Role of the Major Homology Region of Human Immunodeficiency Virus Type 1 in Virion Morphogenesis

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Received 4 February 1994/Accepted 21 April 1994

Retroviral capsid (CA) proteins contain a uniquely conserved stretch of 20 amino acids which has been named the major homology region (MHR). To examine the role of this region in human immunodeficiency virus type 1 morphogenesis and replication, four highly conserved positions in the MHR were individually altered by site-directed mutagenesis. Conservative substitution of two invariant residues (glutamine 155 and glutamic acid 159) abolished viral replication and significantly reduced the particle-forming ability of the mutant *gag* gene products. Conservative substitution of the third invariant residue in the MHR (arginine 167) or of an invariably aromatic residue (tyrosine 164) had only a moderate effect. However, removal of the extended side chains of these amino acids by substitution with alanine prevented viral replication and affected virion morphogenesis. The replacement of tyrosine 164 with alanine substantially impaired viral particle production. By contrast, the substitution of arginine 167 with alanine had only a two- to threefold effect on particle yield but led to the formation of aberrant core structures. The MHR mutants which were severely defective for particle production had a dominant negative effect on particle formation by the wild-type Gag product. The role of the MHR in the incorporation of the Gag-Pol precursor was examined by expressing the Gag and Gag-Pol polyproteins individually from separate plasmids. Only when the two precursor polyproteins were coexpressed did processed Gag and Pol products appear in the external medium. The appearance of these products was unaffected or only moderately affected by substitutions in the MHR of the Gag-Pol precursor, suggesting that the mutant Gag-Pol precursors were efficiently incorporated into viral particles. The results of this study indicate that specific residues within the MHR are required both for human immunodeficiency virus type 1 particle assembly and for the correct assembly of the viral core. However, mutant Gag and Gag-Pol polyproteins with substitutions in the MHR retained the ability to interact with wild-type Gag protein.

Retroviral particles are released by budding from the plasma membrane of the host cell, thereby acquiring a plasma membrane-derived lipid envelope (40). The internal structural proteins of human immunodeficiency virus type 1 (HIV-1), like those of other retroviruses, are encoded by the viral *gag* gene and are translated as a polyprotein precursor (Pr55^{gag}) (47). During translation of *gag*, ribosomal frameshifting into the *pol* reading frame occurs at a frequency of 5 to 10% and leads to the synthesis of a Gag-Pol fusion protein (Pr160^{gag-pol}) (26, 49). HIV-1 particle morphogenesis begins with the assembly of Pr55^{gag} and Pr160^{gag-pol} precursors underneath the cell membrane (11). An increasingly spherical structure is formed that eventually pinches off, resulting in virus particle release (11). The cotranslational attachment of myristic acid at the N terminus of Pr55^{gag} is required for particle assembly (3, 18). Expression of Pr55^{gag} alone in the absence of other viral gene products has been shown to be sufficient for particle formation (13, 19, 37). It has also been shown that the small accessory protein Vpu can greatly facilitate the release of nascent HIV-1 virions from the plasma membrane (16, 27, 42, 50). During or subsequent to the release of an immature particle, cleavage of Pr55^{gag} by the viral protease yields the mature Gag products p17 (MA), p24 (CA), p7 (NC), and p6 (22, 23, 31). Proteolytic cleavage of Pr160^{gag-pol} yields the essential viral enzymes

protease, reverse transcriptase, and integrase, in addition to Gag products (23).

Recent studies have shown that the HIV-1 MA domain, which forms an envelope-associated outer shell in the mature virion (12), is required for the incorporation of viral Env glycoprotein during particle assembly (8, 10, 51). Sequences within the MA domain are also critical for the targeting of Pr55^{gag} to the plasma membrane (10, 52). The deletion of MA sequences involved in membrane targeting apparently results in the redirection of viral particle assembly and budding to the membranes of the endoplasmic reticulum (10). The HIV-1 MA domain has also been shown to contain a nuclear localization sequence, which contributes to the ability of HIV-1 to replicate in nondividing cells (4). The NC domain of the HIV-1 Gag precursor, which contains two copies of a highly conserved cysteine-histidine motif, is involved in the specific recognition and incorporation of genomic viral RNA into assembling particles (1, 2, 7, 15, 30). The p6 domain, which forms the C terminus of Pr55^{gag}, can facilitate the release of budding particles from the cell surface (17) and appears to be required for the incorporation of the accessory viral protein Vpr into virions (29, 34).

By comparison, relatively little is known about the function of the CA domain of the HIV-1 Gag polyprotein. Accumulating evidence from the analysis of widely divergent retroviruses suggests that the CA domain contains sequences that play a central role in particle assembly (5, 21, 25, 38, 44, 47). However, it was shown for HIV-1 and Rous sarcoma virus that a large portion of the CA domain is dispensable for particle production (45, 46, 47). In the mature HIV-1 virion, CA forms

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the shell of the cone-shaped core structure which surrounds a complex of genomic RNA and viral *gag*- and *pol*-encoded proteins (12, 24). Apart from the cysteine-histidine boxes in the NC domain, CA contains the only other sequence motif which is highly conserved among the *gag* gene products of distantly related retroviruses. The conserved motif in CA, which has been referred to as the major homology region (MHR), spans 20 residues (47). The MHR is conserved in all replication-competent retroviruses for which the sequence of the *gag* gene has been determined except spumaretroviruses.

The evolutionary conservation of the MHR strongly suggests that it has an important function in retroviral replication. To examine the role of the MHR in the life cycle of HIV-1, the three invariant residues in the MHR were altered by site-directed mutagenesis. In addition, an invariably aromatic position was altered at which the primate immunodeficiency viruses differ from all other retroviruses. The results of these studies indicate a role of the MHR both in particle assembly and core morphogenesis.

MATERIALS AND METHODS

Proviral DNA constructs. For site-directed mutagenesis of the HIV-1 MHR, single-stranded DNA was prepared from plasmid pGEM*gag-pol* (18), which contains a 4.3-kb *SphI-SalI gag-pol* 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 (28). To regenerate full-length proviral clones after mutagenesis, 0.5-kb *SpeI-ApaI* fragments (nucleotides [nt] 1510 to 2009) carrying the desired mutations as confirmed by DNA sequence analysis were inserted into the parental *vpu*⁺ HXBH10 proviral construct (42) in exchange for the wild-type fragment. The following oligonucleotides were used for the construction of the mutant clones: Q155→N (5'-GGACATAAGAAACGGACCAAAGG-3'), E159→D (5'-GGACCAAAGGATCCCTTTAGAG-3'), Y164→F (5'-CCTTTAGAGACTTTGTAGACCG-3'), Y164→A (5'-CCTTTAGAGACGCGGTAGACCGGT-3'), R167→K (5'-CTATGTAGACAAGTTCTATAAA-3'), and R167→A (5'-CTATGTAGACGCGTTCTATAAA-3'). The presence of the mutations in the final proviral constructs was confirmed by DNA sequence analysis or by restriction enzyme digestion. The protease-defective variants Q155→N-PR⁻, E159→D-PR⁻, and Y164→A-PR⁻ were generated by replacing the segment between the unique *ApaI* site at nt 2009 and the unique *SalI* site at nt 5789 with the corresponding segment from HXBH10-PR⁻ (16), which has the codon for Asp-25 of the HIV-1 protease replaced by a codon specifying glutamic acid. The protease-defective construct HXBH5-PR⁻ was created by replacing the segment of HXBH10-PR⁻ between a *BglII* site at nt 2099 and a *PvuMI* site at nt 2486 with the corresponding segment from the BH5 clone (20), which carries a 36-nt duplication in the p6 domain of the *gag* gene (36).

HXBH10-*gag*⁻ is unable to express Pr55^{gag} or Pr160^{gag-pol} because of a premature termination codon in place of codon 8 of the *gag* gene and a frameshift mutation at the position of the unique *SpeI* site in the CA coding region (8). To generate the Pr55^{gag} expression construct HXBH10Δ*pol*, which lacks most of the *pol* gene, an *HpaI* site was introduced into HXBH10 by site-directed mutagenesis 3' of the *gag* gene, and the segment between the *HpaI* site and a *BalI* site (nt 2333 to 4552) was then deleted. To create the Pr160^{gag-pol} expression construct HXBH10-GPfs, an adenine residue was inserted by site-directed mutagenesis into pGEM*gag-pol* between nt 2090 and 2091. As described previously (32), this manipulation positions

the *gag* and *pol* genes into a single reading frame predicted to encode a Gag-Pol precursor identical to the predominant form generated by ribosomal frameshifting. After verification of the frameshift mutation by DNA sequence analysis, a 4.3-kb *SphI-SalI* fragment containing the mutated region was reintroduced into HXBH10 in exchange for the wild-type fragment. GPfs/Q155→N, GPfs/E159→D, and GPfs/Y164→A were obtained by replacing a *SpeI-ApaI* fragment (nt 1510 to 2009) of HXBH10-GPfs with the corresponding mutated fragments. GPfs/Y164→A-*gag*⁻ is identical to GPfs/Y164→A, except for the presence a premature termination codon and a frameshift mutation in the *gag* gene 5' to the region coding for the MHR and was obtained by replacing a *BsmI-SalI* fragment (nt 1630 to 5789) of HXBH10-*gag*⁻ with the corresponding fragment from GPfs/Y164→A.

Cell culture and transfections. Jurkat cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. HeLa cells (CCL2) 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 × 10⁶) were transfected by the DEAE-dextran procedure (35) with 2.5 μg of proviral DNA. Particle-associated reverse transcriptase activity in culture supernatants was determined as described elsewhere (6a). HeLa cells (10⁶) were seeded into 80-cm² tissue culture flasks 24 h prior to transfection. The cells were transfected with proviral plasmid DNA by a calcium phosphate precipitation technique (6).

Viral protein analysis. HeLa cell cultures and aliquots of transfected Jurkat cells were metabolically labeled for 12 h with [³⁵S]cysteine (50 μCi/ml). Labeling of HeLa cells was started 48 h posttransfection. Labeled cells were lysed in 1× radioimmunoprecipitation (RIPA) buffer (140 mM NaCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 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 with sera from individuals infected with HIV-1 and separated in SDS-11.5% polyacrylamide gels as described previously (18). To determine the amount of viral protein that existed in a particulate form, particulate material released into the supernatant was pelleted through a 20% sucrose cushion for 2 h at 27,000 rpm in a Beckman SW41 rotor. Pelleted material was then disrupted in RIPA buffer, and viral proteins were visualized directly by electrophoresis through SDS-11.5% polyacrylamide gels. Under these conditions, HIV-1 surface glycoprotein shed into the medium is excluded from the virion pellet (8).

Electron microscopy. Transfected HeLa cell cultures were fixed in fresh 2.5% glutaraldehyde in phosphate-buffered saline at pH 7.0 and postfixes with 1% OsO₄. After agar block enclosure, the fixed cells were embedded in Epon. Thin sections were made approximately 60 to 80 nm thick and were poststained with 1% uranyl acetate. Specimens were analyzed with a Zeiss CEM 902 electron microscope equipped with a goniometer stage at an accelerating voltage of 80 kV.

RESULTS

Effects of MHR mutations on virus replication. To study the function of the MHR, the three invariant residues of the motif (Fig. 1) were individually altered by site-directed mutagenesis. In an attempt to minimize structural alterations caused by the side chain replacements, conservative substitutions of glutamine 155 to asparagine, glutamic acid 159 to aspartic acid, and arginine 167 to lysine were made. In addition, an invariably

HIV-1 (HXB2)	I	R	Q	G	P	K	E	P	F	R	D	Y	V	D	R	F	Y	K	T	L
HIV-2 (ROD)	I	K	Q	G	P	K	E	P	F	Q	S	Y	V	D	R	F	Y	K	S	L
SIVmac239	V	K	Q	G	P	K	E	P	F	Q	S	Y	V	D	R	F	Y	K	S	L
FIV	L	R	Q	G	A	K	E	D	Y	S	S	F	I	D	R	L	F	A	Q	I
BIV	I	H	Q	G	P	K	E	P	Y	T	D	F	I	N	R	L	V	A	A	L
VISNA	V	K	Q	K	N	T	E	S	Y	E	D	F	I	A	R	L	L	E	A	I
CAEV	V	K	Q	K	T	N	E	P	Y	E	D	F	A	A	R	L	L	E	A	I
EIAV	I	R	Q	G	A	K	E	P	Y	P	E	F	V	D	R	L	L	S	Q	I
HTLV-I	I	L	Q	G	L	E	E	P	Y	H	A	F	V	E	R	L	N	I	A	L
BLV	I	V	Q	G	P	A	E	S	S	V	E	F	V	N	R	L	Q	I	S	L
MPMV	V	K	Q	G	P	D	E	P	F	A	D	F	V	H	R	L	I	T	T	A
MMTV	L	K	Q	G	N	E	E	S	Y	E	T	F	I	S	R	L	E	E	A	V
RSV	I	M	Q	G	P	S	E	S	F	V	D	F	A	N	R	L	I	K	A	V
Mo-MLV	I	T	Q	G	P	N	E	S	P	S	A	F	L	E	R	L	K	E	A	Y
FeLV	V	V	Q	G	K	E	E	T	P	A	S	F	L	E	R	L	K	E	A	Y

FIG. 1. Alignment of a highly conserved region (MHR) in the CA domains of different retroviruses. The region shown represents residues 153 to 172 of the HIV-1 CA domain. The three invariant residues in the MHR are indicated by shaded boxes. An invariably aromatic position is highlighted by an open box. SIVmac239, simian immunodeficiency virus isolate mac239; FIV and BIV, feline and bovine immunodeficiency viruses; CAEV, caprine arthritis-encephalitis virus; EIAV, equine infectious anemia virus; HTLV-I, human T-cell leukemia virus type I; BLV, bovine leukemia virus; MPMV, Mason-Pfizer monkey virus; MMTV, mouse mammary tumor virus; RSV, Rous sarcoma virus; Mo-MLV, Moloney murine leukemia virus; FeLV, feline leukemia virus.

aromatic residue, tyrosine 164, was replaced by phenylalanine. A phenylalanine is present in most retroviruses at the equivalent position of the MHR (Fig. 1). We then reconstructed full-length proviruses that differ from the parental HXBH10 proviral clone only by the presence of the point mutations in the region encoding the MHR.

To determine the ability of the mutant proviruses to initiate viral replication in a permissive cell line, the mutant DNAs were transfected into the human CD4⁺ cell line Jurkat. The sustained expression of viral proteins following transfection of Jurkat cells requires virus replication (18). Equivalent numbers of cells from the transfected cultures were metabolically labeled for 12 h with [³⁵S]cysteine at 4, 8, and 30 days after transfection. The cells were then lysed in RIPA buffer, and viral proteins were immunoprecipitated from the cell lysates with serum from an individual infected with HIV-1.

At day 4 posttransfection, *gag*- and *env*-encoded proteins were detected in the lysate of cells transfected with the parental construct and in lower amounts in cells transfected with the Y164→F and R167→K mutants (Fig. 2A, lanes 1, 4, and 6). Viral protein could not be detected at this time point in cells transfected with the Q155→N and E159→D mutants (Fig. 2A, lanes 2 and 3). At day 8, cultures transfected with the Y164→F and R167→K mutants contained as much viral protein as the culture transfected with the parental construct (not shown). Cultures transfected with the Q155→N and E159→D mutants did not yield detectable viral protein even 1 month after transfection (Fig. 2B, lanes 1 and 2). These results indicate that the invariant residues in the N-terminal portion of the MHR are essential for virus replication. By contrast, conservative substitutions at invariant or highly conserved

FIG. 2. Effects of substitutions in the MHR on viral replication. Jurkat cells were transfected with the parental provirus HXBH10 (wild type [WT]) or with the indicated mutant proviruses. Viral protein expression was measured 4 days (A) and 30 days (B) posttransfection by immunoprecipitation from the lysates of metabolically labeled cells with patient serum N18. (C) Particle-associated reverse transcriptase activity released into the culture supernatant of Jurkat cells transfected with the indicated proviruses.

positions in the C-terminal part of the motif caused only a moderate delay in virus replication (Fig. 2C). However, removal of the extended side chain at these positions by replacement of tyrosine 164 or arginine 167 with alanine also abolished virus replication (Fig. 2A, lanes 5 and 7; Fig. 2B, lanes 4 and 6).

Effect on particle production. To study the effects of the mutations on protein stability and viral particle production, the mutant proviruses were transfected into HeLa cells. HeLa cells do not support replication of HIV-1, as they lack the CD4 receptor. Cells without the CD4 receptor were used to prevent amplification through virus spread. The expression of *gag*-encoded proteins and their release from the transfected cells were monitored by immunoprecipitation from the cell lysate and supernatant fractions.

As shown in Fig. 3A, the Gag precursor Pr55^{gag} was at least

FIG. 3. Effects of substitutions in the MHR on Gag protein release. (A) Effects of conservative substitutions. (B and C) Effects of nonconservative substitutions. HeLa cells were transfected with 30 μ g of the indicated proviral plasmid DNAs and metabolically labeled from 48 to 60 h posttransfection. (A) Viral proteins were immunoprecipitated from the cell lysates with patient serum N21 (lanes 1 to 6) and from aliquots of the cell-free supernatant fractions with patient serum N21 (lanes 7 to 12) or N18 (lanes 13 to 17). (B) Viral proteins were immunoprecipitated from the supernatant fractions with patient serum N18. (C) Particulate material released into the culture supernatant was pelleted through 20% sucrose, disrupted in RIPA buffer, and directly analyzed by SDS-PAGE.

as abundant in cells transfected with the replication-defective mutants Q155 $\rightarrow$ N and E159 $\rightarrow$ D (lanes 3 and 4) as in cells transfected with the parental construct (lane 2). However, in cells transfected with the Q155 $\rightarrow$ N or E159 $\rightarrow$ D mutant viral DNA, no CA protein was detectable and the levels of MA protein were more than fivefold lower than those in cells transfected with the parental construct (Fig. 3A, lanes 2 to 4), suggesting that the mutations caused a defect in Gag polyprotein processing. Immunoprecipitation of the supernatant fractions with two different patient sera revealed a major defect in Gag protein release. The only Gag products detectable in the supernatant of cells transfected with the Q155 $\rightarrow$ N and E159 $\rightarrow$ D mutants were MA and NC. The levels of MA and NC observed after transfection of the E159 $\rightarrow$ D mutant (lanes 10 and 15) were at least 15- to 20-fold lower than those obtained with the wild-type construct (lanes 8 and 13). Transfection of the Q155 $\rightarrow$ N mutant resulted in even lower levels of MA and NC (lanes 9 and 14).

The Y164 $\rightarrow$ F and R167 $\rightarrow$ K mutations had only a minor effect on the amount and profile of Gag products in the cell lysates (lanes 5 and 6) and supernatant fractions (lanes 11, 12, 16, and 17). However, replacement of tyrosine 164 by alanine caused a defect in Gag protein release which was similar to the defect caused by the Q155 $\rightarrow$ N mutation (Fig. 3B, lane 3). By contrast, replacement of arginine 167 by alanine had only a moderate effect on the amount of viral protein released (Fig. 3B, lane 5). In several independent experiments, the R167 $\rightarrow$ A mutation caused a two- to threefold reduction in the amount of MA or NC protein detectable in the supernatant fraction. Similar to the other mutations that prevented virus replication, the R167 $\rightarrow$ A mutation led to a disproportionate reduction in the amount of CA protein detectable by immunoprecipitation.

To determine whether Gag protein synthesized by the R167 $\rightarrow$ A mutant was released in a particulate form, [35 S]cysteine-labeled particulate material shed into the culture medium was centrifuged through a 20% sucrose cushion and directly analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The amount of MA, NC, or gp120 envelope glycoprotein in the pellet was two- to threefold lower than that obtained with the parental construct (Fig. 3C). Mutant CA protein was present at only about twofold-lower levels than the other Gag products, indicating that the significantly lower levels of mutant CA detected by immunoprecipitation could be accounted for by the loss of an epitope (compare Fig. 3B and C). Viral particles produced by the R167 $\rightarrow$ A mutant contained similar amounts of viral RNA as wild-type particles, as measured by RNase protection analysis (data not shown).

Mutant Gag products that interfere with particle production by wild-type Gag protein. To examine whether Gag products defective for particle production retained the ability to interact with wild-type Gag molecules, mutant and wild-type Gag polyproteins were coexpressed. Proteolytic processing of the Gag polyproteins was avoided by replacing the codon for aspartic acid 25 of the viral protease in each mutant by a codon specifying glutamic acid, thereby generating Q155 $\rightarrow$ N-PR $^{-}$, E159 $\rightarrow$ D-PR $^{-}$, and Y164 $\rightarrow$ A-PR $^{-}$. For the expression of wild-type Gag polyprotein, the protease-defective proviral construct HXBH5-PR $^{-}$ was used. HXBH5-PR $^{-}$ was created by replacing the p6 domain of HXBH10-PR $^{-}$ with that of the BH5 molecular clone. Since the *gag* gene of BH5 encodes an additional 12 amino acids as a result of a duplication in p6 (36), HXBH5-PR $^{-}$ has the potential to synthesize a slightly larger Gag precursor than the MHR mutant constructs. The *gag* gene of HXBH5-PR $^{-}$ is functional, since a variant that encodes an intact protease is replication competent (data not shown).

Transfection of these constructs into HeLa cells and immu-

FIG. 4. *trans*-dominant negative effect of mutant Gag products on particle production. (A) HeLa cells were transfected with 15 μ g of protease-defective proviral plasmid DNAs or combinations of DNAs as indicated. After metabolic labeling from 48 to 60 h posttransfection, Gag and Env proteins present in the cell lysates and culture supernatants were immunoprecipitated and separated in an SDS-7.5% polyacrylamide gel. The HXBH5-PR⁻ construct (lane 1) has a wild-type MHR but carries a 36-nt duplication in the p6 domain of the *gag* gene and therefore expresses a Gag polyprotein (Pr56^{gag}) that is detectably larger than the Pr55^{gag} product expressed by the MHR mutants (lanes 2 to 4). (B) HeLa cells were transfected with 10 μ g of the parental HXBH10 proviral DNA (wild type [WT]; lane 1) or cotransfected with 10 μ g of HXBH10 DNA and 10, 20, or 40 μ g of the Y164 $\rightarrow$ A DNA (lanes 2 to 4) or the HXBH10-*gag*⁻ DNA (lanes 5 to 7). After metabolic labeling with [³⁵S]cysteine from 48 to 60 h posttransfection, viral proteins released into the supernatant fractions were immunoprecipitated and analyzed by electrophoresis through an SDS-11.5% polyacrylamide gel.

nonprecipitation of the cell lysates revealed that HXBH5-PR⁻ and each of the protease-defective MHR mutants produced similar amounts of uncleaved Gag protein (Fig. 4A, lanes 1 to 4). As expected, the Gag polyprotein of HXBH5-PR⁻ (Pr56^{gag}) exhibited an electrophoretic mobility in SDS-PAGE slightly slower than that of the HXBH10-based proviruses (Pr55^{gag}). The Gag precursor of HXBH5-PR⁻ was efficiently released into the supernatant (Fig. 4A, lane 9). By contrast, very little Gag protein could be detected in the supernatant of cells transfected with the protease-defective MHR mutants (Fig. 4A, lanes 10 to 12), demonstrating that the defect in particle production caused by the Q155 $\rightarrow$ N, E159 $\rightarrow$ D, and Y164 $\rightarrow$ A mutations is also observed in the absence of Gag polyprotein processing.

In cells cotransfected at a ratio of 1:1 with HXBH5-PR⁻ and either Q155 $\rightarrow$ N-PR⁻, E159 $\rightarrow$ D-PR⁻, or Y164 $\rightarrow$ A-PR⁻, the Pr56^{gag} product of HXBH5-PR⁻ and the Pr55^{gag} products of the MHR mutants were each detected at levels similar to those observed in individually transfected cultures (Fig. 4A, lanes 5 to 7). However, immunoprecipitation from the supernatant fractions revealed that both Pr56^{gag} and Pr55^{gag} were inefficiently released from the cotransfected cells. The release of Pr56^{gag} was reduced more than 10-fold by cotransfection of Q155 $\rightarrow$ N-PR⁻ or Y164 $\rightarrow$ A-PR⁻ and by about 5-fold in the presence of the E159 $\rightarrow$ D-PR⁻ construct (Fig. 4A; compare lane 9 with lanes 13 to 15).

These results suggested that the mutant Gag products, although severely defective for particle production, were still able to interact with other Gag molecules and thereby prevented their incorporation into virions. The dominant negative

effect of the Y164 $\rightarrow$ A mutation was further examined by cotransfection of HeLa cells with wild-type HXBH10 proviral DNA and increasing amounts of Y164 $\rightarrow$ A mutant DNA. As shown in Fig. 4B, a reduction in the amount of Gag proteins released into the medium was observed as increasing amounts of the Y164 $\rightarrow$ A DNA were added (lanes 2 to 4). A 4-fold excess of Y164 $\rightarrow$ A mutant DNA relative to the amount of wild-type proviral DNA transfected resulted in a more than 30-fold reduction in total Gag protein release, as measured by densitometry (Fig. 4B; compare lanes 1 and 4). This effect differed substantially from that seen after cotransfection of increasing amounts of HXBH10-*gag*⁻ mutant DNA, which is unable to synthesize Gag products as a result of the presence of premature termination codons in the *gag* gene (Fig. 4B, lanes 5 to 7). Up to a fourfold excess of HXBH10-*gag*⁻ proviral DNA did not significantly interfere with the expression and release of Gag protein by the wild-type provirus.

Incorporation of mutant Pr160^{gag-pol} into viral particles. The incorporation of Pr160^{gag-pol} into budding particles is thought to be mediated through interactions of its amino-terminal *gag* domain with Pr55^{gag} (47). To examine whether the residues in the MHR that were shown to be critical for particle production are also important for the incorporation of Pr160^{gag-pol}, complementation experiments were performed in which Pr55^{gag} and Pr160^{gag-pol} were independently expressed from different plasmids. To express Pr55^{gag} in the absence of Pr160^{gag-pol}, most of the *pol* gene of HXBH10 was deleted, yielding HXBH10 Δ *pol*. The remaining *pol* sequences in HXBH10 Δ *pol* code for the first 23 amino acids of the viral protease, followed by two missense codons and a premature

FIG. 5. Independent expression of Pr55^{gag} and Pr160^{gag-pol} from separate plasmids. [³⁵S]cysteine-labeled viral proteins were immuno-precipitated with patient serum N18 from the culture supernatant of HeLa cells transfected with individual Pr55^{gag} and Pr160^{gag-pol} expressor plasmids or with a combination of these plasmids. The cells were metabolically labeled from 48 to 60 h posttransfection. (A) HeLa cells were transfected with 25 μ g of the parental HXBH10 proviral DNA (wild type [WT]; lane 1), the wild-type Pr160^{gag-pol} expressor construct HXBH10-GPfs (lane 2), or the designated mutant Pr160^{gag-pol} expressor constructs (lanes 3 to 5). Lane 6, mock transfection. (B) HeLa cells were transfected with 25 μ g of the Pr55^{gag} expressor construct HXBH10 Δ pol (lane 1) or cotransfected with 25 μ g of HXBH10 Δ pol and 2.5 μ g of either HXBH10-gag⁻ (lane 2), HXBH10-GPfs (lane 3), GPfs/Q155 $\rightarrow$ N (lane 4), GPfs/E159 $\rightarrow$ D (lane 5), or GPfs/Y164 $\rightarrow$ A (lane 6). Lane 7, mock transfection; lane 8, transfection of 25 μ g of HXBH10 DNA (wild type).

termination codon. A Pr160^{gag-pol} expression vector was obtained by fusing the *gag* and *pol* reading frames at the position of the ribosomal frameshift site (26). The resulting HXBH10-GPfs mutant was expected to express an authentic Pr160^{gag-pol} precursor but no Pr55^{gag}.

Transfection of HXBH10- Δ pol into HeLa cells resulted in the release of Pr55^{gag} into the culture medium (Fig. 5B, lane 1). Processed Gag products were not detected as expected because HXBH10- Δ pol encodes a truncated viral protease. Consistent with previously published results (39), Pr160^{gag-pol} and a small amount of apparent cleavage products, including CA and MA, could be detected in cells transfected with HXBH10-GPfs but not in the supernatant fraction (Fig. 5A, lane 2, and data not shown). To achieve independent expression of Pr55^{gag} and Pr160^{gag-pol} at a molar ratio which is similar to that observed for the wild-type virus (26, 49), HeLa cells were cotransfected with HXBH10- Δ pol and HXBH10-GPfs at a ratio of 10:1. The cotransfection of 25 μ g of HXBH10- Δ pol and 2.5 μ g of HXBH10-GPfs resulted in the release of mostly completely processed Gag and Pol products (Fig. 5B, lane 3) in amounts similar to those observed after transfection of 25 μ g of wild-type proviral DNA (Fig. 5B, lane 8). Since neither Gag

nor Pol cleavage products were released from cells transfected with either construct alone, these results indicated that independently expressed Pr160^{gag-pol} was incorporated into particles formed by Pr55^{gag}.

To examine the role of the MHR in the incorporation of Pr160^{gag-pol}, the Q155 $\rightarrow$ N, E159 $\rightarrow$ D, and Y164 $\rightarrow$ A mutations were each introduced into HXBH10-GPfs. The amount and profile of Gag and Pol products released into the supernatant fraction was very similar when GPfs/Y164 $\rightarrow$ A was used instead of HXBH10-GPfs in a cotransfection experiment (Fig. 5B; compare lanes 3 and 6), indicating that the Y164 $\rightarrow$ A mutation did not significantly affect the efficiency of Pr160^{gag-pol} incorporation. Similar amounts of processed Gag products but slightly lower amounts of Pol cleavage products were obtained with the GPfs/Q155 $\rightarrow$ N construct (Fig. 5B, lane 4). Proteolytic cleavage of Pr55^{gag} was somewhat less efficient with the GPfs/E159 $\rightarrow$ D construct, but Pol cleavage products could still be detected (Fig. 5B, lane 5). The cleavage products did not arise as a result of homologous recombination between the cotransfected plasmids, since they were not observed upon cotransfection of HXBH10- Δ pol with HXBH10-gag⁻ (Fig. 5B, lane 2) or GPfs/Y164 $\rightarrow$ A-gag⁻, a variant of GPfs/Y164 $\rightarrow$ A that is unable to express a mutant Pr160^{gag-pol} product because of the presence of premature termination codons in the *gag* gene upstream of the Y164 $\rightarrow$ A mutation (data not shown). These results indicate that all three mutant Gag-Pol precursors can be incorporated into virus-like particles formed by wild-type Pr55^{gag}, although the efficiency of incorporation and/or proteolytic processing was somewhat reduced by the E159 $\rightarrow$ D mutation and to a lesser extent by the Q155 $\rightarrow$ N mutation.

Morphological analysis of R167 $\rightarrow$ A mutant virions. Since the R167 $\rightarrow$ A mutation had only a moderate effect on particle production but abolished virus replication, the morphologies of wild-type and R167 $\rightarrow$ A mutant virions were compared by electron microscopy. Virus particles were as readily detected in cultures transfected with the R167 $\rightarrow$ A mutant as in cultures transfected with the parental proviral genome. The morphology of virus particles was examined in different planes of section by goniometer analysis at different tilt angles. Wild-type virus particles appeared to be smaller and more uniform in size than R167 $\rightarrow$ A mutant particles. The diameter of R167 $\rightarrow$ A mutant particles varied between 110 and 220 nm, compared with 100 to 130 nm for wild-type virions. Otherwise, the morphologies of immature wild-type and mutant virus particles were similar (Fig. 6A and C). However, the mutant virions showed a significantly reduced frequency of structurally mature, cone-shaped cores (Table 1). In morphologically mature wild-type virions, a central, cone-shaped core structure was found in vertical sections (Fig. 6B). By contrast, in mutant virions, nonhomogeneous packing of core material was frequently observed as a dense round body adjacent to the viral envelope or in the center of the virion (Fig. 6C and D).

DISCUSSION

The results of this study demonstrate that two of the three invariant residues in the MHR are critically important for HIV-1 particle production. Even conservative alterations at positions 155 and 159 of the CA domain which preserved the nature of the side chains substantially reduced Gag protein release. A similar effect occurred when an invariably aromatic residue at position 164 of CA was nonconservatively substituted with alanine. These mutations all caused a decrease in the ratio of fully processed to unprocessed Gag products in cell lysates, indicating a defect in the activation of the viral protease, possibly as a consequence of impaired polyprotein

FIG. 6. Electron micrographs of HeLa cell cultures transfected with the parental HXBH10 proviral DNA (A and B) or the R167→A mutant DNA (C and D). Panels A and B show budding, immature, and mature wild-type particles with cone-shaped cores in vertical sections. Panels C and D show R167→A mutant particles with aberrant core structures as demonstrated by goniometer analysis. Bars represent 100 nm.

multimerization. The mutant CA proteins, in contrast to MA and NC proteins, were not detectable in cell lysates or in the external medium, suggesting inefficient immunorecognition or a loss of protein stability. However, protein instability does not explain the reduction in viral particle yield, since particle formation was also impaired when Gag polyprotein cleavage was prevented whereas the mutant Gag polyproteins were clearly stable.

The drastic effect of even conservative single amino acid substitutions in the MHR on particle production contrasts sharply with the lack of an effect of a large deletion closer to the N terminus of the CA domain (45). Apparently, N-terminal sequences within the HIV-1 CA domain are largely dispensable for particle formation, whereas the MHR and sequences close to the carboxy terminus (44) are crucial. However, results obtained for other retroviruses indicate that the presence of an MHR is not always required for efficient particle formation. For Rous sarcoma virus, it was shown that a mutant that lacked all of the MHR retained the ability to release particles at the same rate as wild-type virus (47). In the case of simian immunodeficiency virus, the MA domain alone was sufficient for the formation of virus-like particles (14). It is possible that the presence of modified MHR sequences interferes with particle formation, whereas the removal of the entire MHR

does not. Alternatively, certain assembly domains may be dispensable in some retroviruses but not in others.

The importance of specific residues within the MHR for particle formation has also been shown in the case of Mason-Pfizer monkey virus (41). However, random point mutations within the MHR of Mason-Pfizer monkey virus in most cases did not block particle formation but nevertheless abolished virus replication, indicating that the MHR also has an essential role during another stage of the viral replication cycle (41). A comparison of mutants of HIV-1 and Mason-Pfizer monkey virus with equivalent alterations suggests that the integrity of the HIV-1 MHR is less critical for virus replication than that of Mason-Pfizer monkey virus. The substitution of the invariant arginine residue in the MHR with lysine prevented the replication of Mason-Pfizer monkey virus (41) but not of HIV-1. In addition, a conservative change at an invariably aromatic position from phenylalanine to tyrosine was lethal in Mason-Pfizer monkey virus (41). By contrast, in HIV-1, in which the equivalent position is occupied by a tyrosine, the reciprocal change to phenylalanine had only a minor effect on virus replication.

Mutant Gag proteins that are unable to form viral particles can in some cases be efficiently incorporated into virions when coexpressed with wild-type Gag protein (47). This phenotype

TABLE 1. Quantitative analysis of virus core morphology of HIV-1 particles produced in HeLa cells

Virion morphology	% with morphology	
	Wild type	R167→A
Mature particles with a cone shaped core structure ^a	24	1
Immature particles or budding structures	28	22
Particles with a lateral body of round, dense core material	13	55
Particles with a central round core structure ^b	27	12
Other ^c	8	10
Total no. of particles	507	501

^a Vertical sections of structurally mature particles with a cone-shaped core structure.

^b Horizontal sections of the wild-type, cone-shaped core demonstrated a dense structure in the center of the virion, whereas mutant cores appeared round at all tilt angles.

^c Includes particles with no visible defined core structure, particles with a tubular core structure, and particles with two core structures.

appears to be particularly common for mutants with alterations in the MA protein domain and has been described for Gag mutants of both Rous sarcoma virus (48) and HIV-1 (52). By contrast, the results of this study show that mutant Gag products with alterations in the MHR could not be rescued into virions by the wild type HIV-1 Gag protein. Rather, they exhibited a significant inhibitory effect on the production of particles by wild-type Gag protein. This inhibitory effect was even more apparent in the absence of a functional viral protease, perhaps because a lack of proteolytic processing already causes a reduction in the efficiency of particle production (11). A previous study has suggested that even grossly modified Gag precursors can still interact with the wild-type Gag product and exert a *trans*-acting inhibitory effect on HIV-1 replication (43). However, it was not determined whether the block in replication occurred at the level of particle formation. The *trans*-dominant negative effect of Gag products with substitutions in the MHR on particle production suggested that they too retained the ability to interact with other Gag molecules, although in a nonproductive manner. However, it is also possible that the mutant Gag products exerted their inhibitory effect not by interacting directly with the wild-type Gag molecules but by blocking the transport machinery or membrane association of Gag.

Experiments in which Pr55^{gag} and Pr160^{gag-pol} were independently expressed from separate plasmids support further the conclusion that the MHR residues which were altered by mutagenesis are not critical for the establishment of intermolecular Gag-Gag interactions. As observed previously (39), processed Gag and Pol products were released into the external medium only when the two types of precursor molecules were coexpressed, indicating that the independently synthesized precursors coassembled into particles. The substitutions in the MHR had no substantial effect on the appearance of processed Gag and Pol products when present only in Pr160^{gag-pol}, indicating that the mutant Gag-Pol polyproteins were efficiently incorporated into particles formed by wild-type Pr55^{gag}. Previous studies of Moloney murine leukemia virus had suggested that similar Gag domains are needed for both particle formation and Gag-Pol utilization, since mutations in the *gag* gene always had a parallel effect on both processes (25, 38). However, the divergent effects of substitutions in the MHR, in particular the Y164→A substitution, on virion formation and Pr160^{gag-pol} incorporation demonstrate that the domains are

not identical, at least not in HIV-1. Also, recent studies have shown that unmyristylated Pr160^{gag-pol} can be efficiently utilized, whereas the myristylation of Pr55^{gag} is essential for particle formation (33, 39).

The replacement of an absolutely conserved arginine by alanine caused a phenotype that differed from that of the other MHR mutants. The mutation had only a moderate effect on viral particle yield, but most of the mature mutant particles did not have the characteristic cone-shaped core of mature wild-type HIV-1 virions. Rather, the core material condensed into a round structure that was frequently seen at the periphery of the particles. The morphology of the core structure suggested that the mutant CA was unable to form a stable core shell. It is possible that a reduced stability of the mutant protein contributed to the defect in core formation, since the steady-state level of CA in the mutant particles was reduced by about twofold. Also, the single amino acid substitution may have interfered with the protein-protein interactions required for CA protein multimerization. In this case, the reduced levels of CA in the mutant virions could have reflected a destabilizing effect of improper CA protein multimerization. A possible involvement of the MHR in CA protein multimerization was previously suggested by a study of the oligomerization properties of purified HIV-1 CA protein *in vitro* (9).

Noninfectious HIV-1 virions that contain a round, eccentrically located core were previously obtained after disruption of the proteolytic cleavage site between the MA and CA domains (18). The mutation apparently prevented the liberation of CA from a peripheral location beneath the viral envelope, so that it was not available for the formation of an elongated core shell. A comparison of the biological characteristics of virions that lack a conical core because of cleavage site mutations or mutations in the MHR may further elucidate the role of the core shell in HIV-1 replication.

ACKNOWLEDGMENTS

We thank Dana Gabuzda and Joseph Sodroski for critically reviewing the manuscript.

F.M. is supported by a fellowship from the Istituto Superiore di Sanità (Rome, Italy). S.H. and Å.Ö. are supported by the Wiberg's Foundation (Sweden). This work was supported by National Institutes of Health grants AI29873, AI28691 (Center for AIDS Research), and CA06516 (Cancer Center) and by a gift from the G. Harold and Leila Y. Mathers Charitable Foundation.

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