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Identification of a Membrane-Binding Domain within the Amino-Terminal Region of Human Immunodeficiency Virus Type 1 Gag Protein Which Interacts with Acidic Phospholipids

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Retroviral Gag proteins are targeted to the plasma membrane, where they play the central role in virion formation. Several studies have suggested that the membrane-binding signal is contained within the amino-terminal matrix sequence; however, the precise location has never been determined for the Gag protein of any retrovirus. In this report, we show that the first 31 residues of human immunodeficiency virus type 1 Gag protein can function independently as a membrane-targeting domain when fused to heterologous proteins. A bipartite membrane-targeting motif was identified, consisting of the myristylated N-terminal 14 amino acids and a highly basic region that binds acidic phospholipids. Replacement of the N-terminal membrane-targeting domain of pp60$^{v\text{-}src}$ with that of human immunodeficiency virus type 1 Gag elicits efficient membrane binding and a transforming phenotype. Removal of myristate or the basic region results in decreased membrane binding of Gag-Src chimeras in vitro and impaired virion formation by Pr55^{gag} in vivo. We propose that the N-terminal Gag sequence functions as a targeting signal to direct interaction with acidic phospholipids on the cytoplasmic leaflet of the plasma membrane.

The Gag proteins of retroviruses play the central role in virion assembly and budding. Most Gag proteins are initially synthesized as myristylated polyprotein precursors, which are targeted to the inner face of the plasma membrane where they can direct particle formation, even in the absence of other viral proteins (3 and reviewed in reference 58). For the type C and lentivirus retroviruses, virion assembly is first morphologically evident at the plasma membrane where electron-dense patches of Gag molecules can be visualized by electron microscopy (13). As the buds emerge from the cell surface, the Gag precursor is cleaved by the virus-encoded protease. For human immunodeficiency virus type 1 (HIV-1) Gag, the primary cleavage products include the p17 matrix protein (MA), the p24 capsid (CA) protein, the p7 nucleocapsid protein (NC), and a small peptide termed p6 (27), which are linked in this order in the polyprotein precursor. The p17 MA protein is ultimately localized to the inner surface of the virion membrane, while CA, NC, and p6 are found in the core or internal region of the virus (12).

Membrane association of Gag proteins is essential for the formation of infectious virions, yet the precise mechanism of membrane targeting for HIV-1 or any other retroviral Gag protein remains unknown. Targeting information is believed to reside in the N-terminal MA sequence. The N terminus of Pr55^{gag} (and p17 MA) is myristylated, and myristylation is required for efficient membrane association and virion formation (4, 14, 15). Moreover, the Gag proteins of many other retroviruses are myristylated (45), and inhibition of myristylation has been shown to result in diminished release of infectious virions (4, 19, 36, 41, 46). However, myristylation alone is not sufficient to target Gag proteins (60), or any other proteins, to the membrane (38, 55), and additional signals from within the Gag protein are apparently involved.

In many respects the membrane targeting of Gag proteins appears to be remarkably similar to that of pp60$^{v\text{-}src}$, the transforming protein of Rous sarcoma virus (RSV). Both proteins are initially synthesized on membrane-free ribosomes in soluble form, both are myristylated, and both associate posttranslationally with the inner face of the plasma membrane (38, 58). In addition, the N-terminal Src sequence can complement MA deletion mutants of RSV Gag which are defective in virion assembly (60). It is therefore likely that Src and Gag proteins utilize similar membrane-targeting mechanisms. The membrane-targeting domain of pp60$^{v\text{-}src}$ has been mapped to its myristylated N-terminal region and consists of two motifs: N-terminal myristate plus a cluster of basic amino acids (three lysine residues) (50). The myristate moiety provides hydrophobic interaction with the lipid bilayer (32), while the basic residues strengthen the interaction by forming electrostatic contacts with acidic membrane phospholipids (49).

The presence of acidic phospholipids confers a net negative charge to the inner surface of the plasma membrane. Electrostatic interactions between basic domains and acidic membrane phospholipids have been demonstrated for a growing number of proteins, including vinculin, phospholipase C, myristylated alanine-rich protein kinase C substrate, and protein kinase C (25, 28, 35, 54). Interestingly, HIV-1 Gag also contains a region near its N terminus, which is highly enriched in positively charged amino acids.

In this study, we have investigated the membrane-targeting ability of specific sequence motifs within the HIV-1 Gag N-terminal region. Previous studies have approached the problem of membrane targeting by mutating or deleting regions of the Gag molecule and measuring loss of membrane binding and particle formation (10, 61, 62). Unfortunately, the interpretation of negative results generated by loss-of-function mutants such as these is limited. For example, the failure to

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bud could be due to global defects in folding rather than loss of a single function. We, therefore, have chosen to employ a gain-of-function strategy to ask whether components of the N-terminal sequence of HIV Gag can direct heterologous proteins to the plasma membrane.

Here we demonstrate that the N-terminal region of HIV-1 Gag contains a bipartite membrane-targeting signal, consisting of the myristylated N-terminal 14 amino acids and an additional region of 17 amino acids highly enriched in basic residues. This membrane-targeting sequence can fully substitute for that of Src in conferring membrane binding and a transforming phenotype. Gag sequences can also confer membrane binding to an otherwise soluble heterologous protein, dihydrofolate reductase (DHFR). Moreover, we present evidence that the N-terminal region of HIV-1 Gag interacts with acidic membrane phospholipids.

MATERIALS AND METHODS

Plasmid construction. The plasmid pGEMv-src (9) was used to construct the Src chimeras. pGEMv-src was digested with *Nco*I and *Ngo*MI, resulting in the deletion of 42 nucleotides which encode the first 14 amino acids of v-Src. Oligonucleotides encoding the N-terminal amino acids of HIV-1 Gag (57) and the mutants and the complementary noncoding strands were synthesized, hybridized, and inserted at the site of the pGEMv-src deletion. All mutations were confirmed by DNA sequencing. pGEM315-src was derived from the plasmid pSR-XD315 (8) (a kind gift from H. Hanafusa, Rockefeller University). The plasmids pLJ-v-src and pLJ-src chimeras were prepared by excising v-Src and chimeric Src from pGEM clones by digestion with *Acc*I and *Eco*RI and inserting the fragment made blunt with Klenow polymerase into the retroviral vector pLJ (a kind gift from J. Schwarzbauer, Princeton University), which was linearized by digestion with *Sal*I and similarly made blunt ended. pGEMsrc-DHFR was constructed by replacing the *Ngo*MI-*Bgl*II fragment of pGEMv-src, which includes sequences encoding amino acids 15 to 526 of v-Src, with the entire mouse DHFR (a kind gift from J. Bertino, Sloan-Kettering Institute) coding sequences synthesized by PCR. Two amino acids were introduced at the fusion point. pGEMgag-DHFR chimeras were constructed by replacing the *Nco*I-*Ngo*MI fragment of pGEMsrc-DHFR, which encodes the first 14 amino acids of v-Src, with sequences encoding N-terminal amino acids of HIV Gag as described for construction of pGEMgag-src chimeras.

Construction of mutants in HIV-1 Gag. The wild-type HIV-1 proviral genome, which contains a simian virus 40 origin of replication, was obtained from the infectious clone pHXB2Dgpt (a kind gift from Eric Hunter, University of Alabama at Birmingham). To construct a noninfectious derivative, pΔBalI, the *Bal*I-*Bal*I (nucleotides 2618 to 4550) fragment was deleted, removing most of the *pol* gene but leaving the Gag- and protease-coding regions intact. The D25S mutation, which destroys the active site of the protease, was made by oligonucleotide-directed mutagenesis as previously described (2, 60). The 8N and Δ20-39 mutants (see Fig. 9A) were made in the same manner with the following oligonucleotides, respectively: 5′-GGGGGAGAATTAGATAACTGGGAAAA TATTAACTTAAATCCCGGGGAAACAATAACTATAAT TTAAAACATATAGTATGG-3′ and 5′-GATCGATGGGA AAAAATTGAGCTAGAACGATTC-3′. The mutations were confirmed in two independent clones by digestion with appropriate restriction enzymes (creation of a *Sma*I site in 8N and loss of an *Xmn*I site in Δ20-39) and by dideoxy DNA sequencing. The *Bss*HII-*Spe*I fragment (nucleotides 710 to 1506) containing each mutation was transferred from the recombinant M13 replicative form DNA into pΔBalI and pΔBalI.D25S, which had been digested with *BSS*HII and *Spe*I and agarose gel purified. The presence of both mutations was again confirmed in duplicate clones by restriction mapping. The Myr$^-$ pGAG plasmid (a kind gift of Casey Morrow [31]) has a glycine-to-alanine mutation at position 2 (confirmed by DNA sequencing), preventing myristylation, plus a large *pol* deletion, resulting in nonfunctional protease, reverse transcriptase, and integrase.

Cells, transfection, and soft-agar colony assay. NIH 3T3 cells were maintained in Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories) supplemented with 10% calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. COS-1 cells were maintained in DMEM supplemented with 3% fetal bovine serum and 7% bovine calf serum (Hyclone, Inc.). Transfection and G418 selection of NIH 3T3 cells was done as reported previously (7). Transient transfections of COS-1 cells were carried out by the DEAE-dextran-chloroquine method as previously described (59), with 1.5 μg of DNA per transfection. A soft-agar colony assay was performed as described previously (18).

In vitro membrane-binding assays. mRNA synthesized by in vitro transcription of pGEM clones was translated in rabbit reticulocyte lysates, as previously described (9), in the presence of [^{35}S]methionine. The P100 membrane fraction was prepared as described previously (40), except that the membranes were derived from NIH 3T3 cells and subjected to centrifugation at 10,000 × *g* to rid the fraction of mitochondria before the final spin at 100,000 × *g*. The binding assay was performed as previously described (37, 50). Briefly, after protein synthesis in reticulocyte lysates, Triton X-100 was added to a final concentration of 0.075%. A 20-μl aliquot of the translation mixture, which had been subjected to centrifugation at 110,000 × *g* 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 buffer (protein concentration, 0.5 μg/μl) 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 proteins in each fraction was quantitated by excising the corresponding bands from the dried gels and analyzing radioactivity by liquid scintillation counting.

Liposome competition assay. Liposomes were prepared according to a previously described procedure (1), with modifications. Lipids were purchased from Avanti Polar-Lipids, Inc. Individual phospholipids (phosphatidylcholine) or mixtures of phospholipids (phosphatidylcholine and phosphatidylserine at a 2:1 molar ratio) in chloroform together with a trace amount of [^{3}H]phosphatidylcholine (New England Nuclear Corp.) were stripped of solvent by rotary evaporation. The dry lipids were then suspended in NTE buffer, and 20 μl of the lipid dispersion was taken for scintillation counting. Lipid dispersions were sonicated (Braun-Sonic 2000U sonicator) intermittently at 0°C for 4 to 5 min and then cooled for a 5-min period for a maximum sonication time of 30 min. Following sonication, the vesicle dispersion was centrifuged for 1 h at 110,000 × *g* to remove large multilamellar liposomes. The upper half of the supernatant, which was translucent and contained small unilamellar liposomes, was transferred to a new tube.

In vitro membrane binding was performed as described above, except Triton X-100 was added to the in vitro translation mixture to a final concentration of 0.09%. After a 1-h spin at 130,000 × *g* to remove aggregates, a 20-μl aliquot of the

translation mixture was incubated with 15 μg of P100 membranes from NIH 3T3 cells in 40 μl of NTE buffer in the presence of increasing amounts of liposomes for 30 min at 20°C. The total assay volume was kept constant at 60 μl. The binding mixture was then overlaid onto a 15% (wt/wt) sucrose (in NTE) cushion, which prevents liposome sedimentation, and centrifuged at 100,000 × *g* for 1 h. The amount of material which was pelleted (membrane bound) or stayed in the supernatant (liposome bound plus free) was quantitated as described for the in vitro membrane-binding assay.

Immunofluorescence. G418-resistant cells were grown on glass coverslips (25 by 25 mm). All subsequent steps were performed at room temperature. The cells were rinsed with phosphate-buffered saline (PBS) and then 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 then washed with PBS. Reaction with the primary antibody was carried out in PBS containing 10% calf serum and a 1:200 dilution of polyclonal anti-Src antibody (40) for 45 min, by 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-rabbit 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 microscopy 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 by using Kodak TMAX 400 film.

Cell fractionation and immunoblotting. The subcellular fractionation of proteins expressed in vivo has been previously described (40). 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 the supernatant (S100). Each fraction was adjusted to the same volume and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as previously described (24). The corresponding bands on the film were quantified by using a densitometer (Millipore).

Metabolic labeling and immunoprecipitation. COS-1 cells were labeled 52 h after transfection with L-[^{35}S]methionine (1,220 Ci/mmol) or [^{3}H]leucine (60 Ci/mmol; NEN Research Products). For pulse-labeling, cells were starved in methionine-free or leucine-free DMEM for 30 min and then labeled with 0.15 mCi of [^{35}S]methionine per ml or 0.38 mCi of [^{3}H]leucine per ml for 20 min. Duplicate plates were washed with methionine-free DMEM and then incubated with 0.06 mCi of [^{35}S]methionine per ml for a total of 18 h. After 2 h of labeling, 1/10th volume of serum-free DMEM was added to prevent starvation. After the labeling period, the growth medium was removed from each culture dish, centrifuged to remove loose cells and cellular debris, and mixed with lysis buffer containing protease inhibitors (59). Cells were lysed as previously described (59), and immunoprecipitation of lysates and media was performed with human HIV-1 immune globulin (34) (National Institutes of Health AIDS Research and Reference Reagent Program). The lysate and medium samples were resolved by SDS–12% PAGE and fluorography (59).

RESULTS

Construction of chimeras and mutants. In order to map the membrane-targeting information within the HIV Gag protein, a series of chimeric proteins were constructed by fusing N-terminal Gag sequences onto a heterologous protein. The transforming protein of RSV, pp60$^{v\text{-}src}$, was chosen as a carrier because membrane targeting of this polypeptide is primarily dependent on its myristylated N-terminal sequence. The first 14 amino acids of pp60$^{v\text{-}src}$ have been shown to constitute a membrane-targeting domain and confer membrane binding when fused to heterologous proteins (33). Removal of this sequence from pp60$^{v\text{-}src}$ abrogates myristylation and membrane association, both in vitro and in vivo (8, 37), and completely inhibits the transforming activity of the v-Src oncoprotein (20). We first assessed the ability of N-terminal Gag sequences to restore membrane-binding capacity to Src.

N-terminal Gag sequences confer membrane binding to Gag-Src chimeras. We began our analysis by constructing chimeric Src proteins in which the first 14 amino acids of v-Src were replaced with 14 N-terminal amino acids from HIV-1 Gag (Gag14-Src) (Fig. 1A). Wild-type pp60$^{v\text{-}src}$ and the chimeric Gag-Src protein were synthesized by in vitro transcription from cloned cDNAs, followed by translation of the corresponding mRNA in a reticulocyte lysate. Both proteins were synthesized to similar levels, as judged by incorporation of [^{35}S]methionine (Fig. 2A), and were efficiently myristylated (data not shown). For a control, we also constructed a point mutant (A-Gag) in which the glycine residue, which is the acceptor for myristate, was changed to alanine. The Gly-to-Ala mutation prevents recognition by *N*-myristyl transferase and thereby blocks myristylation of all known myristylated proteins, including HIV-1 Gag (4, 15). An additional construct which lacked N-terminal amino acids 2 to 15 of v-Src (315-Src) was utilized (8). As expected, neither A-Gag14-Src nor 315-Src was myristylated (data not shown).

Membrane binding was evaluated by incubating the radiolabeled proteins in the presence or absence of plasma membrane-enriched fractions from NIH 3T3 cells. Upon ultracentrifugation, membrane-associated material sediments with the pellet fraction. As depicted in Fig. 2, removal of N-terminal amino acids 2 to 15 from pp60$^{v\text{-}src}$ resulted in an 80% decrease of membrane binding in vitro (315-Src). Addition of the N-terminal 14 amino acids from HIV-1 Gag restored membrane binding to approximately 60% of the level achieved with Src sequences (Gag14-Src). The importance of the myristate moiety was revealed by the failure of the nonmyristylated mutant (A-Gag14-Src) to bind to membranes.

Previous studies have demonstrated that the first 10 amino acids of Src can replace the membrane-binding domain of RSV Gag (60). This region of Src contains three basic amino acids at positions 5, 7, and 9 which are critical for membrane binding (50). In contrast, the first 10 amino acids of HIV Gag can suppress most, but not all, RSV Gag deletions (2), suggesting that additional sequences within Gag might contain important membrane-targeting information. Further examination of the HIV-1 Gag sequence revealed a prominent cluster of basic residues between amino acids 15 to 31. We next tested whether this basic region contributed to the membrane-binding properties of HIV-1 Gag. Addition of the N-terminal 31 amino acids from HIV-1 Gag (Gag31-Src) (Fig. 1A) resulted in a two- to threefold increase in membrane binding over that of Gag14-Src and exceeded levels obtained with wild-type v-Src (Fig. 2). To assess the role of the basic residues, the 8N-Gag31-Src mutant in which eight positively charged amino acids between positions 15 and 31 of HIV-1 Gag were replaced with asparagine residues was constructed. The membrane-binding capacity of 8N-Gag31-Src was reduced to approximately that of Gag14-Src (Fig. 2). We therefore conclude that the N-terminal region of Gag contains at least two important elements for

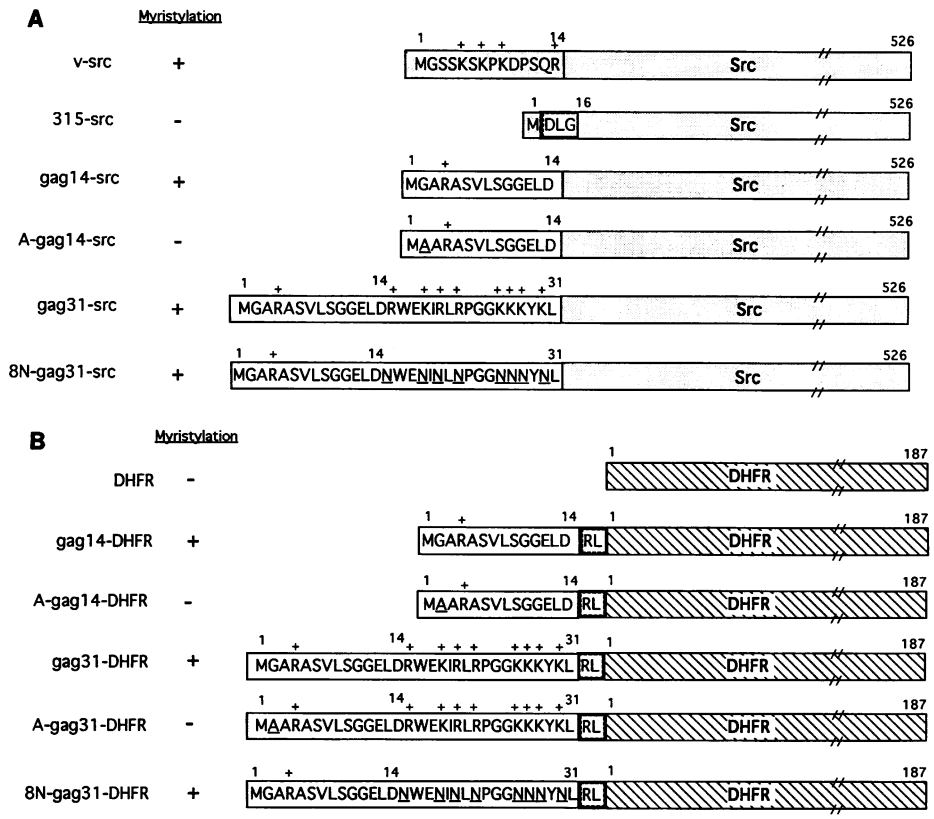


FIG. 1. Schematic representation of constructs. (A) Chimeric Src constructs. v-Src, the transforming protein of RSV pp60$^{v\text{-}src}$; 315-Src, a nonmyristylated variant of v-Src in which amino acids 2 to 15 were deleted and three other amino acids were introduced by linker insertion; Gag14-Src, the first 14 amino acids of v-Src were replaced with 14 N-terminal amino acids from HIV-1 Gag; A-Gag14-Src, Gly at position 2 of Gag14-Src was mutated to Ala; Gag31-Src, the first 14 amino acids of v-Src were replaced by 31 N-terminal amino acids of HIV-1 Gag; 8N-Gag31-Src, eight positively charged amino acids within HIV-1 Gag sequence of Gag31-Src were replaced with Asn. (B) Chimeric DHFR constructs. DHFR (mouse); Gag14-DHFR, the first 14 amino acids of HIV-1 Gag were fused to the entire sequence of DHFR, with two additional residues introduced at the fusion point; A-Gag14-DHFR, Gly at position 2 of Gag14-DHFR was mutated to Ala; Gag31-DHFR, the first 31 amino acids of HIV-1 Gag were fused to the entire sequence of DHFR, with two additional residues introduced at the fusion point; A-Gag31-DHFR, Gly at position 2 of Gag31-DHFR was mutated to Ala; 8N-Gag31-DHFR, eight positively charged amino acids between positions 15 and 31 of Gag31-DHFR were replaced by Asn. The numbers on the top of each box indicate the amino acid positions in the original proteins. +, positive charges. The mutated amino acids are underlined.

membrane binding: the myristylated N-terminal 14 amino acids and a basic domain.

The N terminus of HIV-1 Gag is sufficient to direct cytosolic DHFR to membranes in vitro. Association of v-Src with cellular membranes is complex and involves multiple regions of v-Src, which function in conjunction with the N-terminal sequences (21, 39). Although the results for the Gag-Src membrane-binding assay clearly demonstrate that the N-terminal region of HIV-1 Gag can substitute for that of v-Src in conferring membrane association, it was not clear whether this region is sufficient to promote membrane association.

To further support the conclusions obtained with Gag-Src chimeras, a non-membrane-bound protein was chosen as a carrier. DHFR is present in the cytosol of rodent cells and has been used as a carrier to identify several mitochondrial targeting sequences (17). As depicted in Fig. 1B, Gag-DHFR chimeras were constructed by fusing a series of N-terminal amino acid sequences of HIV-1 Gag with the entire coding sequence of DHFR, with two amino acids introduced at the fusion point. The ability of Gag-DHFR chimeras to bind to membranes was then assessed. As expected, wild-type DHFR behaved as a soluble protein, remaining in the supernatant of the in vitro membrane-binding assay (Fig. 3). Addition of the N-terminal 31 amino acids of HIV-1 Gag targeted the chimeric DHFR to membranes (Gag31-DHFR). The cluster of basic amino acids between 15 and 31 was one important component of this membrane-targeting signal, since either elimination of amino acids 15 to 31 (Gag14-DHFR) or replacement of eight basic amino acids within this sequence (8N-Gag31-DHFR) decreased membrane binding by approximately fourfold. Mutation of the myristylation site significantly reduced the membrane-binding capacity of both Gag14-DHFR and Gag31-DHFR (A-Gag14-DHFR and A-Gag31-DHFR). Thus, the Gag-Src and Gag-DHFR results concur and identify a membrane-targeting domain within the HIV Gag N-terminal region.

The basic domain within the N-terminal region of HIV-1 Gag confers binding to acidic phospholipids. We next tested whether the cluster of basic amino acids between amino acids 15 and 31 of HIV-1 Gag promoted specific interaction with acidic membrane phospholipids. Increasing amounts of liposomes of defined phospholipid composition were added to the binding assays, and the amount of Gag-DHFR protein bound to the liposomes (which float under the conditions of the assay)

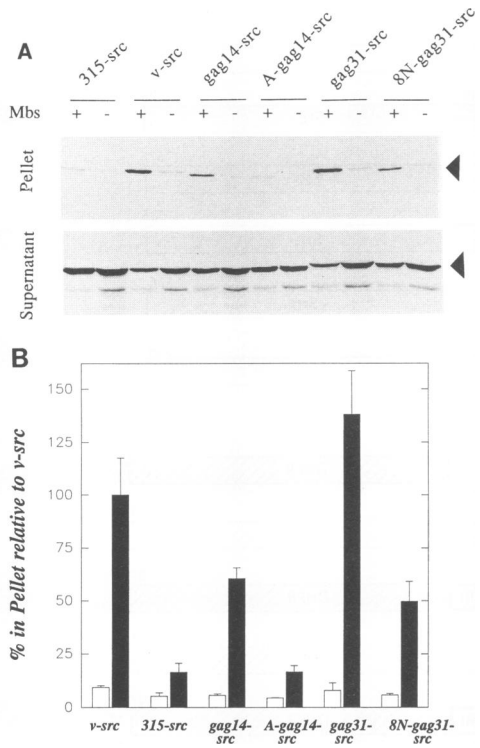


FIG. 2. Membrane association of in vitro-translated Gag-Src chimeras. In vitro-translated, [^{35}S]methionine-labeled v-Src and Gag-Src chimeras were incubated with (+ Mbs) (solid bars) or without (− Mbs) (open bars) plasma membrane enriched fractions (P100) from NIH 3T3 cells for 30 min at 20°C. Following 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. pp60$^{v\text{-}src}$ and chimeric Src products are indicated by the arrowheads. No other radiolabeled bands were evident on the autoradiogram. (B) Graphic representation of the results obtained from four independent experiments. Membrane binding of chimeric Src molecules is represented relative to that of pp60$^{v\text{-}src}$, which has been normalized to 100%.

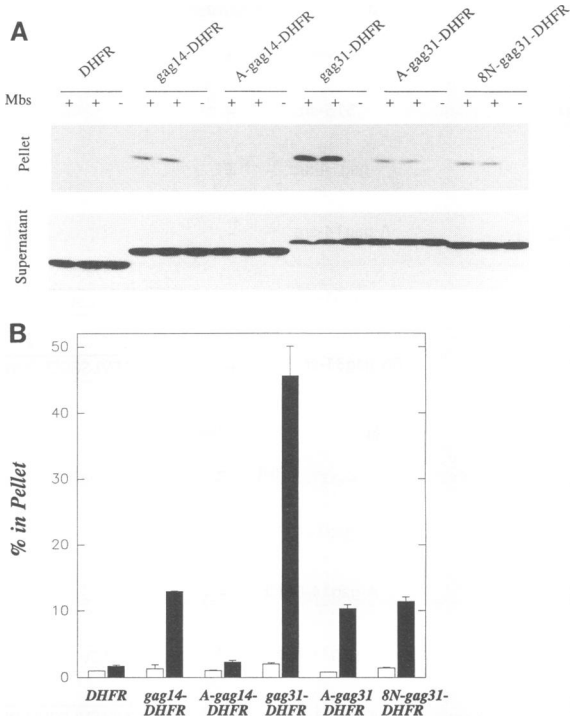


FIG. 3. Membrane association of in vitro-translated Gag-DHFR chimeras. In vitro-translated, radiolabeled DHFR and Gag-DHFR chimeras were subjected to in vitro membrane-binding assays as described in the legend to Fig. 2. (A) Typical autoradiogram. Mobility differences reflect the presence of different numbers and compositions of HIV-1 Gag N-terminal amino acids in the chimeras. The molecular masses range from 22 to 25 kDa. (B) Graphic representation of results obtained from three independent experiments. Symbols are defined in the legend to Fig. 2.

or to membranes (which sediment) was quantitated (Fig. 4). The addition of liposomes composed solely of neutral phospholipid (phosphatidylcholine) had little effect on the binding of either Gag31-DHFR or 8N-Gag31-DHFR to membranes. In contrast, liposomes containing the physiological 2:1 ratio of neutral to acidic phospholipid competed for membrane association of Gag31-DHFR, with no observable effect on 8N-Gag31-DHFR. As the amount of acidic liposomes was increased, nearly 80% of the Gag31-DHFR protein partitioned with the vesicles. These results suggest that the N-terminal polybasic domain of HIV-1 Gag promotes membrane association through interaction between positively charged amino acids and acidic membrane phospholipids.

The N terminus of HIV-1 Gag confers plasma membrane binding to Gag-Src in vivo. To test whether the membrane-binding assay results obtained in vitro reflected the behavior of the chimeric proteins in vivo, v-*src* and *gag-src* were introduced into NIH 3T3 cells by retrovirus-mediated gene transfer, utilizing resistance to the aminoglycoside, G418, as the selectable marker (7). The subcellular localization of Gag-Src proteins was revealed by indirect immunofluorescence microscopy and subcellular fractionation studies (Fig. 5 and 6).

As previously reported (8), 315-Src exhibited diffuse cytoplasmic staining and a predominant presence in the S100 (soluble) fraction, confirming that 315-Src is a cytosolic, nonmembrane-bound protein. A similar staining pattern was observed for nonmyristylated A-Gag31-Src (data not shown). In contrast, v-Src, Gag14-Src, Gag31-Src, and 8N-Gag31-Src all exhibited a characteristic punctate membrane staining. For pp60$^{v\text{-}src}$, this pattern has been shown to be indicative of localization to adhesion plaques, the sites of contact between the plasma membrane and actin stress fibers (43). We also performed subcellular fractionation studies which showed that, compared with v-Src (60%), significant amounts of Gag14-Src (44%), Gag31-Src (47%), and 8N-Gag31-Src (37%) were present in P100 fractions enriched in the plasma membrane. However, discrete differences in subcellular distribution existed among the different chimeras. As shown in Fig. 5, more cytoplasmic staining for Gag14-Src and 8N-Gag31-Src was observed than for Gag31-Src and v-Src. These observations are consistent with the subcellular fractionation results. Larger percentages of Gag14-Src (25%) and 8N-Gag31-Src (39%) than of Gag31-Src (9.8%) and v-Src (3%) were found in the S100 fractions, indicating that the presence of the basic domain increases the degree of membrane binding.

The N terminus of HIV-1 Gag confers transforming activity to Gag-Src in vivo. Association of v-Src with plasma membranes is required for cellular transformation. Mutant v-Src proteins that fail to associate with the plasma membrane are unable to induce transformation despite the fact that they retain normal kinase activity (8, 20). Use of v-Src as a carrier

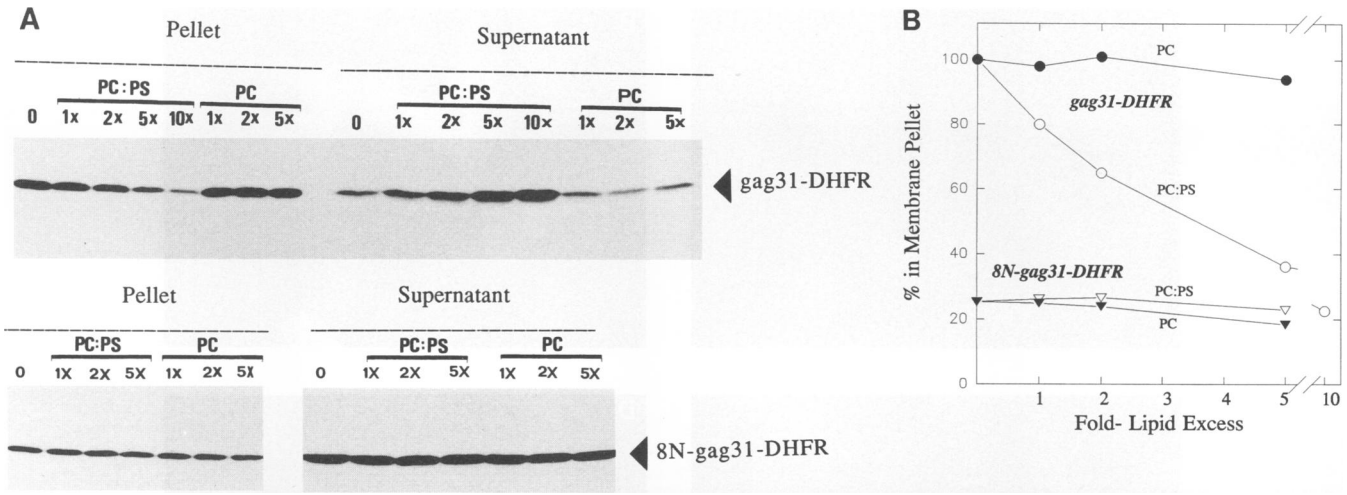


FIG. 4. Effects of liposomes containing neutral or acidic phospholipids on Gag31-DHFR and 8N-Gag31-DHFR membrane binding. In vitro-translated, radiolabeled Gag31-DHFR and 8N-Gag31-DHFR were incubated with P100 membrane fractions derived from NIH 3T3 cells in the presence of increasing amounts of small unilamellar liposomes containing either a 2:1 ratio of phosphatidylcholine to phosphatidylserine (PC:PS) or phosphatidylcholine (PC) alone (see Materials and Methods). Liposomes were added in multiples of the weight of the membrane (P100) used in the experiment. The amount of material in the pellet (membrane bound) and supernatant (liposome-bound plus free) fractions was quantitated as described in the legend to Fig. 2. (A) Autoradiogram from a typical experiment. To facilitate visualization for 8N-Gag31-DHFR, the amount of pellet fractions loaded onto the gel was twice that of supernatant fractions. (B) Graphic representation of results obtained from two independent experiments. Gag31-DHFR bound to membranes in the absence of liposomes was normalized to 100%.

to identify plasma membrane-targeting signals is therefore advantageous, in that one can test the ability of defined sequences to restore transforming activity to a truncated version of v-Src. All constructs tested retained tyrosine kinase activity (data not shown). As shown in Fig. 7 and 8, cells expressing the 315-Src mutant maintained a normal, flat phenotype and failed to grow on soft agar. Likewise, the nonmyristylated A-Gag31-Src-expressing cells were not transformed, on the basis of their flat morphology and lack of growth in soft agar (data not shown). In contrast, v-Src-expressing cells became fully transformed, with a characteristic phase-bright morphology, and were capable of growth in soft agar. As judged by these same two criteria, all three Gag-Src chimeras (Gag14, Gag31, and 8N-Gag31) (Fig. 7D, E and F, and 8D, E, and F, respectively) were able to cause cellular transformation, implying that these chimeras were capable of interacting with v-Src target proteins at the plasma membrane. However, it is important to consider that only small amounts of Src oncoprotein are required for cellular transformation (18) and that the use of overexpressed proteins allows even the more weakly membrane-bound chimeras to transform cells in vivo. We conclude that the myristylated first 14 amino acids of HIV-1 Gag contain membrane-targeting information. These amino acids together with positively charged amino acids between amino acids 15 and 31, which enhance association with the plasma membrane, form a plasma membrane-targeting signal.

Alterations of the N-terminal basic region of HIV-1 Gag decrease budding in vivo. The experiments described above clearly suggest that the N-terminal basic region is important for membrane binding. If so, mutations in this region should reduce or eliminate budding. Two HIV-1 Gag mutants were constructed to address this question (Fig. 9A). In the 8N mutant, all positively charged residues between amino acids 14 and 31 have been replaced with asparagines in a manner identical to that of the 8N-Gag31-Src chimera. In the Δ20-39 mutant, a large deletion removes a slightly different set of eight basic residues. Both constructs were also made with a protease mutation (D25S) so that the synthesis and release of the polypeptide precursor, Pr55, could be compared directly. A previously reported, nonmyristylated form of HIV-1 Gag (Myr^- pGAG) (31) was included to show the phenotype of a Gag protein which is not efficiently released in particles.

The mutants and wild-type HIV-1 Gag (pΔBalI) constructs were transiently expressed in duplicate plates of COS-1 cells. Pulse-labeling of one set of plates revealed that all constructs expressed similar levels of Gag proteins (Fig. 9B, lanes 2 to 8, and C, lanes 2 to 7). After 18 h of labeling of the other set, the wild-type Gag protein was readily detected in the medium along with cleavage products p41 and p24/25 (Fig. 9B, lane 19, and C, lane 16). The D25S protease mutant also was released efficiently but was not processed (Fig. 9B, lane 22, and C, lane 19). As expected, the nonmyristylated Gag protein was released into the medium at very low levels, illustrating the requirement for this modification (Fig. 9B, lane 18 compared with lane 22). In duplicate experiments, Myr^- pGAG was released at 14.9% (± 8.1%, standard deviation) of the wild-type level.

Extended labeling revealed much less Gag protein in the medium for the 8N and Δ20-39 mutants than for the wild-type Gag (Fig. 9B, lanes 19 to 24, and C, lanes 16 to 21). This result is most readily seen by comparing the D25S versions of the 8N and parental (wild-type) constructs (Fig. 9B, lanes 22 to 24). Four independent experiments revealed the level of budding of the 8N mutant to be 5.1% (± 2.1%) of the wild-type level. Thus, budding is diminished when the basic residues are altered as much as or more than it is when the myristic acid moiety is removed. Interestingly, the deletion mutant assembled into particles with slightly better efficiency than did mutant 8N. A possible explanation for this observation is that an arginine at position 43 is moved into close proximity of two other basic residues (positions 15 and 18), which may bring enough positive charges together to reconstitute a weak membrane-binding domain. When the 18-h-incubated cell lysates

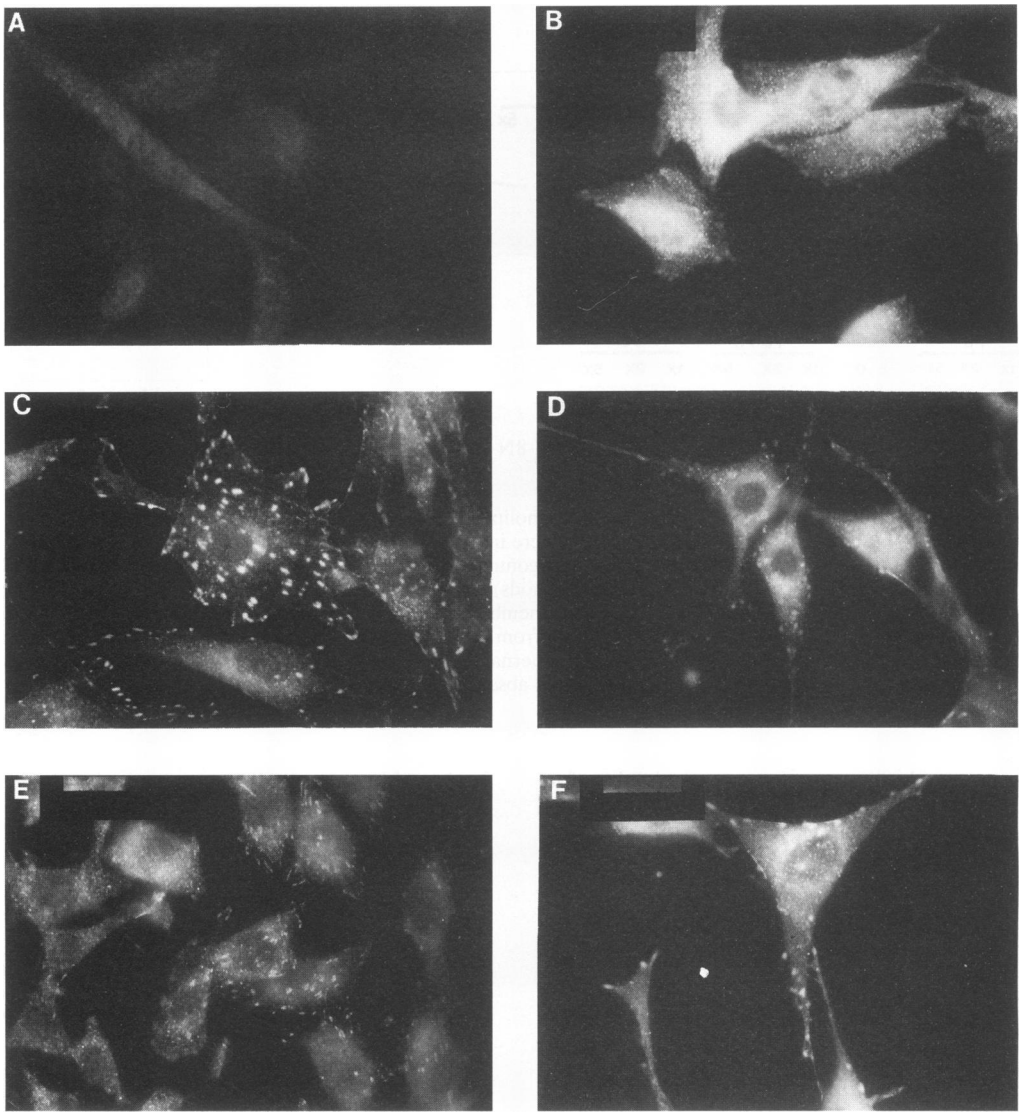


FIG. 5. Subcellular localization of Gag-Src chimeras by immunocytochemistry. v-*src* and *gag-src* genes were introduced into NIH 3T3 cells by retrovirus-mediated gene transfer. Clones of G418-resistant cells were expanded for analysis. (A) NIH 3T3 cells; or cells expressing 315-Src (B), v-Src (C), Gag14-Src (D), Gag31-Src (E), or 8N-Gag31-Src (F). v-Src and Gag-Src proteins were detected by indirect immunofluorescence microscopy with anti-Src polyclonal antibodies.

were examined, the 8N and deletion mutants revealed decreased amounts of Gag proteins, indicating that the mutant Gag proteins are less stable than the wild type (Fig. 9B, lanes 10 to 16, and C, lanes 9 to 14). Thus, the reduced levels of budding observed with the mutants did not result in an accumulation of Gag protein within the cell.

In spite of the mutants' low efficiency of budding, they appear to have preserved other important characteristics of Gag proteins. In particular, envelope gp120 was detected in the immunoprecipitates of the medium samples (Fig. 9B, lanes 18 to 24, and C, lanes 16 to 21), indicating that packaging of viral glycoproteins into particles was not affected by the MA mutations. Moreover, proteolytic processing of the Gag precursor also occurred normally, as indicated by the presence of p41 and p24/25 in both the cell lysates and the retroviral particles (Fig. 9B, lanes 11 to 13 and 19 to 21, and C, lanes 9 to 11 and 16 to 18, respectively).

DISCUSSION

In this report, we show that the first 31 amino acids of Gag can function independently as a membrane-targeting domain when they are fused to heterologous proteins. Several other studies have attempted to probe the sequence elements important for membrane targeting of HIV-1 Gag and subsequent virion production (10, 61, 62). In each case, deletions were made within the coding sequence of Gag, and the effect on intracellular localization was examined. Deletion of amino acids 16 to 99 or 22 to 32 reduced virus budding into the extracellular medium by 10-fold, consistent with our findings that the basic region of HIV-1 Gag (amino acids 15 to 31) is important for plasma membrane targeting. Interestingly, the large Gag deletion mutant directed significant particle assembly at intracellular membranes (10), rather than the plasma membrane, implying that an additional targeting signal(s) is

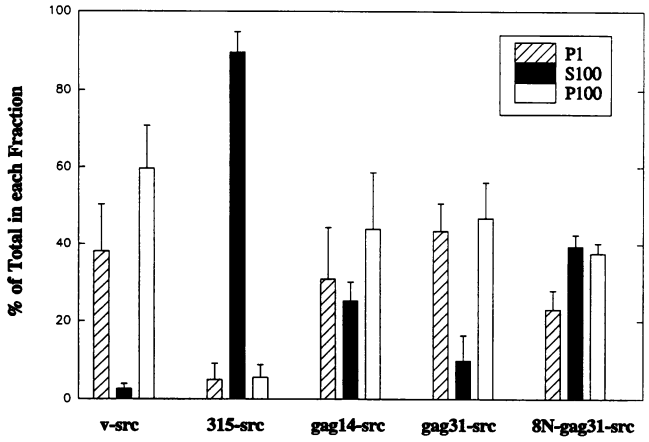


FIG. 6. Subcellular localization of Gag-Src chimeras. Cells expressing v-Src or Gag-Src chimeras were lysed and fractionated by differential centrifugation into a 1,000-×-*g* pellet (P1), a 100,000-×-*g* pellet (P100), and supernatant (S100). Each fraction was subjected to SDS-PAGE, immunoblotting, and densitometry quantitation. Each bar represents the average of three independent experiments.

contained within internal sequences of the MA domain. This study is the first to undertake a molecular dissection of the N-terminal HIV-1 Gag-targeting signal, independent of other Gag domains.

Myristylation is necessary but not sufficient for HIV-1 Gag membrane binding. To date, nearly 70 viral and cellular proteins have been shown to be N myristylated (40, 45, 55). Since myristylated proteins are found in many different cellular locations, including the plasma membrane, Golgi apparatus, endoplasmic reticulum, and cytosol, it is clear that myristate alone is insufficient to serve as a subcellular targeting signal. However, for the subset of myristylated proteins that are membrane bound, deletion of the myristate attachment site reduces or eliminates membrane binding (4, 20, 55). Likewise, membrane association and virion formation are severely reduced when the myristylation site of HIV-1 Gag (4, 14, 15) or other retroviral Gag proteins (19, 36, 41) is mutated. Moreover, use of myristate analogs has revealed that HIV-1 replication is extremely sensitive to substitutions of sulfur or oxygen within the N-terminal fatty acid moiety (5).

The data depicted in Fig. 2, 3, and 9 further support a critical role for myristate in membrane binding of HIV-1 Gag in vitro and particle formation in vivo. However, it is important to note that the Gag polyproteins of several retroviruses, e.g., RSV and visnavirus, do not contain a myristylation site at their N termini (see below) yet are fully capable of membrane targeting and budding. It is likely that myristate is not the sole determinant for membrane binding and that additional membrane-targeting signals are present within the Gag sequence. In support of this notion, membrane association is reduced, but not eliminated, in the nonmyristylated A-Gag31-DHFR mutant (Fig. 3). Likewise, in HIV-1-infected cells, nonmyristylated $Pr55^{gag}$ is still bound to the membrane, although less tightly than the wild-type protein (4), and budding occurs at reduced but detectable levels when the mutant protein is overexpressed in COS-1 cells (Fig. 9). We suggest that the presence of myristate promotes membrane binding by providing hydrophobic interaction with the lipid bilayer. Biophysical measurements estimate the membrane-binding energy contributed by myristate to be 8 kcal/mol (ca. 33 kJ/mol) (32). This corresponds to a K_d of 10^{-4} M and is scarcely enough energy to allow a myristylated protein to stably attach to a cellular membrane.

Myristate plus the first 14 amino acids of HIV-1 Gag form a weak membrane-targeting signal. Replacement of the first 14 amino acids of $pp60^{v\text{-}src}$ with those of HIV-1 Gag (Gag14) resulted in membrane association of the Gag-Src chimera, in vitro and in vivo. Since, in the absence of the first 14 amino acids, $pp60^{v\text{-}src}$ behaves as a soluble protein, these data imply that Gag14 contains membrane-binding information. Results with Gag-DHFR chimeras are consistent with this conclusion. Clearly, Gag14 is not as strong a membrane association signal as Gag31, either in vitro (Fig. 2 and 3) or in vivo (Fig. 5 and 6). However, the amount of Gag14-Src that is correctly targeted is apparently sufficient to elicit transformation of NIH 3T3 cells (Fig. 7 and 8). This is not surprising, as it is known that cellular transformation can occur with levels of $pp60^{v\text{-}src}$ far below those produced by RSV infection (18).

What features of the first 14 amino acids of HIV-1 Gag are important for membrane binding? The Gag14 sequence contains a myristylation consensus motif (GxxxS/T), and as discussed above, myristate is required for membrane binding of Gag14-Src. However, myristylation per se is not sufficient, as chimeric Src proteins containing N-terminal myristylated sequences from other proteins (calcineurin and VP2 of simian virus 40) are not membrane bound in vitro (50) or in vivo (63). Hydrophilicity analysis reveals that the Gag14 sequence is moderately hydrophobic (data not shown), which may enhance interaction with the lipid bilayer. There is no obvious cluster of charged residues, and the arginine at position 4 can be mutated without significantly affecting membrane binding (63). However, in the Gag14-Src chimera, juxtaposition of Gag sequences next to two arginines at positions 15 and 16 of Src could partially reconstitute a basic domain that may contribute to the membrane-binding affinity. Indeed, Gag14 provides a slightly stronger membrane-binding signal in the context of Src (44% of Gag31-Src) than in the context of DHFR (28% of Gag31-DHFR), which lacks the two adjacent arginine residues.

The first 31 amino acids of HIV-1 Gag form a strong membrane-targeting signal: role of the basic domain. The data in Fig. 2 to 8 indicate that Gag31 contains a membrane-targeting signal that fully substitutes for that of Src in conferring membrane binding and cellular transformation. Compared with Gag14, the additional 17 amino acids in Gag31 provide a two- to fourfold increase in the extent of membrane binding in vitro for Src as well as for DHFR chimeras. Several lines of evidence indicate that the cluster of basic residues between amino acids 15 to 31 is important for HIV-1 Gag function. Mutation of eight basic residues (8N-Gag31) to asparagine reduces membrane binding of Gag-Src and Gag-DHFR chimeras in vitro (Fig. 2 and 3). When the protein is expressed in NIH 3T3 cells, a greater percentage of 8N-Gag31-Src protein than of Gag31-Src is found in the cytosolic fraction (Fig. 5 and 6). Moreover, the 8N mutation, when introduced directly into HIV-1 Gag, reduces budding efficiency by 95% (Fig. 9). While this paper was in preparation, a similar observation was reported by Yuan et al. (62), who observed that mutation of eight basic residues between positions 18 and 32 in HIV-1 Gag severely reduced virus production.

The work presented in this study is the first to demonstrate that the basic domain of HIV-1 Gag mediates binding to acidic membrane phospholipids (Fig. 4). Liposomes containing the physiological 2:1 ratio of neutral to acidic phospholipids were effective competitors of Gag31-DHFR membrane binding, while neutral liposomes were without effect. A similar result has been obtained for $pp60^{v\text{-}src}$, which also contains a cluster of

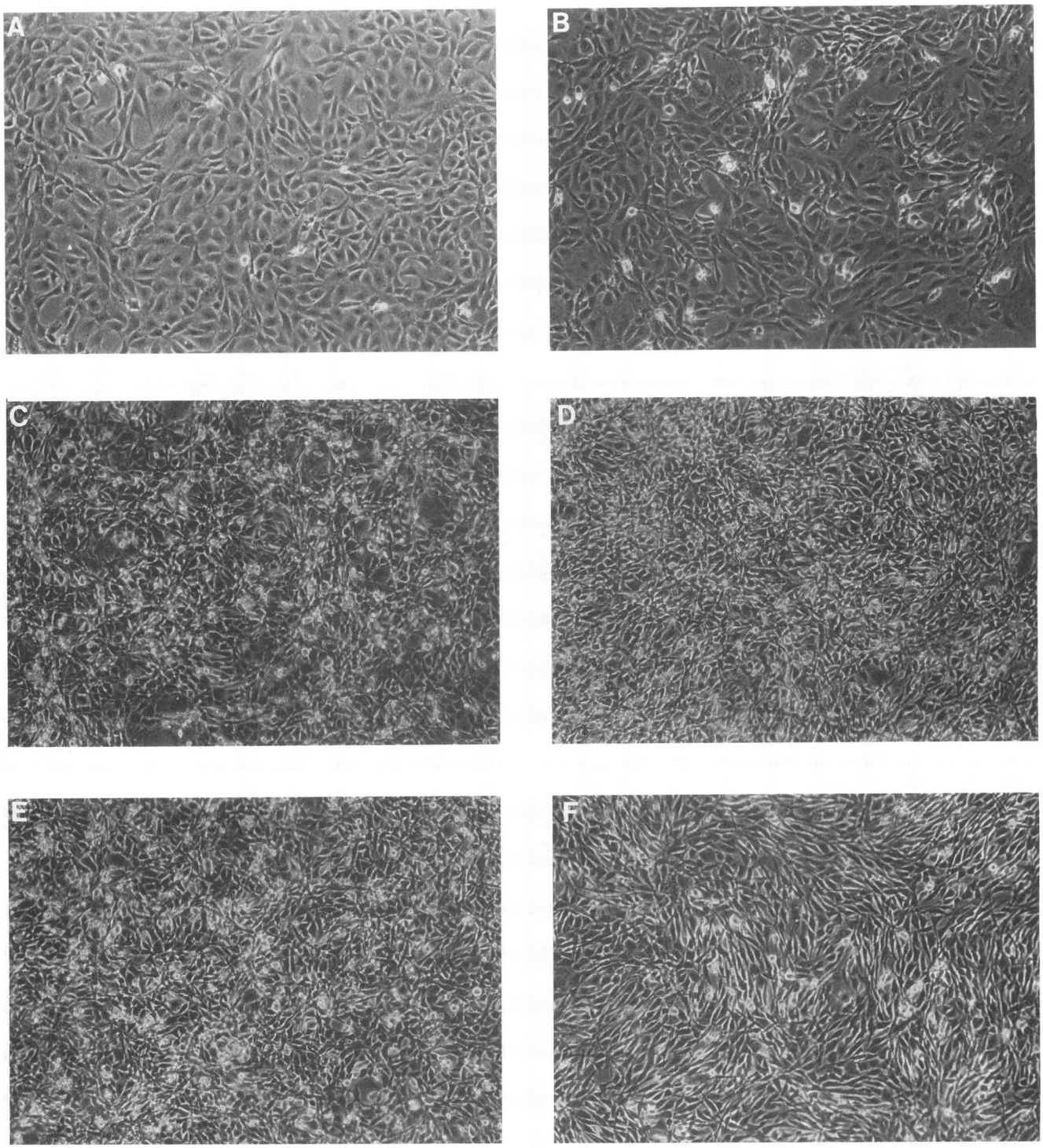


FIG. 7. Morphology of NIH 3T3 cells overexpressing Gag-Src chimeras. Cells expressing v-Src and Gag-Src proteins were obtained as described in the legend to Fig. 5 and photographed under phase-contrast microscopy. (A) NIH 3T3 cells; or cells expressing 315-Src (B), v-Src (C), Gag14-Src (D), Gag31-Src (E), or 8N-Gag31-Src (F).

basic residues at its N terminus (49). Experiments are currently in progress to measure the binding affinity of HIV-1 Gag for acidic phospholipids. However, studies performed with model peptides indicate that the electrostatic interaction between a lysine residue and an acidic phospholipid such as phosphatidylserine contributes 1.4 kcal (ca. 5.9 kJ) of binding energy per mol (23). The eight basic residues contained in the Gag31 chimeras should provide 11 kcal (ca. 46 kJ) of binding energy per mol versus 8 kcal/mol (ca. 33 kJ/mol) for myristate (32). The data in Fig. 2 and 3, showing the relative contributions of

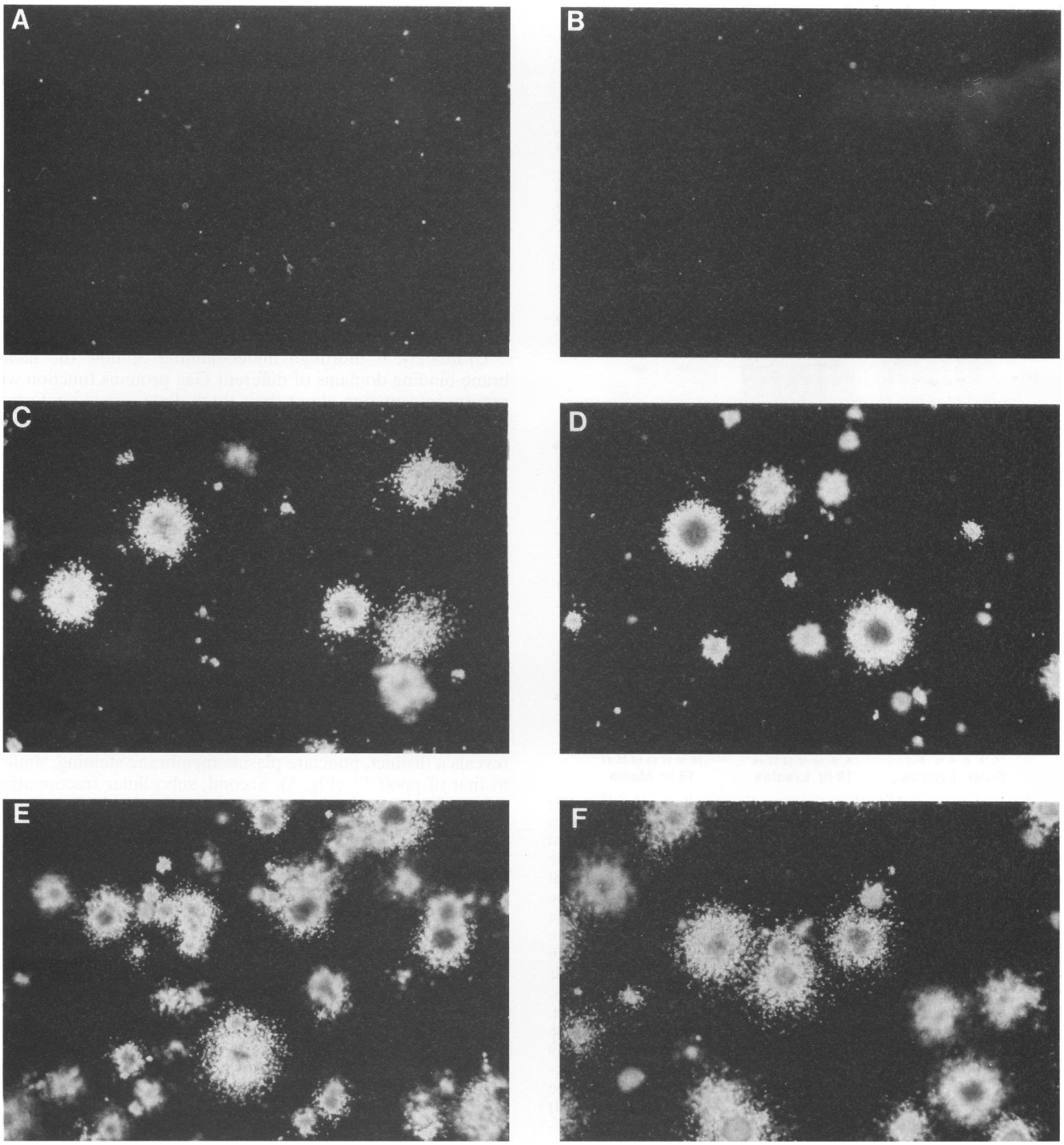


FIG. 8. Growth of Gag-Src transformed cells in soft agar. Soft-agar colonies induced by v-Src and Gag-Src chimeras were photographed under phase-contrast microscopy. (A) NIH 3T3 cells; or cells expressing 315-Src (B), v-Src (C), Gag14-Src (D), Gag31-Src (E), or 8N-Gag31-Src (F).

myristate and the basic domain to membrane binding, agree remarkably well with these calculations.

Basic protein domains are found in other retroviral Gag proteins. Computer analysis of the amphilicity index of HIV-1 Gag predicts a strong propensity for residues 17 to 30 to form an amphiphilic β sheet (Fig. 10A). A model for this predicted structure reveals that seven of the eight basic residues would be found on the same surface of the sheet. It is likely that such a structure would promote efficient interaction of the HIV-1 Gag N terminus with acidic phospholipids on the cytoplasmic face of the lipid bilayer or potentially on other accessible membrane surfaces (see below).

Clusters of basic residues are found within the Gag sequences of other type C and lentivirus retroviruses (Fig. 10B). Myristylated Gag proteins of simian immunodeficiency virus, feline immunodeficiency virus, Moloney murine leukemia vi-

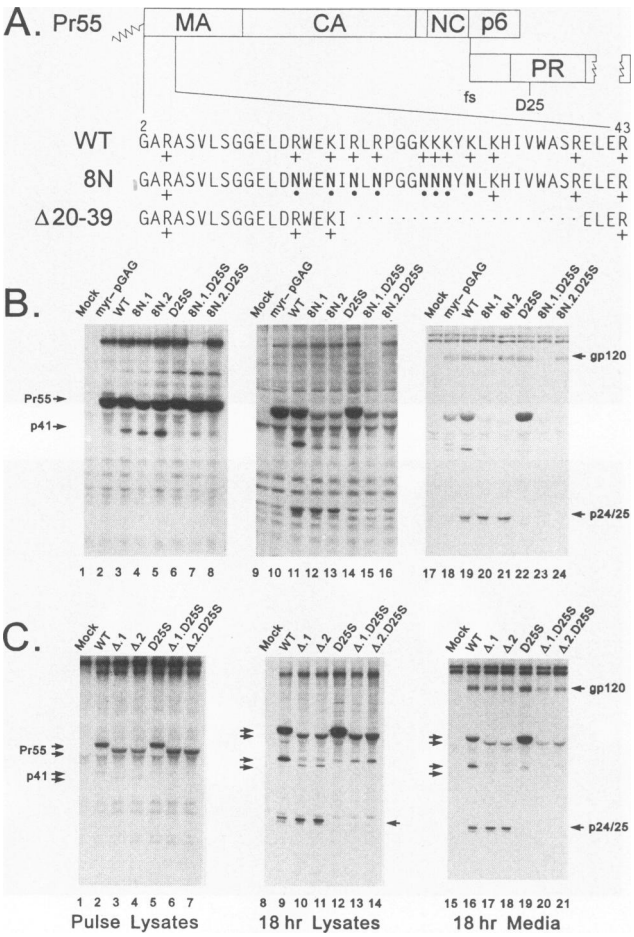


FIG. 9. Effects of MA alterations on Gag-mediated budding. (A) Schematic representation of the HIV-1 Gag polyprotein precursor, Pr55, with the major cleavage products denoted as MA, CA, NC, and p6. The squiggle represents myristic acid, which is added to the glycine at position 2 after removal of the initiator methionine. A ribosomal frameshift (fs) occurs within the coding sequence of p6, resulting in the Gag-Pol fusion protein, which contains the viral protease (PR), reverse transcriptase, and integrase proteins. The aspartic acid in the active site of PR, D25, is noted. In mutant D25S, this residue is replaced with serine, which inactivates PR. All of the HIV-1 Gag expression plasmids have a large deletion in the *pol* gene, as depicted by the broken box. The wild-type (WT) sequence of the first 43 residues of MA is shown. Basic amino acids have a + beneath them. In mutant 8N, eight of these residues (noted by dots) are replaced with asparagine. The Δ20-39 mutant lacks the 20 residues denoted by the dashed line. (B) Characterization of the 8N mutant. COS-1 cells were transfected with the indicated plasmid DNAs and labeled for either 20 min (pulse-labeled) or 18 h with [^{35}S]methionine. HIV-1 proteins were immunoprecipitated from cell lysates and growth media with human HIV-1 immunoglobulin, separated by SDS-PAGE on a 12% gel, and detected by fluorography. 8N.1 and 8N.2 are identical clones, as are 8N.1.D25S and 8N.2.D25S. Arrows indicate the positions of Pr55, p41 (MA-CA fusion), p24/25 (CA), and gp120 (SU glycoprotein). (C) Characterization of the MA deletion mutant. Transfected cells were labeled in the same way as described for panel B, except [^{3}H]leucine was used in the place of [^{35}S]methionine in the pulse-labeled plates. Duplicate clones of the Δ20-39 deletion mutant are designated Δ.1 and Δ.2, and those with the protease mutation are named Δ.1.D25S and Δ.2.D25S. Arrows indicate the positions of full-length Pr55 and p41 as well as the precursors of Δ20-39, which have increased electrophoretic mobility because of the deletion.

rus, and Mason-Pfizer monkey virus and have a large proportion of basic residues close to the N terminus in an arrangement reminiscent to that of HIV-1. It is tempting to speculate that basic domains within these other Gag proteins also function in a manner similar to that observed for HIV-1 Gag. However, it is important to point out that some myristylated Gag proteins have relatively few basic residues within their N-terminal regions (human T-cell leukemia virus type I and bovine leukemia virus) while others, such as RSV, equine infectious anemia virus, and visnavirus contain basic residues within a nonmyristylated N-terminal region. Mapping of the membrane-binding domain of RSV Gag has revealed it to be much larger than that of HIV, approximately 90 amino acids (56). The basic residues within the first 40 amino acids of RSV are insufficient for budding, even when a myristylation site is created artificially (60).

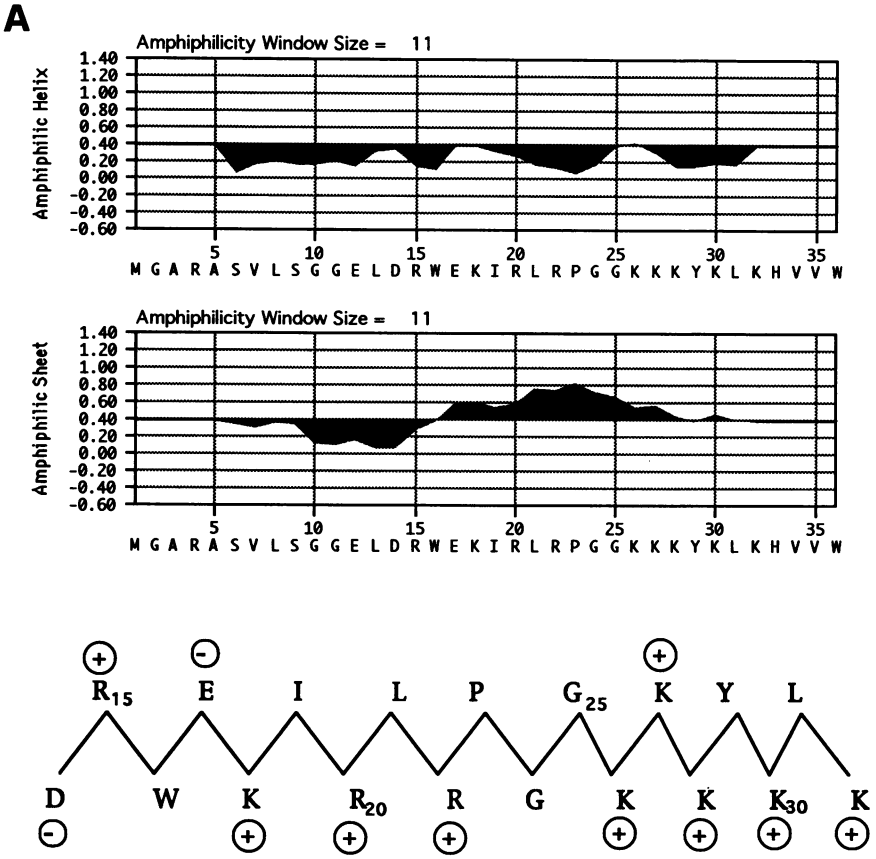
Ultimately, a thorough understanding of how the membrane-binding domains of different Gag proteins function will require information about their three-dimensional conformation. In this regard, it is interesting to note that the Gag molecules of Mason-Pfizer monkey virus assemble into particles in the cytosol, prior to interaction with any membrane. The Mason-Pfizer monkey virus Gag protein is myristylated and contains eight basic residues within the first 40 amino acids. Mutation of a nearby arginine at position 55 to tryptophan redirects particle assembly and budding to the plasma membrane (42). Presumably, Mason-Pfizer monkey virus contains a cryptic or regulated plasma membrane-targeting signal, perhaps utilizing basic residues, which is unmasked by either the mutation or an as-yet-undefined signal.

How is membrane-targeting specificity achieved? Retroviral Gag proteins direct virion formation specifically at the plasma membrane. Several lines of evidence suggest that basic domains may target HIV-1 Gag specifically to the plasma membrane. First, immunofluorescence localization of Gag31-Src reveals a distinct, punctate plasma membrane staining, similar to that of pp60$^{v\text{-}src}$ (Fig. 5). Second, subcellular fractionation shows that Gag31-Src fractionates with the P100, plasma membrane-enriched fraction (Fig. 6). Third, deletions or mutations of the basic residues within HIV-1 Gag prevent targeting to the plasma membrane (Fig. 9) and result in inefficient particle assembly at intracellular membrane sites (10). In addition, other proteins, such as p21$^{K\text{-}ras(B)}$ have been shown to utilize a polybasic domain for efficient plasma membrane targeting (16).

It is well established that lipid asymmetry exists in the plasma membrane, with nearly all the acidic phospholipids located on the inner, cytoplasmic leaflet of the bilayer (30). The enrichment of acidic phospholipids at this location may serve to localize proteins with basic domains to the cytoplasmic face of the plasma membrane. Acidic phospholipids could thus serve as receptors for HIV Gag, which bind the N-terminal basic domain and potentially other downstream basic domains. It remains possible that a cellular plasma membrane protein containing a cluster of negatively charged residues may serve as the receptor. Clearly, the envelope glycoproteins encoded by the virus do not serve this role, as they are dispensable for budding (58).

It is not clear why other intracellular membranes, which also contain acidic phospholipids, do not serve as binding sites for retroviral Gag proteins such as HIV. Presumably, additional targeting specificity is provided through protein-protein interactions involving downstream Gag domains, analogous to pp60$^{v\text{-}src}$ (39).

Basic domains have been shown to function as nuclear localization signals for a variety of proteins (11). In fact, a



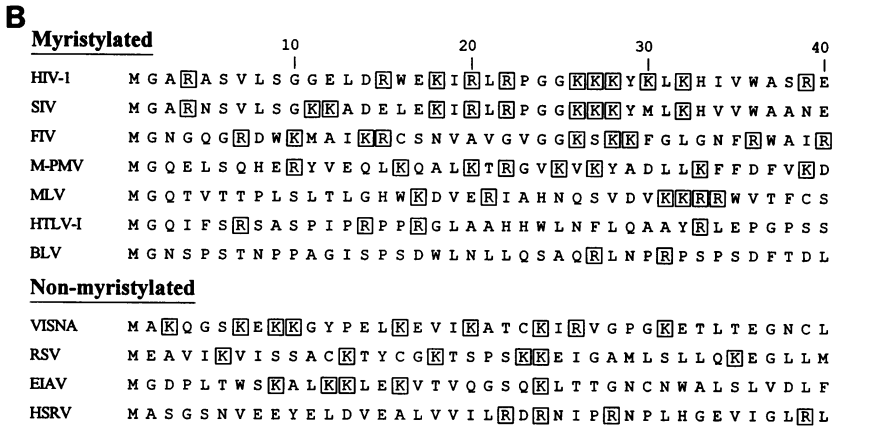


FIG. 10. (A) Potential formation of an amphiphilic β sheet by N-terminal HIV-1 Gag sequences. The first 36 amino acids of HIV-1 Gag were analyzed for their potential to form an amphiphilic α helix or β sheet. A hypothetical β sheet is depicted at the bottom, showing nearly all the basic residues located on the same face of the sheet. (B) Presence of basic amino acids within the N-termini of other retroviral Gag proteins. The N-terminal sequences of HIV-1 (57), simian immunodeficiency virus (SIV) (57), human T-cell leukemia virus type I (HTLV-I) (48), feline immunodeficiency virus (FIV) (53), Mason-Pfizer monkey virus (M-PMV) (52), bovine leukemia virus (BLV) (44), Moloney murine leukemia virus (MLV) (57), RSV (47), equine infectious anemia virus (EIAV) (22), human spumaretrovirus (HSRV) (26), and visna virus (VISNA) (51) Gag proteins are shown, with basic residues boxed.

recent report demonstrated that fusion of the basic domain of HIV-1 Gag (amino acids 25 to 33) to a carrier protein (bovine serum albumin) could direct partial localization of the conjugated protein to cell nuclei (6). Since Gag31-Src does not exhibit noticeable nuclear staining (Fig. 5E), the polybasic domain does not function as a nuclear targeting signal in this context. In this regard, it is interesting to note that a subset of $Pr65^{gag}$ of Moloney murine leukemia virus has been found in

the nucleus and that the amount of nuclear Gag increases when the myristylation site is mutated (29). In this study, we have shown that the myristylated first 14 amino acids, plus the basic domain, coordinately function as a plasma membrane-targeting signal. We propose that the bipartite myristylated Gag31 sequence within HIV-1 Gag serves as a dominant targeting signal, which overcomes the potential for nuclear targeting (by the basic domain) or intracellular membrane targeting (by downstream domains).

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