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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.

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

Abbreviations: CA, capsid; MA, matrix; NC, nucleocapsid; PR, viral protease; VLP, virus-like particle

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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 alanines. 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′-CTATTAGATTCCGGAGCAGAT-3′; A28S:5′-TAGATA CAGGATCCGATGATTAC-3′) and a reverse primer, 2577-51 (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 G80 (5′-ATGAGAGAACCAAGGGGAAGTG TGA-3′). The PCR products were then digested with Spe1 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 harvest 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-$p24^{gag}$ 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 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 RNAase-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