HIV-1 matrix domain removal ameliorates virus assembly and processing defects incurred by positive nucleocapsid charge elimination

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ABSTRACT

Human immunodeficiency virus type 1 nucleocapsid (NC) basic residues presumably contribute to virus assembly via RNA, which serves as a scaffold for Gag–Gag interaction during particle assembly. To determine whether NC basic residues play a role in Gag cleavage (thereby impacting virus assembly), Gag processing efficiency and virus particle production were analyzed for an HIV-1 mutant NC15A, with alanine serving as a substitute for all NC basic residues. Results indicate that NC15A significantly impaired virus maturation in addition to significantly affecting Gag membrane binding and assembly. Interestingly, removal of the matrix (MA) central globular domain ameliorated the NC15A assembly and processing defects, likely through enhancement of Gag multimerization and membrane binding capacities.

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1. Introduction

In most retroviruses, Gag precursor polypeptide expression is sufficient for mediating virus particle assembly [1]. During or soon after virus budding, HIV-1 Gag precursor Pr55 is cleaved by viral protease (PR) into matrix (MA; p17), capsid (CA; p24), nucleocapsid (NC; p7) and p6 domains [1,2]. This PR-mediated virus maturation process is essential for acquiring viral infectivity [3–6]. In addition to PR, enzymes required for virus replication are encoded by pol. Due to its partial overlap with the gag coding sequence, HIV-1 Pol polypeptide is initially translated as a Gag-Pol fusion protein via a ribosomal frameshifting event occurring at a frequency of approximately 5% [7], resulting in a Gag-Pol/Gag expression ratio of approximately 1:20. Maintenance of a low PR-associated Gag-Pol expression level is considered critical, since the artificial overexpression of Gag-Pol or PR triggers reduced virion yield due to the enhanced cleavage of Gag precursors prior to virus assembly [8–14].

In addition to playing a key role in mediating viral genomic RNA packaging, NC contains an interaction domain that promotes Gag–Gag interaction [15–18]. Specific NC mutations either reduce overall virus particle production, or trigger the production of low-density virus particles [1]. NC-associated RNA may serve as a scaffold facilitating NC–NC interaction and Gag assembly [19–26]. Deleting the NC domain or decreasing the number of positively charged NC residues via alanine replacement has been shown to markedly reduce virion yields [17,18,27–29]; reduced virion yields associated with NC mutants may also be attributed, at least in part, to a release defect [30,31]. One research group has suggested that decreased virion production tied to NC mutants is the result of released particle instability following cell budding [28]. However, results from a separate study indicate that HIV-1 PR activity inhibition enhances virion production by NC-deletion mutants, suggesting that substantial amounts of assembly-defective Gag molecules are cleaved by PR prior to virus particle formation [27]. Accordingly, Gag that is slowly assembled may be more susceptible to cleavage by PR, thus further reducing virus release. Since NC possesses dimerization potential [21], it may contribute to PR activation by promoting Gag-Pol dimerization. Although results from an in vitro study suggest that the NC domain enhances
PR-mediated Gag cleavage [32], it is unclear whether the NC contribution to Gag cleavage, if any, also impacts virus assembly.

We have two motivations for the present study: (a) to determine whether positively charged NC basic residues involved in RNA binding are required for PR-mediated Gag cleavage, and (b) to clarify whether the impacts of NC mutations on Gag cleavage (if any) affect Gag assembly and virion yields. We found that blocking NC-RNA association via the alanine replacement of all NC basic residues (NC15A) reduced Gag cleavage efficiency and significantly impaired Gag assembly. PR activity inhibition resulted in partial restoration of NC15A virion yields, suggesting that the virion deficit was in part affected by PR activity. We also observed that removal of the central MA globular domain (ΔMA) markedly reduced NC15A-induced Gag assembly and processing defects.

2. Materials and methods

2.1. Plasmid construction

The NC15A, as described previously [33], had the 15 NC basic NC residues replaced with alamines. The MA mutation was constructed by deleting a fragment from nt 831 to nt 1147 and replacing it with a SalI linker [34]. To construct substitution mutants, T26S and A28S, DNA fragments containing the point mutations were first generated by the mutation-containing primers (T26S: 5′-CTATTAGATCCGAGCAGAT-3′; A28S: 5′-TAGATA CAGGATCCGAGTATTAC-3′) and a reverse primer, 2577-5′ (5′-ACT GGTACAGTCTCAATAGGGCTAATG-3′), using an Env-deficient HIV-1 vector, HIVgpt [35] as template. Each of the resulting PCR products was then used as a megaprimer for a second round of PCR by using the forward primer (5′-ATGAGAACCAAGGGGAAGTG CAGGATCCGAGTATTAC-3′). The PCR products were then digested with SpeI and BclI and ligated into HIVgpt. As described previously, the ΔMA was constructed by deleting a fragment from nt 831 to nt 1147 and replacing it with a SalI linker [34]. The myristylation-minus (Myr−) mutant, in which the second glycine residue has been replaced by alanine, blocks Gag membrane binding and virus production [36]. Each of the gag mutations was also introduced into a PR-inactivated HIV-1 expression vector, HIVgptD25 [14], yielding a set of PR-defective versions. In D25, Arg is substituted for the PR catalytic residue Asp [14]. All mutation constructs were analyzed using restriction enzymes or DNA sequencing, and each mutation was subcloned into the HIV-1 expression vector HIVgpt [35].

2.2. Cell culture and transfection

293T and cells were maintained in DMEM supplemented with 10% fetal calf serum. Twenty-four hours before transfection, confluent 293T cells were trypsinized, split 1:10, and seeded onto 10-cm dishes. For each construct, 293T cells were transfected with 20 μg plasmid DNA using the calcium phosphate precipitation method, with addition of 50 μM chloroquine to enhance transfection efficiency.

2.3. Western immunoblot analysis

Unless otherwise indicated, cells and culture supernatants were harvested for proteins analysis at 48–72 h post-transfection. Cell and supernatant samples were prepared and subjected to Western immunoblot analysis as described previously [42]. For detection of HIV Gag proteins, the primary antibody was an anti-p24Gag monoclonal antibody (mouse hybridoma clone 183-H12-5C) from ascites used at a dilution of 1:5000. The secondary antibody was a sheep anti-mouse horseradish peroxidase (HRP)-conjugated antibody (HRP-conjugated antibody diluted at 1:15,000. An enhanced chemiluminescence (ECL) kit (Pierce) was used to visualize the membrane-bound Gag proteins.

2.4. Membrane flotation assays

We followed the protocol as described previously [42]. Briefly, cells were pelleted and resuspended in TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA) containing 10% sucrose and Complete Protease Inhibitor Cocktail (Roche). Cell suspensions were subjected to sonication followed by low-speed centrifugation to remove nuclei and cell debris. Postnuclear supernatant (200 μl) was mixed with 1.3 ml 87.5% sucrose in TE buffer containing Complete Protease Inhibitor Cocktail and placed on the bottom of a centrifuge tube. Solutions of 7.5 ml 65% sucrose and 3 ml 10% sucrose in TE buffer were layered on top of the 1.5 ml mixture. The gradients were centrifuged at 100,000 g for 16–18 h at 4 °C and then ten fractions were collected from the top of the centrifuge tube. Proteins in each fraction were precipitated with ice-cold 10% trichloroacetic acid (TCA) and analyzed by Western immunoblot.

2.5. Velocity sedimentation analysis of cytoplasmic Gag proteins

As described previously [42] and above, postnuclear supernatant (500 μl) was prepared and mixed with an equal amount of TEN buffer (50 mM Tris–HCl, pH 7.4, 2 mM EDTA, 150 mM NaCl) containing Complete Protease Inhibitor Cocktail. The mixture was then centrifuged through a pre-made 25–45% discontinuous sucrose gradient at 130,000 g for 1 h. Five 0.8-ml fractions were collected from the top of the centrifuge tubes. Proteins present in aliquots of each fraction were precipitated with ice-cold 10% TCA and subjected to Western blot analysis.

2.6. Virus-associated RNA quantification

Virus-containing supernatants were collected and centrifuged through a 20% sucrose cushion. Virus–associated RNA was then purified using a QIAamp Viral RNA Mini Kit (QIAGEN). Viral RNA, eluted in RNase-free buffer, was treated with RQ1 RNaseA-free DNase (Promega) at 37 °C for 30 min. As described previously [42], total viral RNA was then quantified using a RiboGreen RNA Assay Kit (Invitrogen) according to the manufacturer’s protocols. Ribosomal RNA provided in the assay kit was used to establish a RNA standard curve in parallel. Ratios of RNA concentrations to Gag immunoblot band density units were determined for each mutant and normalized to that of wt in parallel experiments.

2.7. Statistical analysis

Differences between control (wt) and experimental (mutant) groups were assessed using Student’s t-tests. Data are expressed as mean ± standard deviation. Significance was defined as: *p < 0.05, **p < 0.01.

3. Results

3.1. NC positive charge neutralization significantly affects HIV-1 Gag assembly and processing

To determine whether NC basic residues are required for Gag assembly and processing, we inserted a NC15A mutation (with all NC basic amino acid residues replaced with alanine [17,35]) into a HIV-1 proviral expression vector (Fig. 1A). Initial results indicate barely detectable Gag products in culture supernatants following the transient expression of NC15A in 293T cells, suggesting that NC positive charge elimination significantly reduced VLP yields. Given the likelihood that the PR-mediated cleavage of assembly-defective Gag molecules may contribute to virion decreases [17,35], PR activity suppression may lead to increased VLP
production. We therefore tested Gag assembly and processing (a)
in the presence of a PR inhibitor, and (b) via the expression of a
PR activity-diminished backbone resulting from a substitution
mutation (T26S or A28S) at the PR domain. The addition of T26S
and A28S resulted in 4-fold and 50-fold reductions in PR activity,
respectively [36]. As shown in Fig. 1, barely detectable VLP-associ-
ted NC15A became clearly detectable, and wt VLP yields increased
following treatment with saquinavir, a PR inhibitor (Fig. 1C, lower panel, lanes 1–6). These results support the proposal that reduced VLP yield associated with NC mutants is partly due to assembly-defective Gag cleavage; further confirmation comes from NC mutant construct expression in a PR-inactivated (D25) vector (Fig. 1D, lane 3). Combined, our data suggest that PR activity suppression was responsible for increased VLP yields associated with assembly-defective NC15A.

3.2. MA removal alleviates NC15A assembly and processing defects

MA basic residues bind with RNA [37–40], which may affect
Gag–Gag or Gag–Pol/Gag–Pol interaction. We therefore tested the
impacts of MA deletion on Gag assembly and processing, and
repeatedly observed that NC15A exhibited a noticeable increase
in VLP yields following MA removal (Fig. 2A, lane 9 versus lane
10). Notably, low but detectable NC15A VLPs were largely
immature—that is, virus-associated Gag remained unprocessed or
incompletely processed (lane 9). In contrast, mature virus-
associated p24gag associated with DMA/NC15A was readily detectable (Fig. 2A, lane 10). To minimize the impacts of PR activity on VLP assembly, all constructs were expressed in the PR-in-activated (D25) backbone. Our results also suggest that NC15A VLP yields were significantly enhanced by MA removal (Fig. 2C and D).

Although NC15A and ΔMA/NC15A both exhibited similar levels of unprocessed Gag precursor at 48 h post-transfection (Fig. 2A, lanes 4 and 5), results from a time-course analysis of Gag processing suggest that removal of the MA globular domain significantly enhanced NC15A Gag processing efficiency (Fig. 3). It is likely that the Gag mutation that disrupts VLP assembly also affects Gag–Pol molecular interaction, which in turn impairs PR activation. To further determine if MA removal impacts VLP release and maturation, accumulated VLPs in supernatants were collected at different time intervals following treatment with cycloheximide. Our results indicate significant increases in wt virus-associated Gag accumulation at 4 and 8 h post-treatment, but not at 12 h, suggesting that most VLP assembly and release in the Gag molecules was completed by 8 h post-treatment (Fig. 4). Likewise, we did not observe significant changes in virus-associated Gag cleavage profiles, suggesting that PR quickly mediates virus maturation following virus budding. Mutations at HIV-1 NC basic residues can lead to PR-dependent virion instability, with virions tending to fall apart with little...
recovery [28,35]. This may partly explain why ΔMA/NC15A exhibited slight accumulation decreases rather than increases at 8 and 12 h. Some of mature wt VLPs might also be unstable after release, which leads to a decrease accumulated VLPs at 12 h. A faster release rate in ΔMA compared to the wt was not observed in repeat independent experiments. However, we consistently observed that ΔMA/NC15A exhibited faster virus release and processing rates compared to NC15A.

3.3. MA deletion enhances NC15A membrane binding and multimerization capacities

We performed velocity sedimentation centrifugation analyses of cellular Gag molecules, using a myristylation-deficient (myr-) Gag mutant (defective in membrane binding and incapable of multimerizing into high-molecular weight complexes) as a control [34]. Results indicate that wt Gag and the assembly-competent mutant DMA tended to be found in high-sucrose density fractions, while most myr- Gag was recovered in fractions 1 and 2 (Fig. 5); this is in agreement with previously reported results [41,42]. We consistently observed substantial shifts of NC15A to higher sucrose density fractions following MA deletion (Fig. 5, panel 3 versus 4 from top), suggesting that MA removal mitigates the NC15A multimerization defect, which is compatible with improved VLP yields (Fig. 2). It should be noted that some slightly overexposed blots were purposely presented to make it easier for readers to see the detected signals. Not all of the Western blots shown in the figures were used for quantification. Since membrane binding is required for efficient Gag multimerization, we searched for correlations between Gag membrane-binding capacity and multimerization efficiency using membrane flotation assays for each mutant and wt as described in the Section 2. As expected, approximately 70% of the intracellular wt or ΔMA Gag was membrane-bound, compared to 10% of the myr- Gag (Fig. 6); this is consistent with previous research results [41,42]. Markedly decreased membrane-binding capacities were

Fig. 3. NC15A-mediated Gag processing efficiency is significantly enhanced following removal of MA. 293T cells were transfected with designated constructs. At 4 h post-transfection, equal amounts of cells were plated on three dishes. Cell were collected at 8, 16 or 24 h post-transfection and subjected to Western immunoblotting. Cellular Pr55\textsuperscript{gag} and p24\textsuperscript{gag} levels were quantified by scanning immunoblot band densities. Ratios of p24\textsuperscript{gag} to Pr55\textsuperscript{gag} or to mutant Gag precursor were determined for each mutant and normalized to those of the wt in parallel experiments. Values were derived from three independent experiments. Bars indicate standard deviations. *p < 0.05.
found for the multimerization-defective NC15A compared to the wt, but NC15A membrane binding capacity increased significantly following MA removal (Fig. 6B), which is compatible with improvements in both Gag assembly and VLP yield (Figs. 2 and 5).

3.4. RNA binding is not sufficient for efficient virus assembly

Since RNA is required for efficient Gag assembly, virus-associated total RNAs for the NC15A and ΔMA/NC15A mutants (all expressed in the PR-inactivated [D25] backbone) were measured and normalized to those of wt (D25) in parallel experiments. Results indicate that RNA quantities in both mutants were significantly lower than that in the wt (Fig. 7). However, they still contained measurable amounts of RNA, suggesting that the CA domain and/or remaining MA and NC basic residues in the ΔMA region may contribute to RNA association. No positive relationships were noted between RNA packaging and VLP assembly capacity in the assembly-defective mutants. For example, both

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Fig. 4. VLP release and processing kinetics 293T cells grown in 10 cm culture dishes in triplicate were transfected with the designated plasmid, pooled, and divided equally onto four dish plates. At 16 h post-transfection, supernatants were collected and fed medium containing 30 μg/ml of cycloheximide. Cells and supernatants were collected at 0, 4, 8 and 12 h following cycloheximide treatment. Supernatants were pelleted through 20% sucrose cushions and subjected to Western immunoblotting (panel A). Gag proteins were quantified by scanning p24gag-associated band densities from immunoblots. Densitometric units representing total Gag proteins in medium and the ratio of p24gag to Pr55gag band densities were plotted against time (panels B and C, respectively).
MA/NC15A and NC15 contained similar small amounts of RNA, but the former produced VLPs more efficiently than the latter. Our results suggest that RNA binding is insufficient for efficient virus assembly.

4. Discussion

Our data indicate that (a) eliminating HIV-1 NC positive charges by replacing all NC basic residues with alanine (NC15A) produced a significant defect in VLP assembly associated with reduced Gag cleavage efficiency and membrane binding capacity, and (b) removing the MA central globular domain significantly reduced NC15A-incurred assembly and processing defects. That NC15A still contains measurable amounts of RNA provides further support for the proposal that RNA association is insufficient for efficient Gag assembly.

We noted that virus-associated NC15A significantly increased following PR activity inhibition, thus supporting the idea that
Further studies are required to test this possibility. A role in combination with p6gag in virus budding by recruiting hypothesis, Dussupt et al. found that HIV-1 NC basic residues play VLP yields were largely due to a release defect. In support of this cleavage.

MA (Fig. 2A, lanes 9 versus 10), indicating that Gag can still be efficiently produced following the removal of supernatant became readily detectable following the removal of RNA may facilitate PR-mediated Gag cleavage. However, we observed that barely detectable p24gag in NC15A transfectant RNA may interfere with the NC15A Gag conformational change. Although NC15A produced low levels of VLPs, substantial amounts of NC15A Gag were capable of membrane binding and assembly efficiency. However, the extent of improved membrane binding and multimerization capacity does not completely account for the increased NC15A virion yields following MA removal. In conclusion, our findings suggest that in addition to Gag multimerization and membrane binding deficits, a budding defect makes a significant contribution to low NC15A virus particle production.

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