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Human Immunodeficiency Virus Type 1 Virion Density Is Not Determined by Nucleocapsid Basic Residues

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The human immunodeficiency virus type 1 (HIV-1) Gag polyprotein is sufficient for assembly and release of virion-like particles from the plasma membrane. To promote assembly, the Gag polyprotein must polymerize to form a shell that lines the inner membrane of nascent virions. Several techniques have been used to functionally map the domain required for Gag polymerization (the I domain). Among these methods, isopycnic centrifugation has been used under the assumption that changes in virion density reflect impairment in Gag-Gag interaction. If virion density is determined by efficient Gag-Gag interaction, then mutation of basic residues in the nucleocapsid (NC) domain should disrupt virion density, since these residues constitute the I domain. However, we have previously shown that simultaneous disruption of up to 10 HIV-1 NC basic residues has no obvious effect on virion density. To rule out the possibility that HIV-1 NC basic residues other than those previously mutated might be important for virion density, mutations were introduced at the remaining sites and the ability of these mutations to affect Gag-Gag interaction and virion density was analyzed. Included in our analysis is a mutant in which all NC basic residues are replaced with alanine. Our results show that disruption of HIV-1 NC basic residues has an enormous effect on Gag-Gag interaction but only a minimal effect on the density of those virions that are still produced. Therefore, the determinants of the I domain and of virion density are genetically distinguishable.

Expression of the human immunodeficiency virus type 1 (HIV-1) Gag polyprotein is sufficient for assembly and release of particles from the plasma membrane (for reviews, see references 11 and 26). Concurrent with release of nascent virions, the Gag polyprotein is cleaved by the virus-encoded protease to produce the mature *gag* proteins that include the matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), and p6 proteins. Prior to processing by the viral protease, discrete domains within the Gag polyprotein provide particular functions that are essential for assembly. Functional mapping of these signals has identified sequences at the amino terminus that are required for Gag targeting and binding to the plasma membrane (the M domain), basic residues in NC that are required for Gag homomeric interaction (the I domain), and signals at the carboxyl terminus that function at the latest stage of the assembly process, when nascent particles are released from the plasma membrane (the L domain).

A number of experimental approaches have been used to map and characterize the I domain required for Gag-Gag interaction, including the yeast two-hybrid system, in vitro binding assays with recombinant protein, and in vivo rescue assays (1, 4, 5, 9, 16). Isopycnic centrifugation, which allows determination of virion density after migration of particles in a linear sucrose gradient (21), has also been used to map the position of I domains in Gag polyproteins from different retroviruses (1, 2, 24). The significance of virion particle density is unknown, as virion density might be determined by permeability of virions to water, by packing of the Gag molecules within the virions, or by some yet-unknown property (22). However, there is a correlation between the low density of virion particles (which generally shifts from 1.16 g of sucrose/ml in the wild type to 1.14 g of sucrose/ml in mutant virions) and impairment of Gag-Gag interaction in deletion mutants of NC (1, 2). This correlation has led to the belief that changes in virion density are caused by impairment in Gag-Gag interaction (1, 2, 24). If this hypothesis is correct, mutations in NC basic residues would be predicted to affect virion density, since NC basic residues have been shown to mediate interaction among Gag polyproteins (7, 8).

We have previously shown that HIV-1 Gag multimerization and virion assembly are impaired when multiple NC basic residues are replaced with alanine (7). Surprisingly, we observed that the mutant virions that were produced had normal density. Prompted by these results, we sought to determine if basic residues other than the ones previously mutated are determinants of virion density. These residues were mutated and examined either individually or in combination with other complex basic residue mutations. Our results show that HIV-1 NC basic residues are required for Gag-Gag interaction, but that they make only a minor contribution to virion density.

MATERIALS AND METHODS

Plasmid DNAs and generation of NC mutations. NC mutations were introduced into either the replication-competent HIV-1 proviral construct NL4-3/HX or the hemagglutinin (HA)-Gag expression construct, both of which have been described previously (6, 7). In the latter, the HA epitope tag is present at the N terminus of Gag. As a result, the HA-Gag polyprotein is myristylation deficient. Mutant Gag expression constructs were generated in an NC coding sequence by using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and standard PCR-mutagenesis protocols. Mutants 2N and 2C were generated using wild-type proviral DNA as a template and the following oligonucleotides: for 2N, 5′-GATGCAGGCAGGCAATTTTGCGAACCAAAGAAAG-3′ and 5′-CTTTCTTTGGTTCGCAAAATTGCCTGCCTGCACATTATGG-3′, and for 2C, 5′-CCAAATGGCAGATTGTACTGAGGCACAGGCTAA-3′ and 5′-TTAGCCTGTGCCTCAGTACAATCTGCCATTTGGTGTC-3′. Mutant 2N/2C was generated using 2N mutant proviral DNA as a template and 2C oligonucleotides for mutagenesis. Mutants 2N/M1-2/BR, 2C/M1-2/BR, and 2N/2C/M1-2/BR were generated using the same oligonucleotides described above and M1-2/BR proviral DNA as template (20). Mutant 15A was generated using oligonucleotides 5′-CGAACCAAGCAGCGACTGTTAAGTGTTTC-3′ and 5′-GAAACACTTA

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ACAGTCGCTGCTTGGTTCG-3′ and mutant 2N/2C/M1-2/BR as template. In all cases, the external oligonucleotides used in the PCRs were 5′-GCGCCTGC AGAATGGGATAGATTGCATCCA-3′ and 5′-CATTGTACTGATATCTAA TCCC-3′. The amplified products obtained after mutagenic PCR were digested with *Sph*I-*Apa*I (nucleotides 1443 to 2001) and used to replace the corresponding fragment of wild-type proviral DNA. Fragment sequences were confirmed by dideoxy sequencing.

NC mutations were introduced into the HA-Gag construct (4) by substitution of a *Pst*I-*Xho*I fragment (nucleotides 1412 to 2289, according to reference 17) with the corresponding fragments obtained after PCR using mutant proviral DNAs as template and the following oligonucleotides: 5′-GCGCCTGCAGAA TGGGATAGATTGCATCCA-3′ and 5′-GCGCGCTCGAGTTATTGTGACG AGGGGTCG-3′. The mutation ΔNC-p6, which removes the spacer peptides NC and p6, was introduced into the HA-Gag construct in the same way, using the upstream oligonucleotide described above and the following downstream oligonucleotide: 5′-GCGCCTCGAGCAAAACTCTTGCTTTATGG CCGG-3′.

Plasmid pEF GagΔNC-p6 contains a deletion that removes the spacer peptides NC and p6 in the context of a rev-independent Gag polyprotein. This deletion was obtained by PCR using a rev-independent wild-type Gag polyprotein as template (4) and the following oligonucleotides: 5′-CGCGCCATGGGTGCGA GAGCGTCA-3′ and 5′-GCGCCTCGAGCAAAACTCTTGCTTTATGGCCG G-3′. The fragments obtained after PCR were digested with *Nco*I and *Xho*I and cloned into the corresponding sites of plasmid pEF/myc (Invitrogen).

Mutant ΔNC contains an in-frame deletion of NC in the context of a complete NL4-3 provirus. This deletion was obtained by PCR using the following oligonucleotides: 5′-TAAAAAATTAGCCTGCATTATGGTAGCTGGATTTGTTA C-3′ and 5′-ACCATAATGCAGGCTAATTTTTTAGGGAAGATC-3′. External oligonucleotides were as described above. The amplified products obtained after PCR were digested with *Sph*I-*Eco*RV (nucleotides 1443 to 2977) and used to replace the corresponding fragment of wild-type proviral DNA in plasmid pUC19NL4-3. This plasmid contains an *Sph*I-*Eco*RI fragment from NL4-3 (nucleotides 1443 to 5743) and has been previously described (7). The entire *Sph*I-*Eco*RI fragment containing the NC deletion was then cloned back into pNL4-3. The resulting Gag and Gag-Pol polyproteins encode only three amino acids of NC (QAN), which were maintained between SP1 and SP2 to retain the proper frameshift sequence.

The protease-negative NL4-3 construct bears the protease-inactivating mutation D25R and is wild type with respect to its *gag* sequence (25).

Cell lines. The human T-lymphocyte cell line Jurkat (30) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Human 293T fibroblasts were maintained in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal bovine serum.

Viral replication assay. Viral infections were initiated in 10^6 Jurkat cells by DEAE-dextran (250 μg/ml; Pharmacia Biotech Inc., Piscataway, N.J.) by using 2 μg of proviral DNA in 1 ml of serum-free RPMI 1640 medium for 20 min at room temperature. Cells were then washed in serum-free medium and resuspended in 3 ml of conditioned medium. Every 2 days, supernatant was harvested and frozen and cells were passaged. At the conclusion of the experiment, the stored samples were analyzed for exogenous reverse transcriptase (RT) activity as described below.

Exogenous RT assay. Ten microliters of cell culture supernatant or of solution containing virions was added to 50 μl of RT cocktail for 1 h at 37°C. HIV-1-specific RT buffer is 60 mM Tris-HCl (pH 8.0), 180 mM KCl, 6 mM $MgCl_2$, 6 mM dithiothreitol, 0.6 mM EGTA, 0.12% Triton X-100, 6 μg of oligo(dT) per ml, 12 μg of poly(rA) per ml, and 0.05 mM [γ-^{32}P]dTTP (800 Ci/mmol). Moloney murine leukemia virus (MMLV)-specific RT buffer is 60 mM Tris-HCl (pH 8.3), 0.7 mM $MnCl_2$, 75 mM NaCl, 6 mM dithiothreitol, 0.12% Triton X-100, 6 μg of oligo(dT) per ml, 12 μg of poly(rA) per ml, and 0.05 mM [γ-^{32}P]dTTP (800 Ci/mmol). Two microliters of each RT sample was spotted onto DEAE-81 paper and washed three times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (23). A PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) was used to quantify the radioactivity incorporated.

Antibodies and Western blot analysis. Rabbit polyclonal anti-cyclophilin A antibody was purchased from Affinity BioReagents (Golden, Colo.). Murine monoclonal anti-HA antibody was purchased from Berkeley Antibody Company (Berkeley, Calif.). Murine monoclonal anti-HIV-1 CA antibody was purchased from Intracel (Cambridge, Mass.). Western blot analysis was performed essentially as described previously (15).

Metabolic labeling and immunoprecipitation. Human HeLa fibroblasts were transfected with proviral DNAs using calcium phosphate, as previously described (6). Forty-eight hours posttransfection, cells were incubated for 12 h at 37°C in DMEM lacking methionine and cysteine plus 100 μCi of [^{35}S]Met-Cys (Translabel; ICN). After this period, cells were washed with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-Cl, pH 8.0). Virions were purified from the supernatant by ultracentrifugation for 2 h at 80,000 × *g* through a cushion of 25% sucrose (wt/vol), and the pellet was resuspended in RIPA buffer. Cell lysates were clarified by centrifugation prior to immunoprecipitation. Cell lysate- and virion-associated fractions were incubated with 100 μl of protein A-Sepharose beads (10% slurry in RIPA buffer; Sigma) for 1 h at 4°C. Supernatant was removed from the beads and incubated with 25 μg of total immunoglobulin from an HIV-1-infected individual (serum was obtained through the AIDS Research and Reference Reagent Program; catalog no. 3957) for 2 h at 4°C. Protein A-Sepharose beads (100 μl) were then added, and the mixture was incubated for 1 h at 4°C. Beads were washed three times, and proteins bound to the beads were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and phosphorimager quantification.

In vivo rescue assays. Human 293T fibroblasts were cotransfected with DNAs encoding a myristylation-deficient, HA epitope-tagged, full-length Gag polyprotein and a complete NL4-3 provirus, as previously described (7). The NL4-3 construct bears the protease-inactivating mutation D25R and is wild type with respect to its *gag* sequence (25). NC mutations were expressed in the context of the HA-Gag polyprotein. Virions in the supernatant were purified by ultracentrifugation 48 h posttransfection, as described above. Virion pellets were resuspended in PBS, normalized by exogenous RT activity, and analyzed by Western blotting. The amount of mutant proteins rescued into virions was quantified by densitometric analysis of the bands obtained after Western blotting with the HA-specific antibody.

Determination of virion density by isopycnic centrifugation. HIV-1 virions were obtained by calcium phosphate transfection of proviral DNAs into 293T cells. Wild-type MMLV virions were obtained from chronically infected NIH 3T3 cells. Virions were concentrated by centrifugation through 25% sucrose, as described above. The pellet was resuspended in 200 μl of PBS for 4 h on ice, and virion yield was determined by exogenous RT activity. Solutions containing HIV-1 and MMLV virions were layered onto a linear sucrose density gradient (20 to 60% [wt/vol]) and subjected to ultracentrifugation in a Ti40 rotor (Sorvall) at 80,000 × *g* for 24 h. Thirteen fractions were collected, each of which was analyzed in exogenous RT assays using HIV-1- and MMLV-specific RT buffers (see above) or precipitated with 10% trichloroacetic acid and analyzed by Western blotting. HIV-1 p24 was quantified by enzyme-linked immunosorbent assay using standard procedures. The density of sucrose fractions obtained after isopycnic centrifugation was determined using an ABBE-3L refractometer (Spectronics Instruments, Rochester, N.Y.).

RESULTS

Engineering of HIV-1 NC basic residue mutations and assessment of viral replication. It has been suggested that basic residues in NC are determinants of virion density (2). However, we have previously shown that simultaneous mutation of 10 of the 15 HIV-1 NC basic residues to alanine has no obvious effect on this physical property (7). In our previous studies, we had not engineered mutations of the most amino-terminal or carboxy-terminal basic residues. To determine if the basic residues that had not previously been mutated might regulate virion density, these remaining basic residues were mutated to alanine and expressed in various combinations, as shown in Fig. 1. The most severe mutant, 15A, was engineered so that all basic residues of HIV-1 NC were mutated to alanine.

The effect of these mutations on viral replication was determined first. Jurkat cells were transfected with proviral DNAs bearing the different NC mutations. Supernatant was collected every 2 days, and exogenous RT activity was determined as an indication of viral spread through the culture (Fig. 2). Wild-type accumulation of exogenous RT activity was evident in supernatant of cells that had been transfected with the 2C mutant, while with mutant 2N, exogenous RT activity accumulated with a marked delay compared to that of the wild type. The remaining mutants, containing substitutions of 4 (2N/2C), 11 (2C/M1-2/BR), 12 (2N/M1-2/BR), 14 (2N/2C/M1-2/BR), or 15 (15A) basic residues didn't accumulate exogenous RT activity above that of the background and were thus unable to replicate in Jurkat cells.

Virion assembly of HIV-1 NC basic residue mutants. The ability of our HIV-1 NC mutants to assemble was assayed next. 293T cells were transfected with proviral DNAs and labeled for 12 h with [^{35}S]methionine and [^{35}S]cysteine. Cells were lysed in RIPA buffer and proteins were immunoprecipitated with serum from an HIV-1-infected individual. Virion particles were purified from the supernatant of transfected cells by ultracentrifugation through 25% sucrose prior to immunoprecipitation. A fraction of each immunoprecipitate was processed by SDS-PAGE and the signal intensity for each band was determined with a phosphorimager (Fig. 3). The amount of virion-associ-

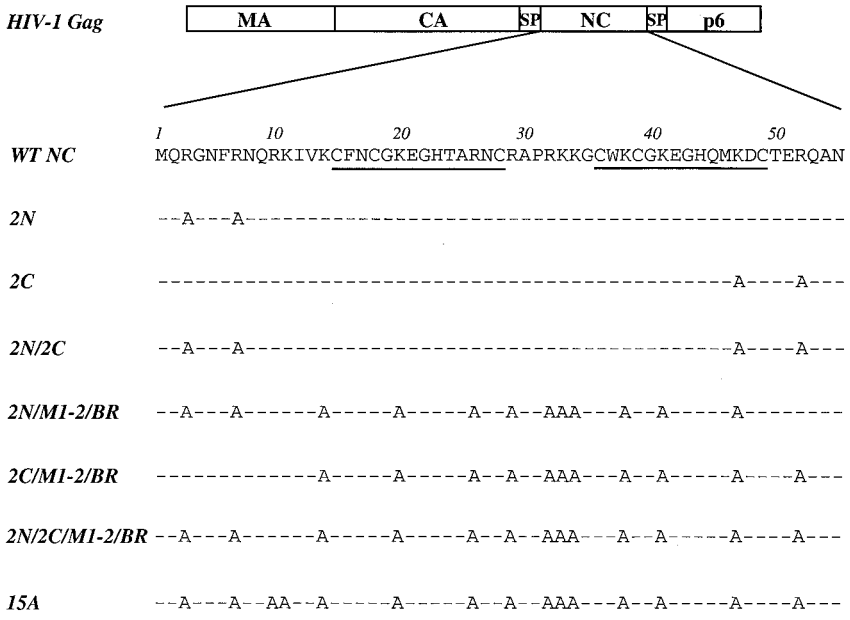

FIG. 1. HIV-1 NC mutants characterized in this study. At the top is a schematic representation of the major domains of the HIV-1 Gag polyprotein. The amino acid sequences of wild-type and mutant NC proteins are given below. The name of each mutant is indicated on the left. Dashes indicate an amino acid residue identical to that of the wild type. Cys-His boxes are underlined.

ated signal obtained for Gag was normalized for the amount of Gag present in the cell-associated fraction. Under these conditions, mutants 2N and 2C assembled as efficiently as the wild type. The remaining mutants exhibited deficiencies in the quantity of virion release, from 14% of that of the wild type for mutant 2N/2C to approximately 5% of that of the wild type for the remaining mutants.

In addition to defects in virion release, abnormal accumulation of incompletely processed Gag products, namely, the p25 CA-spacer precursor, was observed with mutants in which four (2N/2C) or more basic residues had been mutated. Processing of the RT protein was evaluated by Western blotting and appeared normal (data not shown). NC accumulated to wild-type levels in mutants 2N, 2C, and 2N/2C, but no signal was obtained after Western blot analysis of purified virion preparations of mutants 2N/M1-2/BR, 2C/M1-2/BR, 2N/2C/M1-2/BR, and 15A (data not shown). The inability of our polyclonal anti-NC serum to recognize NC proteins from these mutants was probably due to disruption of the epitopes and not to failed processing of NC from the Gag polyprotein, since our serum also failed to recognize unprocessed Gag p55 from these mutants.

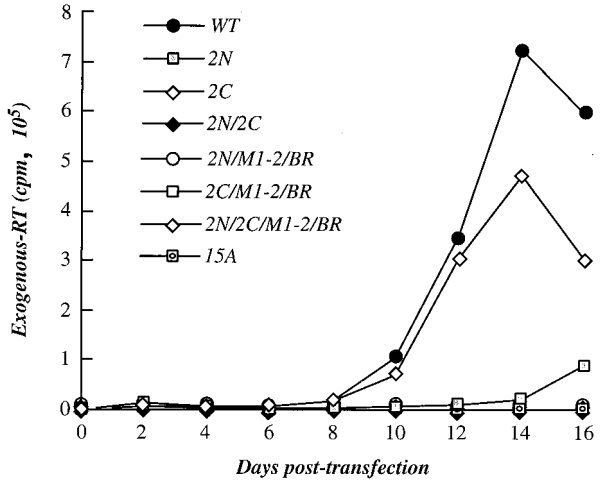

FIG. 2. Replication of HIV-1 wild-type and NC mutants following transfection of proviral DNA into the Jurkat T-cell line. The accumulation of exogenous RT activity in the cell culture supernatant (ordinate) is shown for the indicated day posttransfection (abscissa).

Basic residues in HIV-1 NC mediate interaction among Gag polyproteins. The ability of our mutants to affect interaction among Gag polyproteins was evaluated in an in vivo rescue assay, as previously described (2, 7). NC mutants were expressed in the context of a myristylation-deficient, HA epitope-tagged, full-length Gag polyprotein. Wild-type Gag was expressed from a proviral construct in which the viral protease had been inactivated by a single amino acid substitution (D25R) (25). This allowed us to utilize exogenous RT activity to normalize released virions and to easily detect full-length mutant HA-Gag polyproteins rescued into wild-type virions, precluding concern for possible effects of the mutations on protease processing.

DNAs encoding the various HA-Gag mutants were cotransfected into 293T cells along with the protease-deficient proviral DNA. Virions were purified from the supernatant by ultracentrifugation through 25% sucrose, normalized by exogenous RT activity, and analyzed by Western blotting with anti-HA antibody to determine the amount of HA-Gag mutant proteins rescued into virions (Fig. 4, upper panel). In addition to RT activity, cyclophilin A was used as a marker to normalize particle production. Cyclophilin A is incorporated into virions via interaction with CA (10, 28). The cyclophilin A signal was the same in all the samples, indicating that similar amounts of virions were loaded in all of the lanes (Fig. 4, middle panel).

When expressed by itself, the HA-Gag polyprotein was unable to assemble virions because it is myristylation deficient (Fig. 4, lane 1). When coexpressed with wild-type Gag, HA-

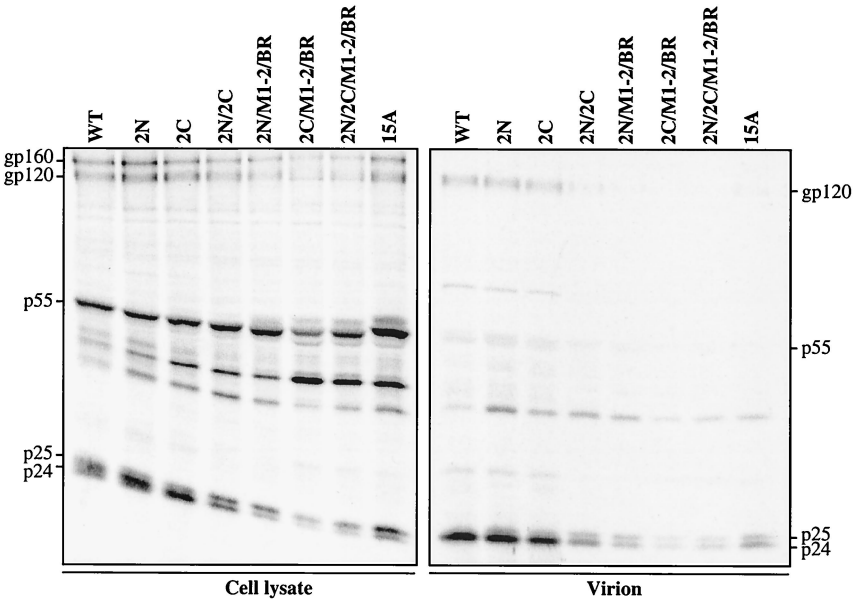

FIG. 3. Effect of HIV-1 NC mutants on virion assembly. HeLa cells transfected with the indicated proviral DNAs were metabolically labeled with [^{35}S]Met-Cys for 12 h. Virion-associated proteins were purified by ultracentrifugation through 25% sucrose. Cell-associated (left) and virion-associated (right) proteins were immunoprecipitated using serum from an HIV-1-infected individual and analyzed by SDS-PAGE and autoradiography. The mobilities of the envelope glycoprotein precursor (gp160), surface envelope protein (gp120), Pr55^{Gag} precursor (p55), incompletely processed Gag precursor (p25), and completely processed CA (p24) are indicated.

Gag was rescued into particles in *trans* (lane 2). Under these conditions, deletion of the entire NC coding sequence (ΔNC-p6) abolished the ability of the HA-Gag mutant to be rescued into virions (lane 10). When expressed in the context of the HA-Gag construct, the less severe mutants, 2N, 2C, and 2N/2C, were rescued as efficiently as the wild type (lanes 3, 4, and 5). Mutants 2N/M1-2/BR and 2C/M1-2/BR were rescued 15-fold less efficiently than the wild type (lanes 6 and 7), and the most severe mutants, 2N/2C/M1-2/BR and 15A, were not rescued to any extent detectable by our assay (lanes 8 and 9). The inability of these mutants to be rescued in *trans* by wild-type Gag polyprotein is not explained by failure of the proteins to be expressed in cells, since all proteins were expressed at similar levels in 293T cell lysates (Fig. 4, lower panel). These results extend our previous studies (7), indicating that NC basic residues are required for detectable Gag-Gag interaction in vitro and in vivo.

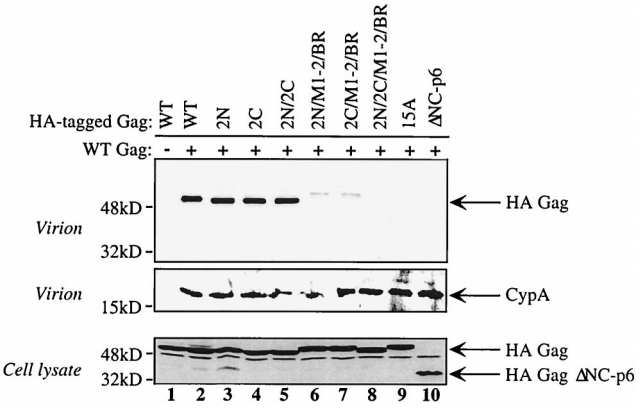

FIG. 4. Determination of the ability of HIV-1 NC basic residue mutants to be rescued in *trans* into wild-type (WT) virions in vivo. Myristylation-deficient, HA-tagged Gag polyproteins bearing the various NC mutations (as indicated) were coexpressed with protease-defective HIV-1 provirus into 293T cells (lanes 2 through 10, as indicated). Virions released in the supernatant were purified by ultracentrifugation through 25% sucrose and normalized by exogenous RT activity. The amount of HA-Gag mutant polyprotein rescued into wild-type virions was determined by Western blotting using anti-HA antibody (upper panel). Virions were also probed with anti-cyclophilin A antibody (a virion-associated protein) to monitor the gel loading (middle panel). Cell lysates were analyzed by Western blotting using anti-HA antibody to monitor mutant protein expression levels (lower panel). An HA-Gag mutant bearing a deletion of the NC and p6 coding sequences was included as a negative control (lane 10, ΔNC-p6). Wild-type HA-Gag polyprotein expressed in the absence of the protease-defective HIV-1 provirus served as a further negative control (lane 1). The positions of migration of the proteins are indicated on the right of the panels. The positions of migration of molecular mass markers (in kilodaltons) are indicated on the left.

Determination of mutant virion density by isopycnic centrifugation. Mutant HIV-1 virions were produced by transfection of proviral DNAs into 293T cells, concentrated by ultracentrifugation, resuspended in PBS, and layered onto a linear sucrose gradient (20 to 60% [wt/vol]). Routinely, virions were concentrated through a 25% sucrose cushion prior to sucrose gradient; however, similar results were obtained if virions were concentrated by low-speed centrifugation through Centricon. Wild-type MMLV virions, purified in the same manner from the supernatant of infected NIH 3T3 cells, were also added onto the gradient, as an internal control for virion density (2). The gradient was then centrifuged at 80,000 × *g* for 24 h. Under these conditions, equilibrium is reached and particles migrate in the gradient according to their density (21); this was demonstrated by showing that identical sedimentation profiles were obtained whether virions were layered on top of the gradient or mixed within the gradient (data not shown).

Thirteen fractions were harvested from the top to the bottom of the gradient. The quantity of virions present in each fraction was determined by measuring exogenous RT activity, though similar results were obtained with enzyme-linked immunosorbent assay or Western blotting (data not shown). Due to different divalent cation preferences, HIV-1 and MMLV RT enzymatic activities can be distinguished by choosing salt con-

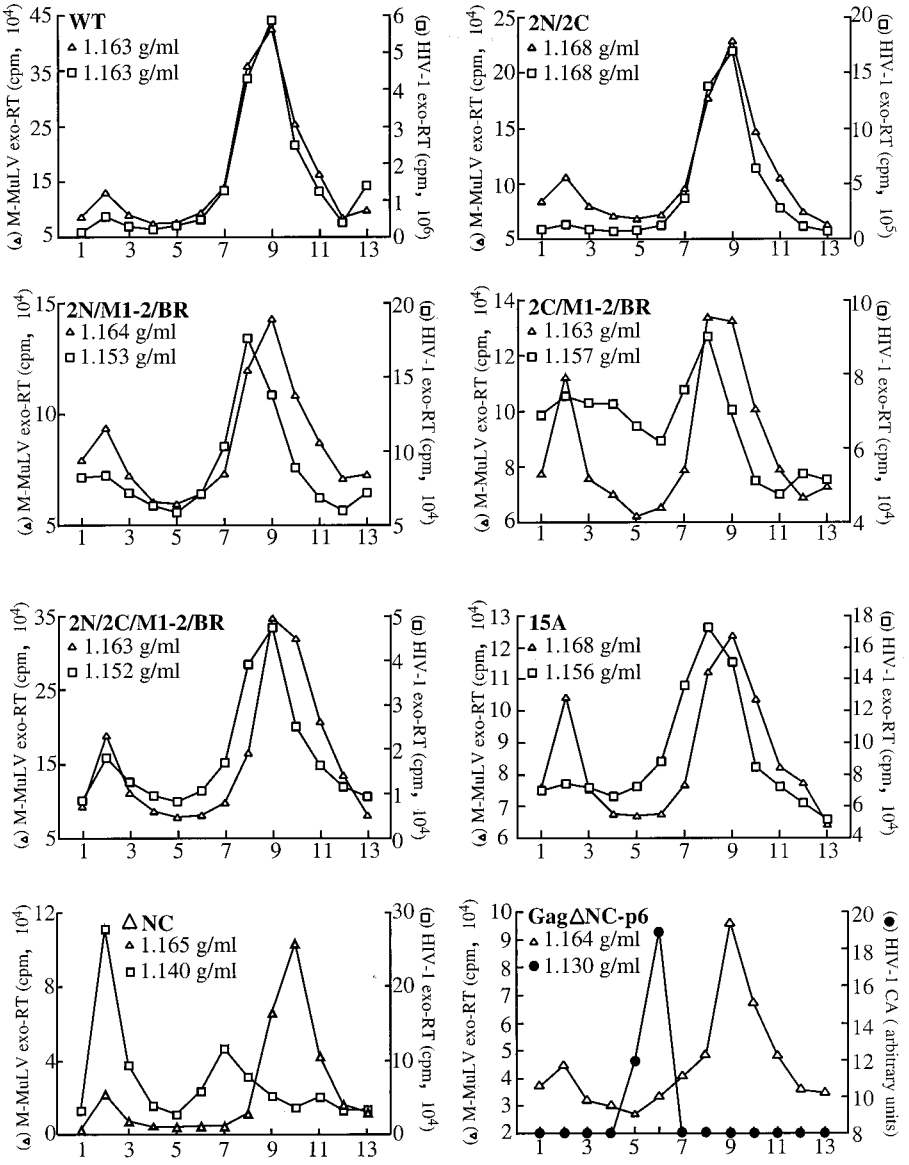

FIG. 5. Determination of HIV-1 NC mutant virion density by isopycnic centrifugation. Wild-type and mutant HIV-1 virions (as indicated) were produced by transfection of proviral DNA into 293T cells. Wild-type MMLV virions were harvested from infected NIH 3T3 cells. Virions were purified separately by ultracentrifugation through 25% sucrose and resuspended in PBS. Purified wild-type MMLV virions were mixed with HIV-1 virions and layered onto a linear sucrose gradient (20 to 60%). After ultracentrifugation for 24 h, 13 fractions were collected from the top to the bottom of the gradient (fractions 1 to 13 along the abscissa). MMLV (M-MuLV) (white triangles, values on left ordinate) and HIV-1 virions (gray squares, values on right ordinate) in each fraction were quantitated by exogenous RT activity using buffers that distinguish the activities from the two viruses. Quantification of virions produced by a deletion mutant of the Gag polyprotein lacking the entire NC and p6 domains (GagΔNC-p6) was performed by Western blotting using an anti-CA antibody (black circles, values on right ordinate). The density of the sucrose in each fraction was determined using a refractometer, and the densities of the peak fractions for MMLV (top value) and HIV-1 (lower value) are shown.

ditions specific for each enzyme (reference 27 and data not shown). Also, identical profiles were obtained for all the HIV-1 NC mutants whether density was determined in the presence or absence of MMLV virions (data not shown).

Wild-type MMLV virions had similar sedimentation profiles in all samples examined that were between 1.163 and 1.168 g/ml (Fig. 5). The density of HIV-1 virions bearing two or four substitutions of basic residues in NC (Fig. 5, 2N2C; 2N and 2C data not shown) was identical to that of the wild type (between 1.163 and 1.168 g/ml). Virions containing increasing numbers of substitutions of basic residues had a slight decrease in their density: 2N/M1-2/BR, 1.1534 g/ml; 2C/M1-2/BR, 1.1576 g/ml; 2N/2C/M1-2/BR, 1.1523 g/ml; and 15A, 1.1560 g/ml.

To compare the density of virions containing point mutations of NC basic residues with the density of virions bearing a deletion of NC, an in-frame deletion of NC that removes all but three C-terminal amino acids of NC was introduced into pNL4-3. In contrast to the effect of substitutions of basic residues, a pronounced shift in density was observed in virions bearing the in-frame deletion of NC (1.140 g/ml), consistent with previous reports (2, 8, 24). A large quantity of the total exogenous RT activity present in the sample (approximately

50%) failed to enter the sucrose gradient after ultracentrifugation. We believe that proteins contained in this fraction represent free protein or subvirion complexes rather than intact virions, since wild-type virions that had been disrupted with detergent prior to ultracentrifugation have a similar migration pattern (data not shown). Deletion of the NC and p6 domains in the context of a Gag polyprotein expression construct also resulted in virions with light density (1.130 g/ml), in agreement with previous results (2, 23).

DISCUSSION

It has been proposed that virion density is determined by the packing density of Gag polyprotein monomers within the virion. As a consequence, virion density and Gag multimerization were believed to be specified by the same domain of Gag, the I domain. This hypothesis sprang from the analysis of NC deletion mutants, in which it was observed that impairment of Gag-Gag interaction correlated with the production of virions of low density (1.13 to 1.14 g/ml, as shown in reference 1). Subsequent observations supported this model (1, 24) and suggested that within NC, basic residues are key determinants for both Gag multimerization and virion density. Indeed, NC basic residues mediate Gag-Gag interaction via nonspecific binding to RNA (7, 8) and addition of a stretch of basic residues to NC deletion mutants restores normal virion density (2). In contrast, NC Cys-His boxes are dispensable for both Gag-Gag interaction and virion density (7, 12, 13).

However, these previous reports described the phenotype of NC deletion mutants (1, 2, 8, 24) and never directly examined the effect of NC point mutations on virion density. When we replaced NC basic amino acids with alanine, we were surprised to see minimal effect on virion density (7), compared with what we observed after deletion of the entire NC coding sequence. In this report we have shown that replacement of up to 15 NC basic residues with alanine—the total basic residues present in HIV-1 NC—has only a minor effect on virion density.

Contrary to the small effect on virion density, replacement of HIV-1 NC basic residues with alanine dramatically impairs Gag-Gag interaction, as indicated by virion assembly defects and by failure of the mutant Gag proteins to be rescued into particles by wild-type Gag expressed in *trans*. Thus, while NC basic residues constitute the HIV-1 Gag polyprotein I domain and determine efficient Gag multimerization, they cannot be considered the sole determinants of virion density, since in their absence virion density is maintained. One might hypothesize that in the absence of NC basic residues, other regions of Gag are sufficient for formation of virions of proper density. The activity of these other regions may have been undetected in previous deletion mutagenesis studies showing that deletion of NC decreases virion density. A possible explanation is that NC deletions impose structural changes that affect other regions in Gag also required for proper density. In this respect, use of point mutations might result in a less dramatic conformational change and might allow mapping of these other putative regions. CA, p6, and Gag spacer peptides can be mutated individually with no obvious consequences on virion density (1, 14, 18). However, in light of our observations, it will be interesting to determine the effect of mutations in these domains when combined with our NC basic residue mutations.

Among the factors that might determine virion density are permeability to water or gross conformational defects induced by NC deletions. Cellular RNAs incorporated into virions may also determine virion density, although this possibility is unlikely, since RNA constitutes approximately only a few percent of the total mass of the virion. Alternatively, mutations in NC might influence Gag membrane binding, as recently proposed (19, 24), and direct Gag to a particular location on the plasma membrane which contains or lacks a particular lipid composition. Microdomains in the plasma membrane with different compositions of lipids have been described (3); thus, lipids, which constitute up to 30% of the total mass of the virus (29), may well influence virion density. Clearly, discrimination among the above-mentioned possibilities will require further biochemical and genetic analysis.

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