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Role of the Matrix Protein in the Virion Association of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein

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The matrix (MA) protein of human immunodeficiency virus type 1 (HIV-1) forms an inner coat directly underneath the lipid envelope of the virion. The outer surface of the lipid envelope surrounding the capsid is coated by the viral Env glycoproteins. We report here that the HIV-1 capsid-Env glycoprotein association is very sensitive to minor alterations in the MA protein. The results indicate that most of the MA domain of the Gag precursor, except for its carboxy terminus, is essential for this association. Viral particles produced by proviruses with small missense or deletion mutations in the region coding for the amino-terminal 100 amino acids of the MA protein lacked both the surface glycoprotein gp120 and the transmembrane glycoprotein gp41, indicating a defect at the level of Env glycoprotein incorporation. Alterations at the carboxy terminus of the MA domain had no significant effect on the levels of particle-associated Env glycoprotein or on virus replication. The presence of HIV-1 MA protein sequences was sufficient for the stable association of HIV-1 Env glycoprotein with hybrid particles that contain the capsid (CA) and nucleocapsid (NC) proteins of visna virus. The association of HIV-1 Env glycoprotein with the hybrid particles was dependent upon the presence of the HIV-1 MA protein domain, as HIV-1 Env glycoprotein was not efficiently recruited into virus particles when coexpressed with authentic visna virus Gag proteins.

The *gag* gene of retroviruses, which encodes the internal structural viral proteins, is initially expressed in the form of a polyprotein precursor. Following the assembly of an immature capsid, the Gag precursor is cleaved by the viral protease to yield the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins of the mature virus particle and often additional cleavage products of unknown function (40). The N-terminal cleavage product of retroviral Gag polyproteins has been classified as an MA protein on the basis of its close proximity to the viral lipid envelope (23). Evidence for the association of retroviral MA proteins with the lipid envelope was initially obtained from lipid-protein cross-linking studies using avian and murine type C retroviruses (23, 24). Immune electron microscopy of human immunodeficiency virus type 1 (HIV-1) virions has confirmed that the MA protein (p17) is a component of the inner coat visible directly beneath the lipid envelope of the virion (11).

In most mammalian retroviruses, including HIV-1, the MA domain of the Gag precursor is cotranslationally modified at the N terminus by attachment of a myristic acid (27, 33). Addition of the N-terminal fatty acid is required for virus budding and release from the cell membrane (3, 15, 27, 28, 38). MA protein sequences not involved in myristylation have also been found to be critical for retroviral morphogenesis. A point mutation in the MA domain of Mason-Pfizer monkey virus caused capsid assembly to occur at the cell membrane rather than in the cytoplasm, as is usual for type D retroviruses (29). Other mutations in the Mason-Pfizer monkey virus MA domain caused defects in the stability and assembly of mutant Gag precursors, the intracellular transport of assembled capsids, or the interaction of preassembled capsids with the cell membrane (30, 31). Deletions in the Rous sarcoma virus MA protein, which is not myristylated, led to a block in particle formation, a block that could be reversed by fusing the membrane-binding domain of $p60^{src}$ to the N terminus of the mutant proteins (41).

In addition to a role in capsid formation and release, the close association of the MA protein domain with the lipid envelope of the virion suggests that it may establish functionally important contacts with viral Env glycoproteins during particle morphogenesis. The incorporation of retroviral Env glycoproteins into assembling virions appears to be a selective process, since the majority of host cell membrane proteins is excluded (16). However, some cellular proteins have been shown to be associated with retroviral particles in substantial amounts (1). The results presented here show that the stable association of HIV-1 Env glycoprotein with viral particles is critically dependent on the integrity of the HIV-1 MA protein domain, consistent with recent results reported by Yu et al. (45). Moreover, an analysis of chimeric capsids revealed that HIV-1 MA protein sequences are sufficient for the efficient incorporation of HIV-1 Env glycoprotein.

MATERIALS AND METHODS

Proviral DNA constructs. Single-stranded DNA was prepared from plasmid pSK^+gag (15), which contains a 1.3-kb *Sac*I-*Apa*I fragment from the infectious HXBc2 proviral clone of HIV-1, and used as a template for the annealing of oligonucleotides and primer extension with T4 DNA polymerase as described previously (15). Mutations introduced into the MA coding region are shown in Fig. 1. In addition, codons 19 to 21 and 43 to 45 were deleted from the CA protein-coding region of the HIV-1 *gag* gene to generate the CAΔ19-21 and CAΔ43-45 mutations. To regenerate full-length proviral clones after mutagenesis, 1.3-kb *Bss*HII-*Apa*I fragments carrying the

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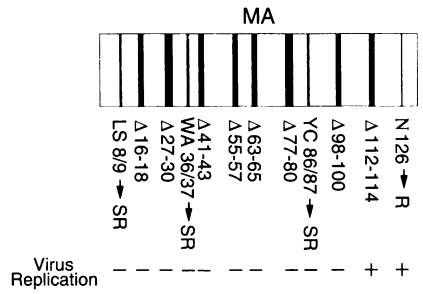

FIG. 1. Location of clustered point mutations in the MA protein-coding region of the HIV-1 *gag* gene. Numbers refer to the positions of the substituted (stippled bars) or deleted (closed bars) amino acids relative to the initial methionine. The effect of the mutations on virus replication as measured by viral protein synthesis in transfected Jurkat cells in indicated. A minus sign indicates that viral Gag and Env proteins could not be detected by immunoprecipitation in the lysates of Jurkat cells for up to 1 month after transfection with the mutant proviruses. A plus sign indicates that the mutations did not impair viral Gag and Env protein synthesis in the transfected cells as measured by sequential immunoprecipitation at 1, 2, and 4 weeks posttransfection.

desired mutations were inserted into the parental vpu^+ HXBH10 proviral construct (37).

The protease-defective variant HXBH10-PR$^-$ (14) has the codon for Asp-25 of the HIV-1 protease replaced by a codon specifying glutamic acid. The HXBH10-*gag*$^-$ proviral construct is identical to HXBH10, except for a premature termination codon that replaces codon 8 of the *gag* gene and a frameshift mutation in the CA-coding region that was generated by inserting 4 bp at the unique *Spe*I site (nucleotide [nt] 1506) by using the Klenow fragment of DNA polymerase I. The envelope-deficient provirus HXBH10-*env*$^-$fs has the *env* initiation codon replaced by ACG and also harbors a frameshift mutation at a *Kpn*I site (nt 6346) early in the *env* gene (43). The HXBH10/$LV_{gag\text{-}pol}$ chimeric provirus (14) represents HXBH10 with 5′ untranslated, *gag*, and *pol* sequences replaced by the corresponding region of the LV1-1KS1 molecular clone of visna virus (35). HXBH10-MA/$LV_{gag\text{-}pol}$ is identical to HXBH10/$LV_{gag\text{-}pol}$, except that the 5′ untranslated and MA protein-coding regions are from HXBH10 rather than from LV1-1KS1. To obtain HXBH10-MA/$LV_{gag\text{-}pol}$, a *Sfc*I site was introduced by site-directed mutagenesis into the visna *gag* gene immediately downstream of the predicted MA-CA cleavage site by changing nucleotide 924 of LV1-1KS1 from T to A. The point mutation does not alter the predicted amino acid sequence of the visna virus CA protein. A naturally occurring *Sfc*I site at the equivalent position in the HIV-1 *gag* gene at nt 1186 was used to join HIV-1 and the visna virus *gag* coding sequences exactly at the junction of the MA and CA domains. A *Nar*I-*Spe*I fragment containing the HIV-1 5′ untranslated and MA protein-coding region (nt 637-1186) linked at its 3′ end to sequences from the visna virus CA protein-coding region (nt 921 to 1067 of LV1-1KS1) was then used to replace the corresponding visna virus-derived fragment in HXBH10/$LV_{gag\text{-}pol}$. The resulting proviral construct (HXBH10-MA/$LV_{gag\text{-}pol}$) contains coding sequences for a chimeric Gag polyprotein with an HIV-1 MA domain joined precisely at the position of the proteolytic cleavage site to the CA and NC domains of the visna virus molecular clone LV1-1KS1.

Cell culture and transfections. Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. HeLa cells (CCL 2) were obtained from the American Type Culture Collection (Rockville, Md.) and grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. For virus replication studies, Jurkat cells (5×10^6) were transfected by the DEAE-dextran procedure (26) with 2.5 μg of proviral plasmid DNA. HeLa cells (10^6) were seeded into 80-cm^2 tissue culture flasks 24 h prior to transfection. The cells were transfected with 30 μg of proviral plasmid DNA by a calcium phosphate precipitation technique (4).

Viral protein analysis. HeLa cell cultures and aliquots of transfected Jurkat cells were metabolically labeled for 12 h with [^{35}S]cysteine (50 μCi/ml). Labeling of HeLa cells was started 48 h posttransfection. Labeled cells were lysed in 1× radioimmunoprecipitation assay (RIPA) buffer (140 mM NaCl–8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]) and virus particles in cell-free supernatant fractions were disrupted by adding 5× RIPA buffer. HIV-1-encoded proteins were immunoprecipitated by using sera from individuals infected with HIV-1 and separated in SDS–11.5% polyacrylamide gels as described previously (15). To analyze particle-associated viral protein, virions released into the supernatant were pelleted through a 20% sucrose cushion for 2 h at 27,000 rpm in a Beckman SW 41 rotor. Pelleted virions were lysed in 1× RIPA buffer, and viral proteins in the pellets were either analyzed directly by electrophoresis or immunoprecipitated with patients' sera prior to electrophoresis.

Quantitation of virion-associated viral RNA. RNA was isolated from virions pelleted through 20% sucrose, and samples were adjusted for the amount of $p24^{gag}$ in the virion pellets as described previously (5). HIV-1 viral RNA in the samples was quantified by RNase protection analysis using a uniformly labeled RNA probe synthesized from *Mro*I-linearized pTZ18U/MB as described previously (5). This plasmid contains 5′ long terminal repeat and untranslated sequences from HXBc2 (nt 308 to 713) in antisense orientation to a T7 RNA polymerase promoter (5). The probe was hybridized to RNA extracted from equivalent amounts of viral particles, and unhybridized portions of the probe were degraded by RNase. RNase-resistant fragments were separated on 4.5% polyacrylamide–8.3 M urea gels, and dried gels were subjected to autoradiography and analysis with a Betascope model 603 Blot Analyzer (Betagen Corporation, Waltham, Mass.).

Electron microscopy. Transfected HeLa cells were processed for thin-section electron microscopy as described previously (15).

RESULTS

Effect of alterations in MA on viral infectivity, particle production, and immunorecognition. To analyze the role of MA in viral morphogenesis, a series of missense and deletion mutations was introduced into the region coding for the MA domain of the HIV-1 Gag precursor (Fig. 1). The majority of the mutations affected two or three adjacent codons. Regions of homology between the MA domains of HIV-1 and HIV-2 were targeted for mutagenesis, and an effort was made to space mutations evenly throughout the MA protein-coding region.

The effect of the alterations in the MA protein domain on virus replication was determined by transfection of full-length mutant proviruses into the human CD4$^+$ T-cell line Jurkat that is permissive for HIV-1 replication. Viral protein expression in the transfected cultures was monitored by immunoprecipitation with serum from an individual infected with HIV-1 after metabolic labeling at 1, 2, and 4 weeks posttransfection. Virus replication, as measured by viral protein synthesis, was not affected by two alterations near the C terminus of the MA domain (Fig. 1). Similar amounts of HIV-1 Gag and Env

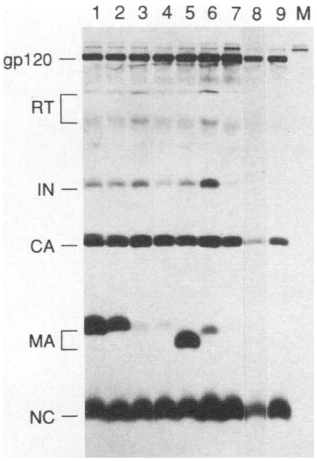

FIG. 2. Immunoprecipitation from the culture supernatant of HeLa cells transfected with MA mutants. The transfected cells were metabolically labeled from 48 to 60 h posttransfection, and viral proteins were immunoprecipitated from the cell-free culture supernatant with patient's serum N18 (5). The transfected proviruses were the parental HXBH10 construct (lane 1) and the MA mutants LS8/9→SR (lane 2), WA36/37→SR (lane 3), YC86/87→SR (lane 4), N126→R (lane 5), Δ16-18 (lane 6), Δ27-30 (lane 7), Δ55-57 (lane 8), and Δ77-80 (lane 9). Lane M, mock transfection. RT, reverse transcriptase; IN, integrase.

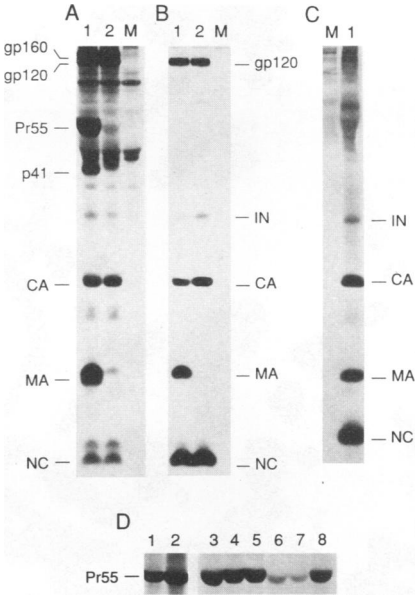

FIG. 3. Effect of a deletion in MA on immunorecognition of MA and Pr55. [^{35}S]cysteine-labeled viral proteins were immunoprecipitated from the cell lysates (A) and culture supernatants (B) of HeLa cells transfected with the parental provirus HXBH10 (lane 1) or the Δ41-43 mutant (lane 2). Lane M, mock transfection. Immunoprecipitations were carried out with the N18 patient's serum. (C) Direct analysis of particle-associated proteins by SDS-PAGE. HeLa cells were mock transfected (lane M) or transfected with the Δ41-43 mutant (lane 1), and [^{35}S]cysteine-labeled particulate material released into the supernatant was pelleted through 20% sucrose, disrupted in RIPA buffer, and separated by SDS-PAGE. (D) Effect of the Δ41-43 mutation on immunorecognition of Pr55. HeLa cells were transfected with the protease-defective proviruses HXBH10-PR$^-$ (lanes 1 and 3 to 5) or HXBH10-PR$^-$/Δ41-43 (lanes 2 and 6 to 8), and [^{35}S]cysteine-labeled particulate material released into the supernatant was pelleted through 20% sucrose and disrupted in RIPA buffer. Aliquots were either analyzed directly by SDS-PAGE (lanes 1 and 2) or immunoprecipitated with patient's serum N18 (lanes 3 and 6), N21 (lanes 4 and 7), or E1 (lanes 5 and 8) prior to SDS-PAGE. IN, integrase.

proteins were detectable in cells transfected with the parental provirus or with the mutant proviruses Δ112-114 and N126→R (Fig. 1). By contrast, viral proteins were not detected in cultures transfected with any of the other MA mutants (Fig. 1). These results indicate that all of the alterations in the MA domain with the exception of the two proximal to the C terminus prevent virus replication.

To determine whether the amino acid changes in the MA protein domain that abolish virus replication affect viral particle assembly or release, the mutant proviruses were transfected into HeLa cells. HeLa cells do not support replication of HIV-1 as they lack the CD4 receptor. Therefore, proteins made in HeLa cells represent products of the original transfected DNA. The release of HIV-1 Gag and Env proteins from the transfected cells was monitored by immunoprecipitation from the cell-free supernatant fractions.

The results of a representative experiment are shown in Fig. 2. Similar amounts of gp120 Env protein were immunoprecipitated from the supernatant of cells transfected with the parental and all of the mutant proviruses. The amounts of CA and NC proteins detectable in the supernatant fractions showed that the changes introduced into the MA protein domain did not significantly impair particle production, with the exception of the Δ55-57 mutation (Fig. 2, lane 8). In some cases, particularly for the Δ16-18 mutant (Fig. 2, lane 6), the amount of *gag*- and *pol*-encoded proteins immunoprecipitated exceeded the levels obtained by using the parental provirus (Fig. 2, lane 1). The Δ16-18 mutant yielded about fourfold-more viral particles than the parental provirus as determined by p24^{gag} capture assay after pelleting of virions through 20% sucrose (data not shown).

Several of the alterations in the MA protein domain led to a selective decrease in the amount of MA protein compared with that of CA or NC protein detectable by immunoprecipitation (Fig. 2 and 3). In addition to the MA protein, products that contain an MA domain such as the Gag precursor Pr55 and the Gag-processing intermediate p41 were selectively reduced in immunoprecipitates from cell lysates as shown in Fig. 3A for the Δ41-43 mutant. Sera from three individuals infected with HIV-1 were used in attempts to immunoprecipitate MA from the supernatant fraction of cells transfected with the Δ41-43 mutant. The three sera efficiently precipitated wild-type MA, but Δ41-43 mutant MA could hardly be detected (Fig. 3B and data not shown). However, the mutant MA protein was detected in amounts equimolar to those of CA and NC protein when Δ41-43 mutant particles were pelleted through sucrose and particle-associated viral proteins were visualized directly by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3C). Evidently, the mutation reduces the amount of MA protein precipitated by sera from individuals infected with HIV-1 but does not decrease the amount of MA protein actually present in virions.

To determine whether the Δ41-43 mutation also affected immunorecognition of the uncleaved Pr55 Gag precursor, the viral protease in the parental and Δ41-43 mutant proviruses was inactivated. Particulate material released into the supernatant of cells transfected with the HXBH10-PR$^-$ and HXBH10-PR$^-$/Δ41-43 strains was pelleted through 20% sucrose, disrupted in RIPA buffer, and analyzed directly by

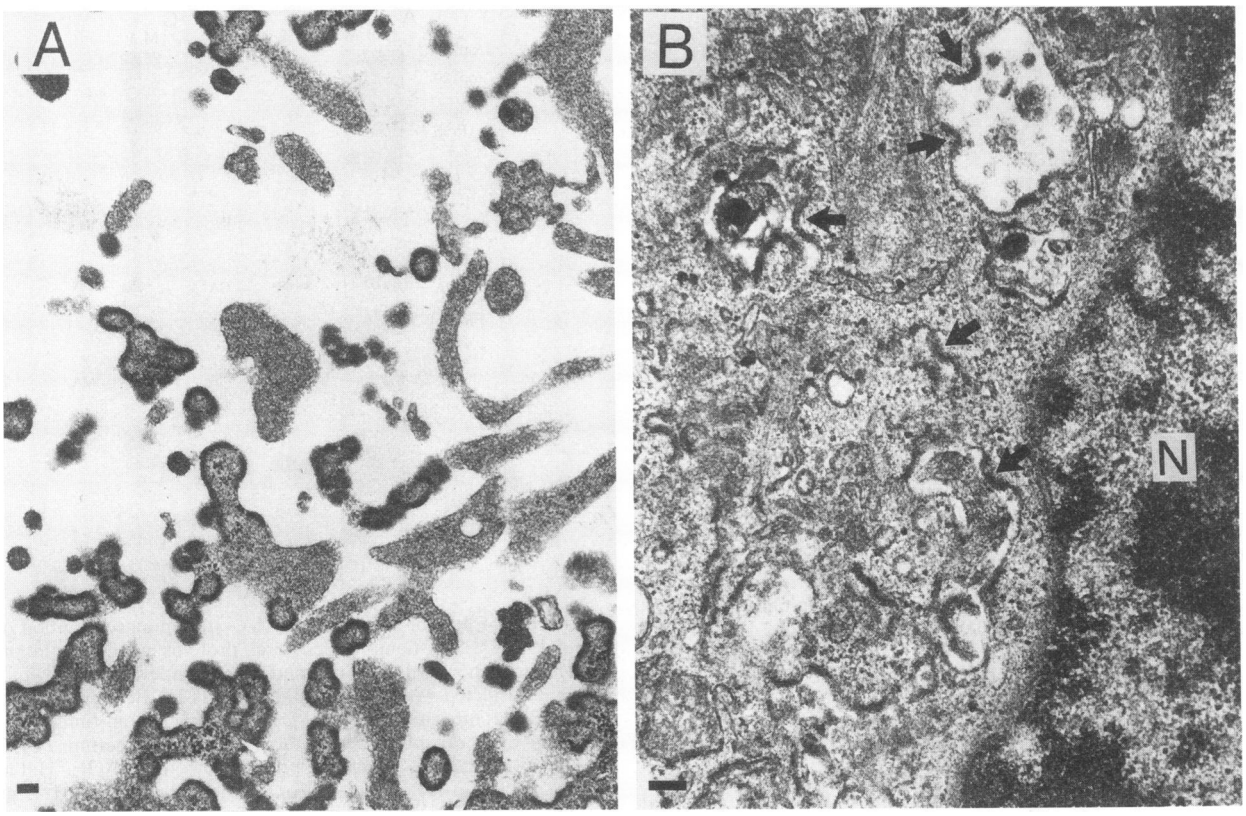

FIG. 4. Thin-section electron micrographs of HeLa cell cultures 60 h after transfection with the MA deletion mutants Δ41-43 (A) and Δ16-18 (B). Panel A shows cellular projections that are densely covered with budding structures. Some of the electron-dense budding structures visible in the cell shown in panel B are marked by arrows. N, nucleus.

SDS-PAGE. As shown previously (14), transfection of HXBH10-PR$^-$ resulted in the release of particulate Pr55 precursor (Fig. 3D, lane 1). The HXBH10-PR$^-$/Δ41-43 construct yielded slightly higher amounts of particulate Pr55 than the parental protease-defective provirus (Fig. 3D, lane 2). Immunoprecipitation from the pelleted material revealed that two of the three serum samples used recognized the mutant Pr55 Gag precursor much less efficiently than wild-type Pr55 (Fig. 3D, lanes 3 to 8). This result suggests that the small amounts of mutant Pr55 detected in cell lysates were, at least in part, a consequence of inefficient immunorecognition.

Electron microscopic analysis of HeLa cell cultures transfected with the Δ16-18 and Δ41-43 mutants revealed the production of particles with a morphology similar to that of mature wild-type virions. However, viral structures at a relatively early stage of budding were overrepresented (Fig. 4A). Occasionally, these structures were also seen at intracellular membranes (Fig. 4B). Aberrant budding at intracellular membranes, in particular the membranes of the endoplasmic reticulum, has recently been reported for a mutant of HIV-1 that lacks codon 16 to 99 of the MA protein-coding region (7). However, in contrast to the Δ16-18 and Δ43-45 mutations, the larger deletion in the MA protein domain significantly reduced virus particle release from the cell membrane (7).

Effect of alterations in the MA protein domain on particle-associated viral RNA and Env glycoprotein levels. A role of the MA domain of retroviral Gag precursors in viral RNA encapsidation has been suggested by the observation of a specific interaction between the MA protein and viral RNA of bovine leukemia virus (17). To determine whether changes in the HIV-1 MA domain affected RNA encapsidation, particles produced by the Δ16-18, WA36/37→SR, Δ41-43, and YC86/87→SR mutants were analyzed for their viral RNA content. RNA extracted from pelleted virions was adjusted for particle yield by measuring p24^{gag} in the pellets and subjected to quantitative RNase protection analysis (5). A probe complementary to the viral long terminal repeats was used to distinguish between viral RNA and potentially contaminating viral DNA (5). Hybridization of the probe to the 5′ and 3′ ends of RNA extracted from wild-type particles yielded the expected protected fragments of 260 and 244 nt (Fig. 5). The mutations analyzed had only a minor effect (less than twofold) on the intensity of the specifically protected fragments (Fig. 5), indicating that they do not significantly impair viral RNA encapsidation.

To compare the amount of Env glycoprotein associated with wild-type and mutant particles, particulate material released from transfected HeLa cells was pelleted through 20% sucrose. Pelleted virions were lysed in RIPA buffer, and viral proteins were immunoprecipitated from the lysates by using serum from an individual infected with HIV-1. In pellets of wild-type virions, significant amounts of the gp120 surface protein were detected, in addition to *gag*- and *pol*-encoded viral proteins (Fig. 6A, lane 1 and B, lanes 1 and 6). The gp41 transmembrane protein was also visible. Most MA mutants yielded amounts of pelletable *gag*- and *pol*-encoded proteins that were comparable to or, in some cases, greater than the amount produced upon transfection of the parental construct (Fig. 6). However, 9 of 11 mutations analyzed resulted in a dramatic reduction in the levels of Env glycoprotein detectable in virion

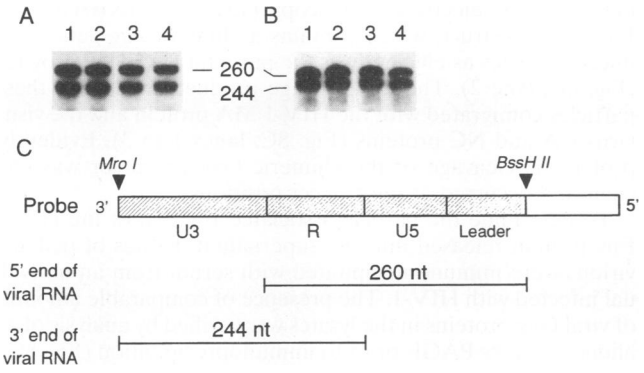

FIG. 5. Viral RNA content of MA mutant virions. Viral RNA extracted from equivalent amounts of viral particles as determined by $p24^{gag}$ radioimmunoassay was quantified by RNase protection analysis. Viral particles were produced in HeLa cells transfected with the parental provirus HXBH10 (A and B, lanes 1 and 2) or the MA mutants Δ16-18 (A, lane 3), WA36/37→SR (A, lane 4), Δ41-43 (B, lane 3), and YC86/87→SR (B, lane 4). Lanes 1 and 2 are duplicate samples. (C) Structure of the probe and expected size of fragments protected from RNase digestion by the 5′ and 3′ ends of the viral RNA. Hatched segments of the RNA probe refer to regions of complementarity between the probe and viral sequences.

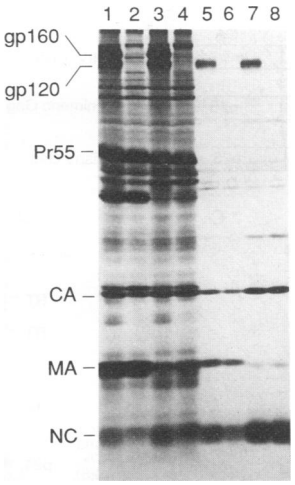

FIG. 7. Env glycoprotein does not influence the efficiency of particle production. HeLa cells were transfected with the parental provirus HXBH10 (lanes 1 and 5), the MA mutant Δ16-18 (lanes 3 and 7), or the isogeneic envelope-deficient variants HXBH10-*env*⁻fs (lanes 2 and 6) and Δ16-18/*env*⁻fs (lanes 4 and 8). Following metabolic labeling with [^{35}S]cysteine, viral proteins were immunoprecipitated from the cell lysates (lanes 1 to 4) and supernatant fractions (lanes 5 to 8) with the N18 patient's serum.

pellets (Fig. 6). The amount of gp120 immunoprecipitated from virion pellets was comparable to the amount obtained after transfection of the HXBH10-*gag*⁻ provirus, a construct which expresses parental levels of Env glycoprotein but is unable to produce viral particles because of the presence of premature termination codons in the *gag* reading frame (Fig. 6B, lane 5). A band corresponding to gp41 was not detected (bottom part of Fig. 6B). All alterations within the amino-terminal 100 amino acids of MA that were tested reduced the amount of Env glycoprotein associated with pelleted virions to background levels. Such alterations included substitutions of two neighboring amino acids (Fig. 6A, lanes 2 to 4) and deletions of three or four amino acids (Fig. 6A, lanes 5 and 6; Fig. 6B, lanes 2, 3, 7, and 8). By contrast, alterations at the C terminus of MA (Fig. 6B, lanes 4 and 9) or in the adjacent CA domain (Fig. 6B, lanes 10 and 11) did not reduce particle-associated Env glycoprotein levels. The effect of alterations in MA on particle-associated Env glycoprotein levels correlated precisely with their effect on virus replication (compare Fig. 1 and 6).

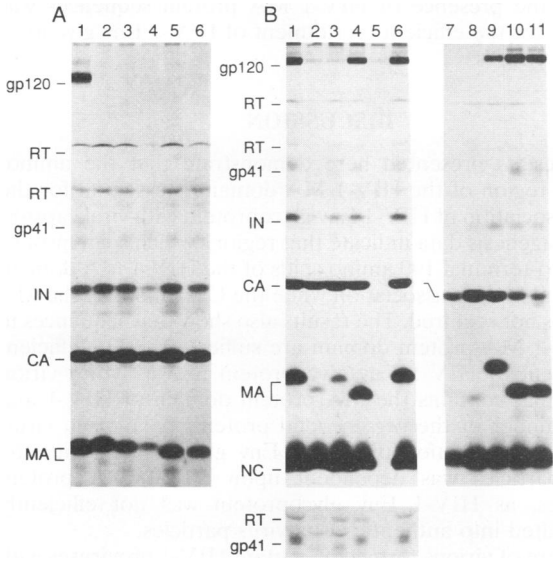

FIG. 6. Analysis of particle-associated HIV-1 Env glycoprotein. HeLa cells transfected with the parental or mutant proviruses were metabolically labeled with [^{35}]cysteine, and particulate material released into the supernatant was pelleted through 20% sucrose. Viral proteins in the pellets were analyzed by immunoprecipitation with the N18 patient's serum. (A) Lanes: 1, HXBH10; 2, LS8/9→SR; 3, WA36/37→SR; 4, YC86,87→SR; 5, Δ16-18; 6, Δ41-43. (B) Lanes: 1 and 6, HXBH10; 2, Δ27-30; 3, Δ77-80; 4, N126→R; 5, HXBH10-*gag*⁻; 7, Δ63-65; 8, Δ98-100; 9, Δ112-114; 10, CAΔ19-21; 11, CAΔ43-45. For lanes 1 to 6 of panel B, a longer autoradiographic exposure for the position occupied by the gp41 transmembrane protein is shown at the bottom. RT, reverse transcriptase; IN, integrase.

Some of the mutations, in particular the Δ16-18 mutation, prevented a stable association of Env glycoprotein with viral particles and also resulted in a higher particle yield. This observation suggested that the capsid-Env interaction may have been rate limiting for virus particle production. To test this possibility, the amounts of wild-type and mutant Gag protein released in the presence and absence of Env glycoprotein expression were compared. As shown in Fig. 7, disruption of the *env* gene in the parental provirus did not affect Gag protein release. The Δ16-18 mutation increased the amount of Gag protein released independent of Env glycoprotein expression. These results demonstrate that the effect of the Δ16-18 mutation on particle production is not a direct consequence of its effect on particle-associated Env glycoprotein levels.

The HIV-1 MA domain is sufficient for the efficient association of HIV-1 Env glycoprotein with heterologous particles. The experiments described above indicate that a domain comprising the amino-terminal 100 amino acids of the HIV-1 MA protein is essential for the incorporation of HIV-1 Env glycoprotein into virions. In an attempt to determine whether the HIV-1 MA domain is sufficient to direct the incorporation of HIV-1 Env glycoprotein, all HIV-1 *gag* sequences except for

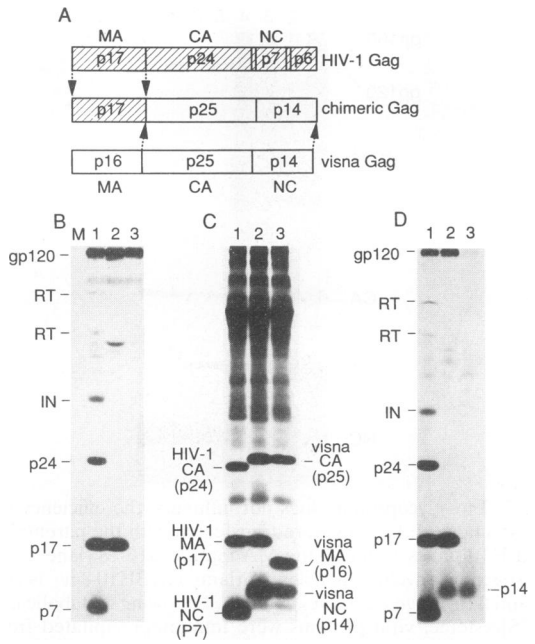

FIG. 8. Association of HIV-1 Env glycoprotein with a chimeric viral particle. (A) Domain organization of the parental and chimeric *gag* gene products. (B) Immunoprecipitation of HIV-1-encoded proteins from the total supernatant fractions of HeLa cells transfected with proviral constructs. (C and D) Analysis of particle-associated viral proteins. Particles released from transfected HeLa cells were pelleted through 20% sucrose and disrupted in RIPA buffer, and aliquots were analyzed directly either by SDS-PAGE (C) or by immunoprecipitation with the N18 serum, followed by SDS-PAGE (D). (B to D) The transfected proviruses were the parental HIV-1 construct HXBH10 (lanes 1), the chimeric *gag* gene construct HXBH10-MA/$LV_{gag\text{-}pol}$ (lanes 2), and the visna *gag* gene construct HXBH10/$LV_{gag\text{-}pol}$ (lanes 3).

those coding for the MA domain of the Gag precursor were replaced by corresponding sequences from visna lentivirus. Visna virus *gag* sequences were chosen as a replacement, as the Gag precursors of HIV-1 and visna virus share a similar domain organization, yet their primary sequences differ considerably (36).

To test whether the authentic visna virus *gag* gene product is capable of a stable association with HIV-1 Env glycoprotein within virion particles, the chimeric HXBH10/$LV_{gag\text{-}pol}$ provirus, which has the *env* gene of HIV-1 and the *gag* and *pol* genes of visna virus, was used (14). The related proviral construct HXBH10-MA/$LV_{gag\text{-}pol}$ also has the *env* gene of HIV-1 but encodes a chimeric Gag precursor in which the HIV-1 MA domain is linked precisely at the proteolytic cleavage site to the CA and NC domains of the visna virus Gag precursor (Fig. 8A). Both the HXBH10/$LV_{gag\text{-}pol}$ and the HXBH10-MA/$LV_{gag\text{-}pol}$ chimeras direct the expression and release of similar amounts of HIV-1 Env glycoprotein upon transfection into HeLa cells as determined by immunoprecipitation from the cell lysates (data not shown) and supernatant fractions (Fig. 8B).

To determine the ability of the chimeras to produce viral capsids, particulate material released by transfected HeLa cells was pelleted through 20% sucrose. The presence of viral proteins in the pellets was analyzed by SDS-PAGE. As shown previously (14), the HXBH10/$LV_{gag\text{-}pol}$ construct efficiently produced particles that contain the visna virus *gag* cleavage products (Fig. 8C, lane 3). Virus-like particles that have the size and morphological appearance of visna virus capsids were detectable by electron microscopy (13). The HXBH10-MA/$LV_{gag\text{-}pol}$ construct, which contains a chimeric *gag* gene, produced particles as efficiently as the parental HXBH10 provirus (Fig. 8C, lane 2). The *gag* cleavage products present in these particles comigrated with the HIV-1 MA protein and the visna virus CA and NC proteins (Fig. 8C, lanes 1 to 3). Evidently, proteolytic cleavage of the chimeric Gag precursor was efficient and occurred at the correct positions.

To determine the particle-associated fraction of the HIV-1 Env protein released into the supernatant, lysates of pelleted virions were immunoprecipitated with serum from an individual infected with HIV-1. The presence of comparable amounts of viral Gag proteins in the lysates was verified by analysis of an aliquot by SDS-PAGE prior to immunoprecipitation (Fig. 8C). Also, similar amounts of HIV-1 MA protein were immunoprecipitated from lysates of particles produced by HXBH10 and HXBH10-MA/$LV_{gag\text{-}pol}$ (Fig. 8D, lanes 1 and 2). Furthermore, a small amount of visna virus NC protein was present in immunoprecipitates from particles produced by both HXBH10-MA/$LV_{gag\text{-}pol}$ and HXBH10/$LV_{gag\text{-}pol}$ (Fig. 8D, lanes 2 and 3), probably as a consequence of a nonspecific association of concentrated NC protein in virion lysates with the Sepharose beads used for immunoprecipitation (data not shown).

The amount of HIV-1 Env glycoprotein that was immunoprecipitated from the lysate of pelleted particles produced by the HXBH10/$LV_{gag\text{-}pol}$ construct did not exceed background levels obtained with the HXBH10-*gag*$^-$ construct, implying that HIV-1 Env glycoprotein was not efficiently incorporated into visna virus capsids (Fig. 8D, lane 3). By contrast, the hybrid particles produced by the HXBH10-MA/$LV_{gag\text{-}pol}$ construct contained as much HIV-1 Env glycoprotein as did wild-type HIV-1 particles (Fig. 8D, lanes 1 and 2), demonstrating that the presence of HIV-1 MA protein sequences was sufficient for the efficient recruitment of HIV-1 Env glycoprotein into virions.

DISCUSSION

The results presented here demonstrate that the amino-terminal region of the HIV-1 MA domain is essential for the stable association of HIV-1 Env glycoprotein with viral capsids. The mutagenesis data indicate that regions which span most of the amino-terminal 100 amino acids of the HIV-1 MA domain are critical for this association while the C terminus of the MA domain is not required. The results also show that sequences in the HIV-1 MA protein domain are sufficient for the efficient recruitment of HIV-1 Env glycoprotein into a hybrid virion particle that contains the MA protein domain of HIV-1 and the remainder of the *gag*-encoded proteins from visna virus. The stable association of HIV-1 Env glycoprotein with the hybrid particles was dependent upon HIV-1 MA protein sequences, as HIV-1 Env glycoprotein was not efficiently incorporated into authentic visna virus particles.

A failure of virions formed by mutant HIV-1 proviruses with larger deletions in the MA domain to incorporate Env glycoprotein was reported previously (45). The results reported here are in agreement with this finding and demonstrate that the HIV-1 capsid-Env glycoprotein interaction is remarkably sensitive even to more subtle alterations within most of the MA domain. Without exception, small in-frame deletion or missense mutations within the coding sequence for the N-terminal 100 amino acids of the MA domain reduced particle-associated Env glycoprotein close to background levels. However, these mutations had no effect on Env glycoprotein synthesis or processing. Neither the incorporation of Env glycoprotein nor

virus replication was significantly affected by two alterations near the C terminus of the MA protein domain. A recent analysis of mutant HIV-1 proviruses with more extensive alterations in this region has suggested that the C terminus of the MA protein is involved in early steps of the virus life cycle, such as virus penetration or uncoating (44).

Mutations throughout most of the MA protein-coding region significantly affected the association of Env glycoprotein with the HIV-1 capsid, suggesting that a three-dimensional structure, rather than sequence specificity, governs the Env-MA protein interaction. Although the outer envelope component gp120 appears to be very labile even in the mature wild-type virion (11, 12), the virtual absence of Env glycoprotein from the mutant HIV-1 particles could not be explained simply as a result of stripping of loosely associated gp120 during centrifugation, since both the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 were affected. For the same reason, it is more likely that the reduction in particle-associated Env glycoprotein levels was a consequence of inefficient incorporation rather than a rapid loss of previously incorporated Env glycoprotein.

The failure of visna virus capsids to stably associate with HIV-1 Env glycoprotein was unexpected in view of the efficient formation of pseudotypes between more distantly related retroviruses (18–21, 34, 42). It is possible that infectious pseudotype viruses can be detected even when low levels of Env glycoprotein are incorporated, but the immunological methods used here may lack the sensitivity of a biological assay. Moreover, although it has been shown that HIV-1 can be pseudotyped by the Env glycoproteins of widely divergent retroviruses (18, 20, 21, 34), the reciprocal incorporation of HIV-1 Env glycoprotein into other retroviral capsids may be less efficient. In comparison with other retroviruses, the transmembrane proteins of lentiviruses like that of HIV-1 have unusually long cytoplasmic domains (9, 16, 32), which may interfere with their incorporation into heterologous capsids. This possibility is consistent with the observation that HIV-1 can be efficiently pseudotyped by murine leukemia virus Env glycoproteins (18, 21, 34), whereas attempts to complement Moloney murine leukemia virus cores with HIV-1 Env glycoprotein have not succeeded (42).

Despite limited sequence homology, the HIV-1 MA domain can fully substitute for the visna virus MA domain in the assembly, release, and proteolytic maturation of viral capsids, suggesting that the HIV-1 and visna virus MA domains share structural similarities. However, the HIV-1 and visna virus MA domains differed considerably in their ability to recruit HIV-1 Env glycoprotein into viral particles. The replacement of the visna virus MA domain by that of HIV-1 led to a significant increase in levels of particle-associated HIV-1 Env glycoprotein, indicating that the MA protein domain confers at least some specificity to the capsid-Env glycoprotein interaction. Alternatively, it is conceivable that the MA domains of HIV-1 and visna virus direct capsid formation to different regions of the cell membrane which contain different amounts of HIV-1 Env glycoprotein. Since the MA domain of HIV-1, in contrast to that of visna virus, is modified by N-terminal attachment of a myristic acid (27), another possible explanation for this apparent specificity is a potential role of the myristic acid moiety of MA in the incorporation of HIV-1 Env glycoprotein.

A close physical association between retroviral MA and Env proteins was suggested by the finding that the MA protein of Rous sarcoma virus can be cross-linked to the viral transmembrane protein (10). Furthermore, point mutations within the MA domain of the Mason-Pfizer monkey virus *gag* gene can affect a late maturation step of the transmembrane Env protein in cell-released virions, providing genetic evidence that the MA and Env proteins of this virus interact (2). In the case of HIV-1, indirect evidence for a specific interaction between Gag and Env proteins was provided by the observation that the Env glycoprotein determines the site of capsid assembly and release in polarized epithelial cells (22). This result also suggested that an interaction between HIV-1 Gag and Env proteins may occur prior to their transport to the cell membrane.

It appears likely that signals in the transmembrane Env protein are involved in the interaction between Env and MA. Some studies indicate that mutations in the cytoplasmic domain of the gp41 transmembrane protein of HIV-1 impair the incorporation of Env glycoprotein into virions (6, 46). In contrast, other studies have concluded that the cytoplasmic domain of gp41 is not required for Env glycoprotein incorporation or virus replication (8, 39). In the latter studies, a decrease in Env glycoprotein incorporation into virions is accompanied by a decrease in cell surface expression of the mutant glycoproteins. It was previously reported that the cytoplasmic tail of the transmembrane protein of Rous sarcoma virus is dispensable for the incorporation of Env glycoprotein into virions (25). These observations point to the hydrophobic anchor domain of the transmembrane protein as a possible site of interaction with the MA domain of the Gag precursor.

The sensitivity of the HIV-1 capsid-Env glycoprotein interaction to even minor alterations in the MA domain suggests that this interaction may be a promising target for antiviral drug development. It will be of interest to determine whether the incorporation of heterologous viral Env glycoproteins or of some cellular proteins into HIV-1 virions is similarly dependent on the integrity of the MA domain or is mediated by a different mechanism.

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