic domain of p17MA and the inner layer of the plasma membrane. However, the phosphorylation sites, Ser-111 and Tyr-132, are located far away from the N-terminal basic domain in the three-dimensional structure (23, 24). Phosphorylation at these sites is therefore unlikely to directly affect the electrostatic interactions of the N-terminal region. Moreover, no difference in the membrane binding of a p17-DHFR construct containing a Ser-111→Ala mutation compared with wild-type p17-DHFR was observed (48).

Instead, phosphorylation may regulate subcellular localization of HIV-1 Gag via other mechanisms. As suggested in one study (45), phosphorylation may influence the conformation of HIV Gag, thereby indirectly governing the N-terminal membrane binding signal. This theory is compatible with our model in which a conformational change is involved in regulating membrane binding affinity. At present, no data are available to assess whether phosphorylation results in such a conformational change.

An alternative mechanism by which phosphorylation could exert an effect has recently been described. Gallay et al. (10, 11) have shown that C-terminal tyrosine phosphorylation of p17MA facilitates nuclear targeting by stimulating phosphorylation-mediated binding to integrase, triggering the redistribution of p17MA from the membrane to the inner region of the virus. Such a redistribution process may occur only after the membrane associations of p17MA become weak, a condition resulting from the myristyl switch proposed in our study. Tyrosine phosphorylation may thus exert an additive effect to the myristyl switch-mediated membrane dissociation of p17MA, with both events being critical for subsequent nuclear targeting.

In conclusion, regulation of subcellular localization of HIV-1 Gag is a complex process, and multiple factors are likely involved. Our work suggests that a myristyl switch mechanism is likely to be partially accountable for this process. Further structural studies are needed to correlate the effects of conformational change, myristyl accessibility, phosphorylation, and cooperativity on the targeting of HIV-1 Gag proteins.

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