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Targeting the HIV entry, assembly and release pathways for anti-HIV gene therapy

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ABSTRACT

Targeting the HIV entry and assembly pathways holds promise for development of novel anti-HIV gene therapy vectors. We characterized discrete dominant negative (DN) Gag and Envelope mutants for their anti-HIV-1 activity. We show here that capsid mutants (Q155N and Y164A) are more potent inhibitors of WT HIV than the matrix mutant 1GA. Both the Envelope mutants tested, V513E and R515A, were equally effective and a combination of Gag and Envelope DN genes significantly enhanced potency. Interestingly, the DN mutants acted at multiple steps in the virus life cycle rather than solely disrupting virus release or infection. Inhibition mediated by R515A could be partially attributed to the Envelope cytoplasmic tail, as deletion of R515A tail partially abrogated its DN effect. Finally, the Y164A/R515A double mutant expressed in a lentiviral vector was effective at inhibiting HIV replication in CD34+ hematopoietic stem cell-derived macrophages, demonstrating the therapeutic potential of our approach.

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Introduction

The rapidly mutating nature of HIV and the adverse effects associated with routine drug therapy suggests the need for development of alternative therapeutic interventions. One of the obvious alternatives to chemotherapy is gene therapy. Recent advances in genetic manipulation of CD34+ stem cells, along with the development of lentiviral vectors capable of delivering desired genes to non-dividing cells,have made HIV-based gene therapy a realistic possibility (Liu and Berkhout, 2009; Rossi et al., 2007a). Moreover, there is always a need for novel anti-HIV drugs with unique mechanisms of action that can be harnessed as salvage therapy in case of intolerance or resistance to antiretroviral drugs.

One of the most commonly studied conventional gene therapy approach for controlling HIV infection is the use of shRNAs targeting viral or host genes (Liu and Berkhout, 2009; Nazari and Joshi, 2008; Rossi et al., 2007a; Unwalla et al., 2004b). Another attractive alternative gene therapy approach is to utilize dominant negative (DN) HIV proteins to target the HIV life cycle which is exemplified by the success of DN rev (RevM10) in inhibiting HIV replication in vitro (Bahner et al., 2007; Woffendin et al., 1996). However, both shRNA-mediated therapy and use of DN Rev comes with the caveat that escape mutants usually arise (Legiewicz et al., 2008). Hence, a combinatorial approach using both shRNA and DN host or viral genes is critical to target multiple viral or host proteins in order to limit the emergence of resistant mutants. Just like long-term shRNA therapy is accompanied by the problem of cell toxicity due to competition with the host micro-RNA processing machinery (Liu and Berkhout, 2009; Rossi et al., 2007a), the constitutive expression of DN HIV proteins could be detrimental to cells due to toxicity or immune recognition and cytotoxic T cell-mediated elimination. Moreover, gene therapy itself is associated with the drawbacks of 1) low transduction efficiency of CD34 + 2) elimination of transduced cells in vivo over a period of time, 3) potential of emergence of virus resistance,and 4) cellular toxicity against the anti-viral gene. Hence, long-term anti-HIV gene therapy solicits the use of conditional vectors that express anti-HIV genes post-wild type (WT) virus infection. Such vectors dependent on HIV Rev and Tat have been described by others (Ding et al., 2002; Unwalla et al., 2004a). This strategy is most likely to be successful by targeting HIV at late steps in the life cycle like assembly and budding. Hence in this study we sought to study the potential of DN HIV Env and Gag mutants that could target the HIV assembly and budding process and limit the infectivity of progeny virions.

The structural proteins of HIV-1, including the Gag and Env polyproteins, are essential for completion of the virus life cycle (Freed,

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1998; Swanstrom and Wills, 1997). HIV-1 Gag is the most abundant viral protein essential for virion morphogenesis. Expression of the Gag polyprotein alone is sufficient for the formation of virus like particles (VLPs) (Freed, 1998; Swanstrom and Wills, 1997). The Env glycoprotein, on the other hand, is important for virus entry into target cells. Hence, targeting the HIV assembly and entry pathways with DN Gag and Env mutants is likely to limit virus spread long-term by imposing severe constraints on the emergence of viral mutants. Currently there are no thoroughly characterized inhibitors of HIV assembly and entry pathways that show promise for anti-HIV gene therapy.

HIV-1 Gag is comprised of distinct structural and functional domains that play multiple essential roles in the virus life cycle. The N-terminal matrix (MA) domain is required for Gag membrane binding, while the capsid (CA) and nucleocapsid (NC) domains are important for Gag–Gag interactions and multimerization (Freed, 1998; Joshi et al., 2006; Swanstrom and Wills, 1997). It is known that certain mutations in the MA and CA domain of HIV Gag are not only non-functional per se, but also act in a DN fashion by inhibiting WT virus (Checkley et al., 2010; Kawada et al., 2008; Lee et al., 2009; Mammano et al., 1994; Muller et al., 2009; Shimano et al., 1999a,b; Trono et al., 1989). Moreover, since the retroviral Gag proteins are highly conserved and multimeric, their DN mutants are likely to exhibit a broad spectrum of inhibition, and hence may be suitable for gene therapy. Mutations in specific residues of HIV-1 Env are also known to interfere with viral integrity (Buchschacher et al., 1992; Inubushi et al., 1998; Iwatani et al., 2001). However, their mechanism of interference and the feasibility of their use in combination with Gag mutants for HIV gene therapy remain undetermined.

To identify the most potent DN inhibitor(s) targeting HIV assembly and entry, we undertook a thorough in vitro characterization of the mechanism of action of DN proteins consisting of a panel of Gag and Env mutants. In the *gag* gene we used the MA mutant 1GA (Freed et al., 1994; Kawada et al., 2008) and CA mutants Q155N and Y164A because of their ability to retain interaction with WT Gag but not be rescued to produce virions (Mammano et al., 1994), while for the *env* gene we selected the cleavage-defective (R515A) or fusion-defective Env mutant V513E (also known as the 41.2 mutant) (Freed et al., 1992, 1990; Iwatani et al., 2001) and a combination thereof. Our data demonstrate that DN mutations in the CA are more potent at inhibiting WT virus than the DN MA mutant 1GA. Combination of Gag and Env mutants Y164A/R515A was the most potent at suppressing WT HIV. Elucidation of the mechanism of inhibition demonstrated that Y164A/R515A acted at multiple steps of the virus life cycle, including assembly and release as well as entry and post-entry of the progeny virions. Interestingly, the inhibition mediated by R515A was partially attributed to the Env cytoplasmic tail. Thus, disrupting HIV's structural proteins can be harnessed for development of potential anti-HIV gene therapy vectors. Furthermore, our approach of targeting multiple HIV structural genes at the same time is likely to enhance potency and limit resistance development.

Results

DN HIV-1 Gag and Env mutants are potent inhibitors of WT HIV-1

The aim of this study was to determine whether targeting the virus assembly and entry pathway holds potential for anti-HIV gene therapy. To this end, we chose the MA mutant 1GA, known to be defective in Gag plasma membrane binding (Freed et al., 1994), and Q155N and Y164A mutations in the major homology region (MHR) of the HIV CA (Mammano et al., 1994) as potential candidates for DN HIV-1 inhibition. With respect to the Env glycoprotein, we selected the cleavage-defective mutant R515A (Iwatani et al., 2001) and the fusion-defective mutant V513E (the 41.2 mutant) (Freed et al., 1992, 1990). All the mutant genes were generated in the pNL4-3 full length HIV as described in Materials and methods. We first determined whether these mutants were capable of inhibiting WT HIV and the relative potency of each mutant. To determine the effect of DN proteins on infectious virus production the virus supernatants derived from HeLa cells transfected with various ratios of WT and DN mutants were used to infect TZM cells. TZM is a reporter cell line that expresses luciferase after infection with HIV and is ideally suited for detection of infectious HIV. The amount of infectious virus was determined by gene reporter readout in TZM cells 24 h post-infection. As shown in Fig. 1A, all of the Gag mutants were capable of inhibiting WT virus, with pNL4-3/Y164A being the most potent and pNL4-3/1GA being the least. Among the Env mutants, both Lai/V513E and Lai/R515A effectively inhibited WT, with the former being a slightly better inhibitor (Fig. 1B). With respect to the Env mutants, we also investigated the potential of each mutant to inhibit cell-to-cell fusion. Env glycoprotein expressed on the surface of infected cells has the potential to mediate fusion with neighboring cells to form syncytia which can be determined by cell-to-cell fusion assay in TZM cells (Garg et al., 2007). Interestingly, the Env/V513E mutant was more potent than Env/R515A in inhibiting WT Env-mediated cell-to-cell fusion (Fig. 1C).

While the preceding data demonstrate that the selected Gag and Env mutants independently are potent DN inhibitors of virus replication we wanted to know whether a combination of Gag and Env DN inhibitors would enhance the overall potency. For this purpose we chose the R515A mutation with the uncleaved Env as a potential therapeutic candidate for combination with various Gag mutants because, unlike the V513E mutant, R515A is not processed into gp120 and gp41 subunits of the Env glycoprotein and hence is not likely to induce toxic side effects due to release of soluble gp120 (Gougeon, 2003; Perfettini et al., 2005). Interestingly, a combination of Gag and Env mutations only increased the potency of inhibition for 1GA Gag (IC_{50} value lowered from ~24 to ~6, Fig. 1D and Table 1) but not Q155N or Y164A Gag (Fig. 1E and F and Table 1). This is most likely due to the saturating anti-HIV activity of the Gag mutants Y164A and Q155N by themselves in our cotransfection assays. Although these data suggest that DN mutations in HIV Gag alone are sufficient to strongly inhibit WT HIV, combining these with DN Env mutants has the advantage of targeting the virus at multiple steps to limit virus resistance.

The Gag MA and CA mutants inhibit release of WT HIV-1

We next determined the mechanism of inhibition of the DN Gag mutants. As Gag expression alone is sufficient for virus assembly and budding we wanted to know the effect of the DN mutants on virus release from cells. For this purpose we conducted virus release assays with the mutants alone or cotransfected with a 1:1 ratio with WT HIV-1 DNA. As shown in Fig. 2A, the MA mutant 1GA and the CA mutants Q155N and Y164A (consistent with Mammano et al., 1994) were severely defective in virus release by themselves. More importantly, when transfected at 1:1 ratio the DN mutants inhibited the release of WT HIV as well. This phenomenon was more pronounced for the Y164A mutant compared to the 1GA and Q155N mutants (Fig. 2B), consistent with the infectious virus production seen previously. Thus, these data suggest that the DN Gag mutants disrupt WT HIV by inhibiting release of virus particles from the cells.

The Env mutant R515A affects gp160 processing, cell-to-cell fusion and apoptosis mediated by WT HIV Env

The Env glycoprotein of HIV mediates virus entry by fusion of cellular and viral membranes. Additionally the role of Env glycoprotein in HIV pathogenesis is also an important factor to consider if it is to be a target for anti-HIV gene therapy. Hence we asked what effect DN Env has on the many functions of the Env glycoprotein. First we looked at the mechanism of R515A mediated inhibition of WT virus infectivity by examining whether it interfered with gp160 processing. As anticipated,

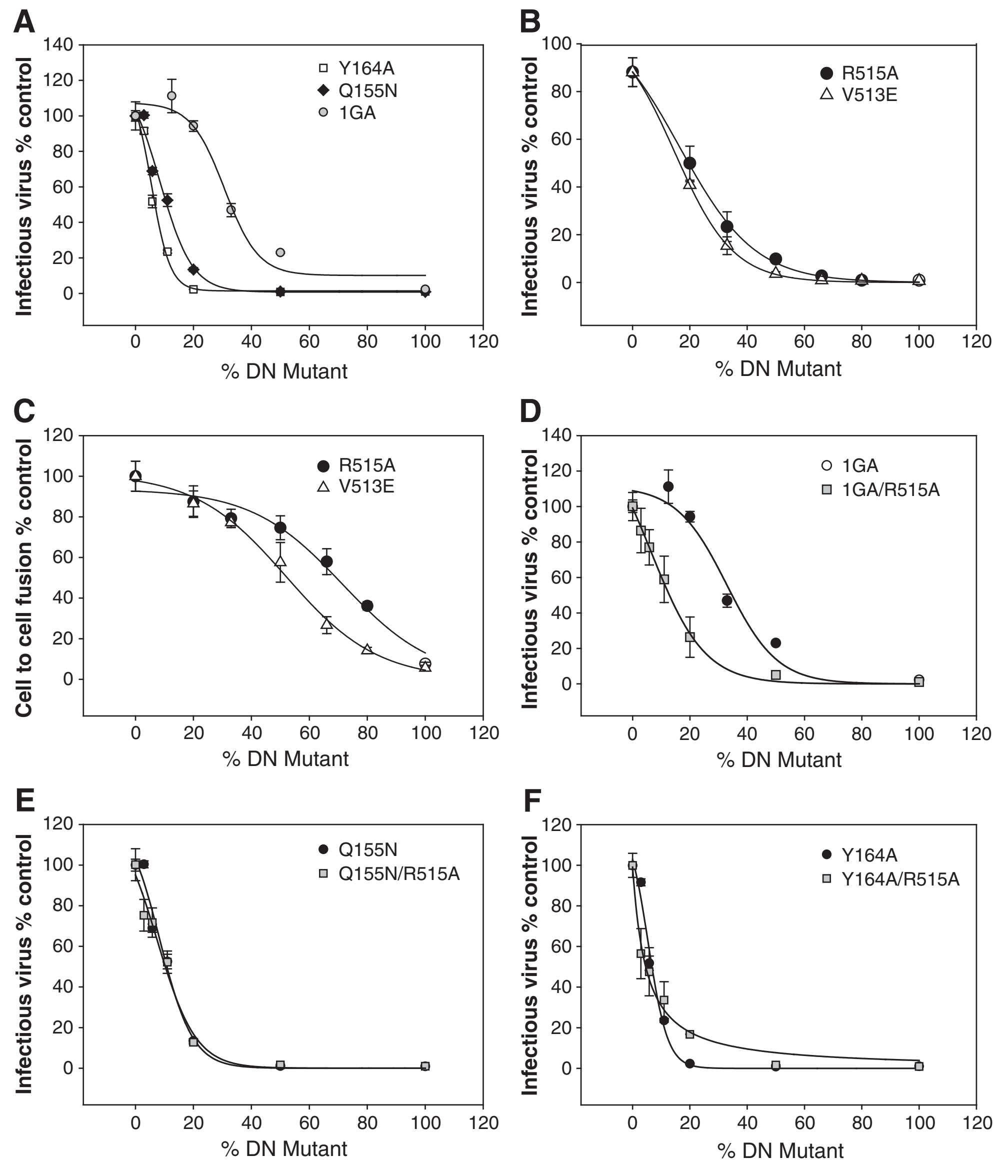

Fig. 1. DN Gag and Env mutants severely inhibit WT HIV. (A and B) HeLa cells were either transfected with WT HIV proviral DNA alone, DN Gag or Env HIV mutant DNA alone or varying ratios of WT HIV along with DN mutants. The total DNA amount for transfection was kept constant by using carrier pUC 18 DNA. Virus supernatants were harvested 48 h post-transfection and centrifuged, and an equal volume of virus input was used to infect indicator TZM cells. TZM cells were lysed 24 h post-infection and read on a luciferase plate reader. Readings were normalized and graphs fitted using the SigmaPlot software. Data are mean ± SE from 3 independent experiments. (C) HeLa cells were transfected with Env expression constructs for the V513E or R515A mutant along with WT HIV Env at various ratios. The total DNA amount for transfection was kept constant by using carrier pCDNA3.1 DNA. Twenty-four hours post-transfection, an equal number of TZM cells were added to each well. Cells were cocultured for 6–7 h followed by analysis on a luciferase plate reader. Readings were normalized and graphs fitted using the SigmaPlot software. Data are mean ± SE from 2 independent experiments with triplicate wells in each experiment. (D–F) HeLa cells were transfected with varying ratios of the double Gag and Env DN mutant DNA along with WT HIV-1 and assayed as in part A and B above.

the R515A mutation has no effect on release of WT virus from cells (Fig. 3A). However, the R515A mutant displayed severely defective gp160 processing, as levels of gp120 were markedly reduced in both cell and viral lysates (Fig. 3A). Interestingly, the levels of virion-associated gp120 were also substantially reduced in cotransfections consisting of WT + R515A DNA (Fig. 3A), suggesting a DN effect.

HIV Env-mediated fusion has been implicated in bystander apoptosis and T cell death during HIV-1 infection (Garg and Blumenthal, 2008; Garg et al., 2007) which is related to the fusogenic activity of the protein. Hence, we wanted to determine the fusion and apoptotic potential of the DN Env mutants themselves and the effect they have on WT Env-mediated apoptosis and fusion. As shown in Fig. 3B, both V513E and R515A Env mutants were severely defective in cell-to-cell fusion and consequently apoptosis induction when compared to WT Env. Moreover, a 1:1 ratio of DN Env mutants with WT Env reduced both cell-to-cell fusion and apoptosis (Fig. 3B), suggesting that the use of the Env mutants for HIV gene therapy will have the added benefit of modestly limiting bystander apoptosis. Taken together, these findings suggest that the Env mutant R515A is multifunctional by affecting WT Env processing, cell-to-cell fusion and bystander apoptosis in addition to inhibiting virus infection.

The MA and CA mutants affect WT Gag plasma membrane localization and virion morphology

We further characterized the DN mutants with respect to defects in plasma membrane (PM) localization and virion morphology using fluorescence and electron microscopy. By cotransfection of various DN

Table 1
IC_{50} values for various DN mutants used in the study

DN mutant	$IC_{50} \pm SE$
1GA	24 ± 4.9
1GA + R515A	6.4 ± 4
Q155N	8.3 ± 3.1
Q155N + R515A	7.4 ± 4.6
Y164A	5.4 ± 1.5
Y164A + R515A	4.6 ± .79
R515A	16.3 ± 2.0
V513E	14.7 ± .70

Inhibitory IC_{50} concentrations for the single Gag and Env mutants or the double DN Gag+Env mutants were determined using the SigmaPlot software. IC_{50} concentration represents percent of mutant DNA required for 50% inhibition of WT HIV.

mutants with WT Gag RFP, we were able to determine defects in PM localization of the WT Gag induced by the various DN mutants. As shown in Fig. S1, the 1GA Gag mutant was severely defective in PM localization as evident by diffuse cytosolic Gag staining as opposed to punctuate PM Gag staining pattern for WT Gag, while the CA mutants Q155N and Y164A showed lower, yet significant defects in membrane recruitment. This defect was also seen when DN mutants were cotransfected at a 1:1 ratio with WT Gag-mRFP and analyzed by fluorescence microscopy. As shown in Fig. 4A, the 1GA mutant most significantly affected membrane localization of WT Gag (diffuse staining) followed by Y164A and Q155N. On the other hand, as expected, the R515A Env mutant showed punctuate PM Gag staining pattern, suggesting that it had no effect on WT Gag membrane localization. We next determined by electron microscopy whether the DN mutants induced defects in virion morphology. The DN Gag mutants induced severe defects in WT HIV-1 virion morphology as evident by eccentric cores and immature as well as aberrantly shaped virions (Fig. 4B and C). The defects in virus membrane localization and budding as well as virion morphology demonstrate that the DN Gag mutants disrupt multiple late steps in the virus replication pathway.

Replication potential of DN mutants

We next sought to examine whether the DN mutants described above were capable of undergoing multiple-round replication in T cell lines like Jurkat and MT4 cells. It is essential to rule out this possibility as a gene therapy vector should not be able to replicate either by itself or upon recombination with WT virus. As shown in Fig. S2, both in Jurkat and MT-4 T cells, WT pNL4-3 and WT Lai replicated to high levels between days 6 and 10. However, none of the other Gag or Env mutants were replication competent even in the highly permissive cell line MT-4, suggesting that they were severely defective. We next tested whether virus particles produced after co-transfection of WT and DN mutants were capable of multiple rounds of infection. For this purpose, we infected Jurkat T cells with equal RT cpm of virus derived

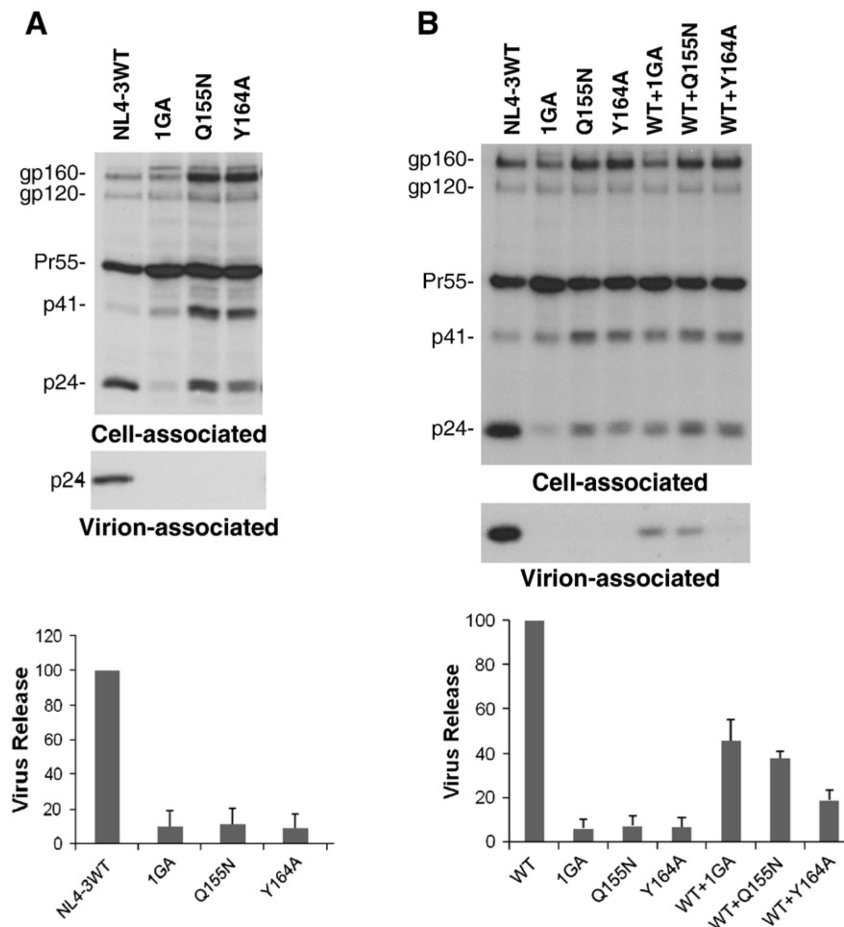

Fig. 2. DN Gag mutants are severely defective in virus release and inhibit WT HIV-1 release. HeLa cells were transfected with (A) HIV DN Gag mutants alone or (B) with a 1:1 ratio WT HIV along with the DN HIV Gag mutants. The total DNA amount for transfection was kept constant by using carrier pUC 18 DNA. Twenty-four hours post-transfection, cells were labeled with $[^{35}S]$Met/Cys, and cell and virus lysates were immunoprecipitated with HIV-Ig followed by resolution on an SDS–PAGE gel and fluorography. Virus release was calculated as the ratio of virion-associated Gag to total cell + virion-associated Gag. Data are mean ± SD from 2 independent experiments.

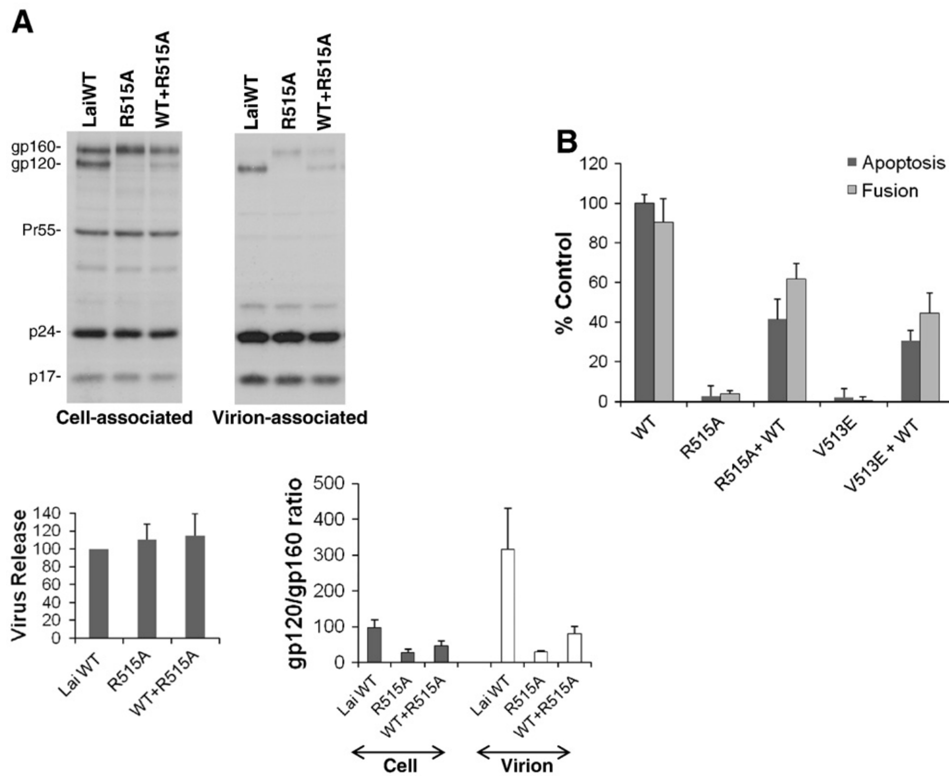

Fig. 3. The HIV Env mutant R515A affects gp160 processing, cell-to-cell fusion and apoptosis. (A) HeLa cells were transfected with WT HIV alone, HIV-R515A mutant alone or a 1:1 ratio of WT HIV and HIV-R515A mutant. Twenty-four hours post-transfection, cells were labeled with [^{35}S]Cys and processed as described in Fig. 2. Data are mean ± SD from 2 independent experiments. (B) HeLa cells were transfected with WT HIV Env construct or the various Env mutants. Twenty-four hours post-transfection, an equal number of TZM (for fusion) or SupT1 (for apoptosis) T cells were added to the wells. Fusion was assayed 5–6 h post-TZM addition using the britelite plus reagent. Apoptosis was measured after overnight coculture of SupT1 cells using annexin V staining. Data are mean ± SD of triplicates. One representative of 2 independent experiments is shown.

from HeLa cells transfected with different ratios of WT and Y164A/R515A mutant. As shown in Fig. 5A, the 1:8, 1:16 and 1:32 Y164A/R515A:WT derived virus replicated with WT kinetics or better. This could be due to the fact that incorporation of R515A inhibits bystander apoptosis, thereby making more cells available for virus replication consistent with previous findings of Garg et al (2007). However 1:4-derived virus replicated with delayed kinetics, peaking at day 30 post-infection, and 1:1 derived virus did not show any replication up to day 50 post-infection. These data suggest that the combination of DN Gag and Env mutants is capable of severely restricting the replication of WT HIV in T cell lines.

The DN Gag and Env mutants affect WT HIV at the entry/post-entry step

The defects in virion morphology induced by the DN Gag mutants seen above suggested that the progeny virions produced may also be infection defective. Furthermore the step at which the Env DN mutants inhibit virus infection also needs to be studied. For this purpose we conducted studies to determine whether the DN effect of Gag and Env mutants was mediated at the entry or post-entry level. To determine if the single and double HIV Gag and Env mutants are themselves defective at the entry step, TZM cells were infected with equal RT cpm (to account for the defective release of the Gag mutants) of the indicated viruses. As shown in Fig. S3, all the DN Gag mutants but not Env mutant R515A were defective in virion release (as determined by released RT activity) but all the Gag and Env mutants were defective in virus infectivity (luciferase activity, Fig. 5B). To determine if the defect in infectivity was only at the entry level or also at some post-entry step, TZM cells were infected with equal RT cpm of the indicated viruses complemented with VSV-G. Interestingly, all of the mutants were defective even when complemented with VSV-G, suggesting a defect at a post-entry stage, although we cannot exclude the possibility that the DN mutants may also have an effect on VSV-G-mediated entry. We then determined whether the DN mutants inhibited WT virus at an entry or post-entry step. To this end, we transfected HeLa cells with a 1:1 ratio of HIV-WT and indicated HIV DN DNAs and collected the virus in the supernatants. Subsequently, TZM cells were infected with normalized RT cpm of the virus stocks derived from HeLa cells and infectivity was determined by luciferase activity. As shown in Fig. 5C, in the presence of the DN mutants, the WT virus was defective both in entry (−VSV-G) and potentially at post-entry stages (+VSV-G). These data indicate a potential for both the DN Gag and Env mutants to inhibit WT HIV at multiple steps in the virus life cycle.

The Env R515A mutant is severely defective in early stages of reverse transcription

As shown in Fig. 5, the VSV-G-mediated viral infectivity data suggested that the Gag and Env mutants may have defects at a post-entry step. To determine whether they harbor defects in reverse transcription, we conducted real-time PCR from cells infected with equal input RT cpm of various Gag and Env mutant viruses or a 1:1 ratio of WT and mutant viruses to determine if the DN mutants affected reverse transcription of WT virus. Real-time PCR for early reverse transcription products using *env* gene primers (Lee et al., 2009) showed that the R515A mutant was severely defective in initiation (Fig. 5D) of reverse transcription and inhibited WT virus reverse transcription process. A similar defect was also seen with the Y164A Gag mutant but to a lesser extent. The 1GA and the Q155N

mutants also showed varying degrees of defects for early reverse transcription products but to a lesser extent. These data corroborate the findings from VSV-G rescue experiments suggesting a defect in the DN Gag and Env mutants at a post-entry step as well.

The cytoplasmic tail partially contributes to the inhibitory effect of R515A Env

The fact that VSV-G complementation assays failed to rescue R515A virus suggests defects in a post-entry step, raising questions regarding the mechanism by which the R515A Env affects virus uncoating. It has been shown that the viral Gag and Env remain associated in a mature virion via residues in the MA in Gag and the gp41 cytoplasmic tail. Dissociation of Env from Gag is important for proper virion infectivity (Freed and Martin, 1995a,b, 1996; Murakami and Freed, 2000a,b; Wyma et al., 2000). Since the R515A Env could only be minimally rescued with VSV-G, we speculated that defects in Gag/Env dissociation post-fusion may be responsible for the apparent post-entry defects. To test this, we constructed a tail-deleted version of HIV R515A (CTDel/R515A) and looked at its infectivity in TZM cells in the presence or absence of VSV-G. It needs mention here that HIV-CTDel mutant by itself shows significantly reduced Env incorporation and hence lower infectivity when compared to WT HIV-1. As shown in Fig. 6A, while HIV R515A and CTDel/R515A were severely impaired in

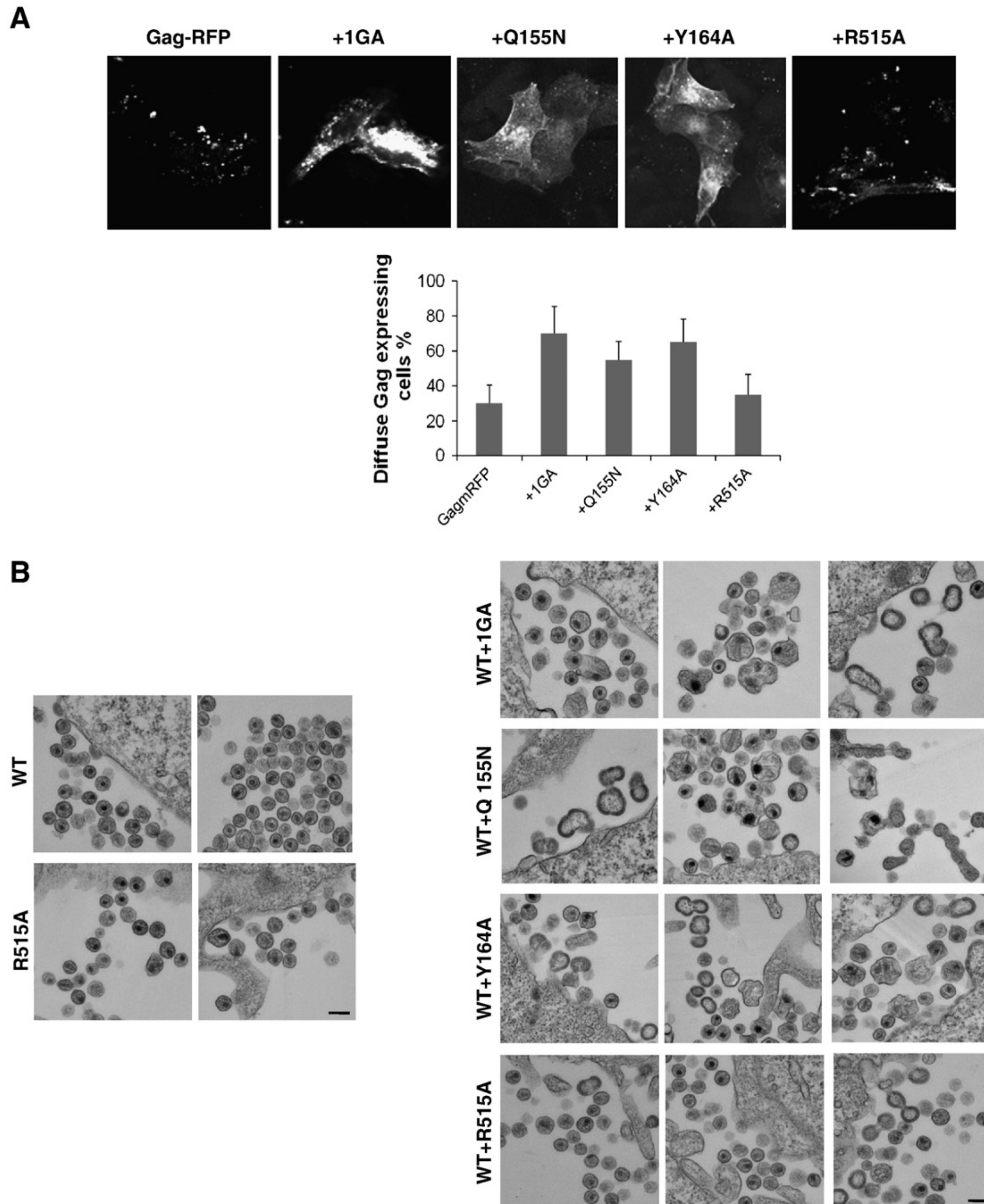

Fig. 4. DN Gag mutants are defective in Gag membrane binding. (A) HeLa cells were transfected with 1:1 ratio of DN HIV Gag or Env mutant proviral DNA along with HIV Gag-mRFP. Cells were fixed 24 h post-transfection and analyzed by fluorescence microscopy. Graph represents number of cells showing diffuse pattern of Gag RFP expression. (B) HeLa cells were transfected with DN HIV Gag or Env mutant proviral DNA alone or a 1:1 combination of WT and indicated DN mutants. Cells were fixed 24 h post-transfection and analyzed by electron microscopy. (C) Graph represents number of virus particles with normal, immature or aberrant morphology from 15–20 images for each mutant. Note: No virus particles were observed in cells transfected with 1GA, Q155N or Y164A Gag mutants alone. Scale = 100 nm.

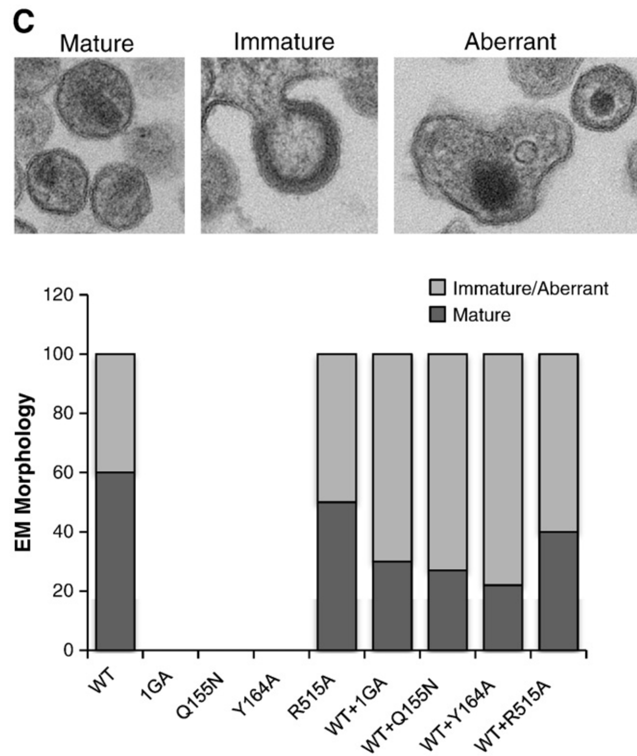

Fig. 4 (*continued*).

infectivity assays in TZM cells, the infectivity of CTDel/R515A but not R515A alone could be rescued partially when complemented with VSV-G. Having seen that the CTDel/R515A is partially restored in infectivity, we looked at its DN inhibitory potential in virus cell fusion assays. As shown in Fig. 6B, the dominant negative effect of HIV-CTDel/R515A was significantly less than R515A in virus cell fusion assays. These data suggest that the DN inhibitory effect of R515A Env is partially dependent on the cytoplasmic tail.

The lentivirus Y164A/R515A inhibits HIV replication in MDMs derived from transduced stem cells

The long-term objective of our study is to develop anti-HIV vectors that can be used to transduce CD34+ hematopoietic stem cells for reconstitution in AIDS patients. Before conducting in vivo studies, ex vivo testing of the vectors can be conducted in cord blood derived CD34+ cells differentiated into macrophages and challenged with HIV. For these ex vivo studies we cloned the Gag-Y164A and Env-R515A mutant into a lentivirus vector (Fig. S4). As shown in Fig. 7A, expression from the lentivirus expressing Gag-Y164A alone was significantly reduced compared to the lentivirus expressing Gag and Env Y164A/R515A together presumably due to lack of Rev protein and Rev responsive element (RRE) that functions in regulating the transport of the full-length and partially spliced HIV RNAs out of the nucleus (Felber et al., 1989; Hadzopoulou-Cladaras et al., 1989). CD34+ stem cells isolated from human cord blood (purity of 94%, Fig. 7B) were expanded in vitro and subsequently transduced with either a lentiviral vector expressing GFP (TyEFeGFP) or the Y164A/R515A DN mutant. The transduction efficiency was ~19% and ~16% for the TyEFeGFP versus Y164A/R515A mutant, respectively, as assessed by GFP and HIV p24 expression (Fig. 7C). The transduced stem cells were then differentiated into MDMs using human-M-CSF. In a separate experiment, stem cells were first differentiated into MDMs using M-CSF prior to transduction with the above-mentioned lentiviral vectors (transduction efficiency ~20%). Following MDM differentiation, cells were infected with HIV NL-AD8 and monitored for virus replication over a period of time. As shown in Fig. 7, MDMs derived from transduced stem cells (Fig. 7D and Fig. S5C) or directly transduced (Fig. 7E and Fig. S5D) showed significantly reduced virus replication over time with the lentivirus vector expressing the Y164A/R515A mutant when compared to cells transduced with TyEFeGFP vector in 2 independent experiments. At the end of the experiment, stem cells transduced with TyEFeGFP vector in 7D were fixed and analyzed for GFP expression by microscopy. As shown in Fig. 7F, GFP expression was evident in approximately 20–40% of MDMs, suggesting that gene expression was maintained over a prolonged period of time. Moreover, transduction with the lentiviral vectors led to normal differentiation of stem cells into MDMs following M-CSF treatment (Fig. S5A and B). These data suggest that the DN Gag and Env-expressing lentiviral vector described in this study can induce stable stem cell transduction and holds promise as a gene therapy tool for HIV inhibition.

Discussion

Gene therapy for HIV has gradually become a realistic possibility with the advances in CD34 stem cell transduction and repopulation in humans (Deeks and McCune, 2010; Holt et al., 2010; Kohn, 1999; Kohn and Sarver, 1996; Rossi et al., 2007b; Urnov et al., 2010; Weaver, 2010). The recent findings by Hutter et al. (2009), in which CCR5Δ32 homozygous stem cells were transplanted into an HIV patient resulting in undetectable virus replication for 20 months even in the absence of antiretroviral therapy, strengthen the notion that stem cell-mediated gene therapy holds potential. These advances have paved the way for establishing much needed novel and potent anti-HIV gene therapy approaches, especially for patients failing the currently available drug regimens. In this respect, DN Gag and Env proteins, either alone or in combination, represent an unexploited novel approach for anti-HIV gene therapy.

The use of DN Gag and Env mutants to inhibit WT HIV is unique as currently there are few chemotherapeutic drugs or other forms of

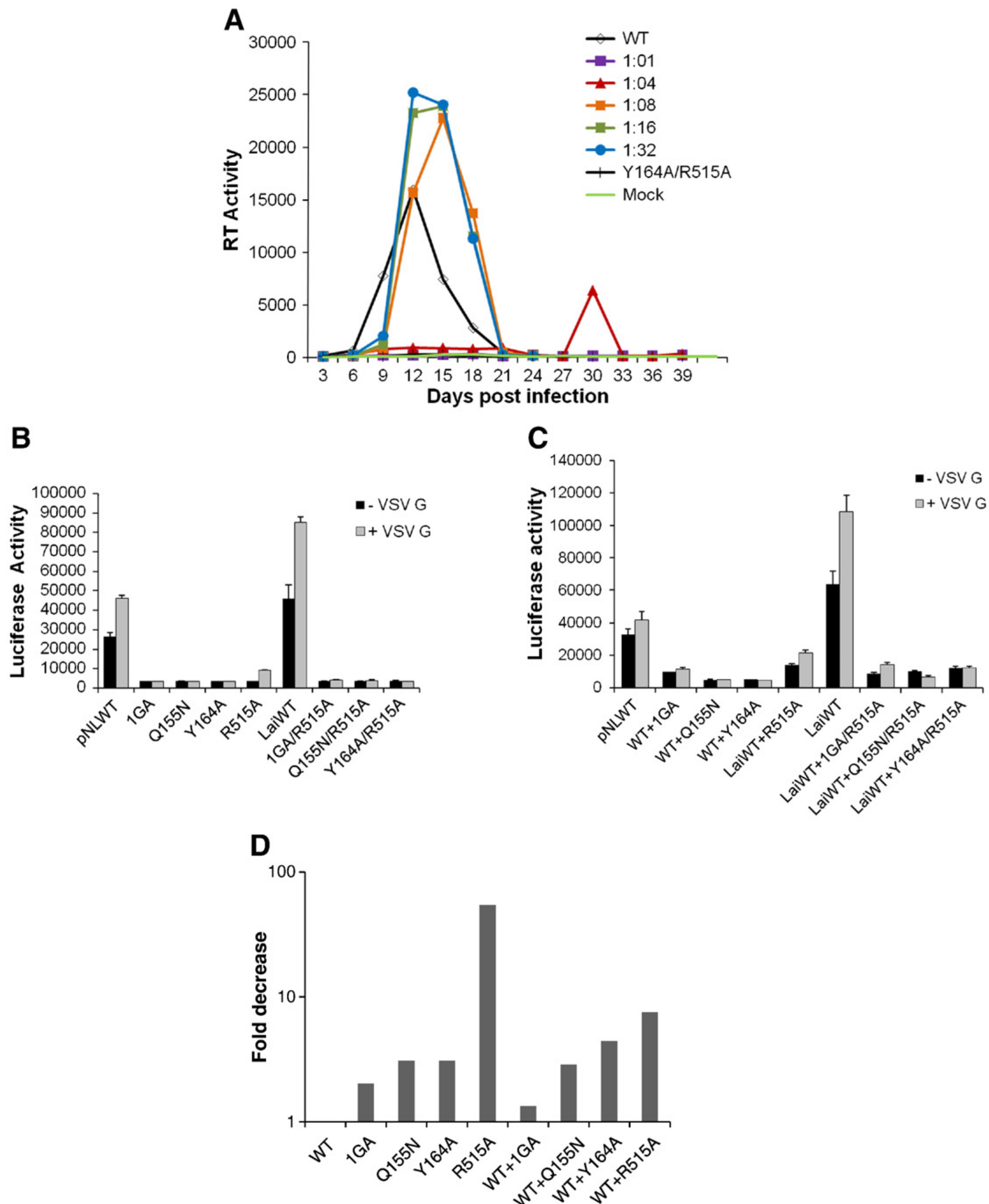

Fig. 5. HIV DN Gag and Env mutants restrict replication of WT virus. (A) Jurkat T cells were infected with equal RT cpm (20,000) of virus derived from HeLa cells transfected with different ratios of WT and Y164A/R515A mutant. Culture supernatants were harvested every 3 days for determination of RT activity. One representative from 3 independent experiments is shown. The 1:4 ratio derived virus always replicated with a delay although the virus peak varied between experiments. (B and C) The DN Gag and Env mutants affect WT HIV at the entry/post-entry step. HeLa cells were transfected with indicated DN Gag and Env single or double mutant plasmid DNA alone (B) or with the indicated DN Gag and Env single or double mutants along with WT HIV plasmid at a 1:1 ratio (C) in the presence (+) or absence (−) of VSV envelope expression plasmid. Culture supernatants were harvested 48 h post-transfection, assayed for RT activity and used to infect TZM cells with 20,000 RT cpm of the indicated virus stocks. Plates were read for luciferase activity 24 h post-infection. Data represent mean ± SD from triplicate observations. One representative from 2 independent experiments is shown. (D) The Gag Y164A and Env R515A DN mutants exhibit defects in early stages of reverse transcription. Jurkat T cells were infected with equal RT cpm (20,000) of virus stocks derived from HeLa cells transfected with DN Gag and Env single mutants alone or with the mutants transfected with a 1:1 ratio with WT HIV-1. DNA was isolated 24 h post-infection and quantitated, and an equal DNA amount was used for real-time PCR using primers specific for the Env gene. The copy number was calculated based on HIV-1 plasmid DNA standard with known copy number. Equal input DNA amount was verified by PCR for the CCR5 gene. One representative of 2 independent experiments is shown.

therapy targeting the Gag protein (Blair et al., 2010; Vozzolo et al., 2010). Another aspect of these structural proteins is that the DN effect is via protein–protein interaction rather than protein–RNA interaction as in the case of DN Rev and Tat. This suggests that resistance against these mutants would be less likely. Another attraction of using DN Gag mutants for anti-HIV therapy, especially those derived from the CA, is that amongst the various DN mutants of Gag, Pol, Env, Tat and Rev, the Gag CA mutants are the most potent at inhibiting WT virus (Shimano et al., 1999a). A combination of Gag and Env would not only increase the potency but make it difficult for the virus to attain breakthrough resistance.

We characterized several Gag and Env mutants for DN inhibition of HIV replication. Mammano et al. (1994) described the Q155N and Y164A CA mutations and their DN effect while studying the role of

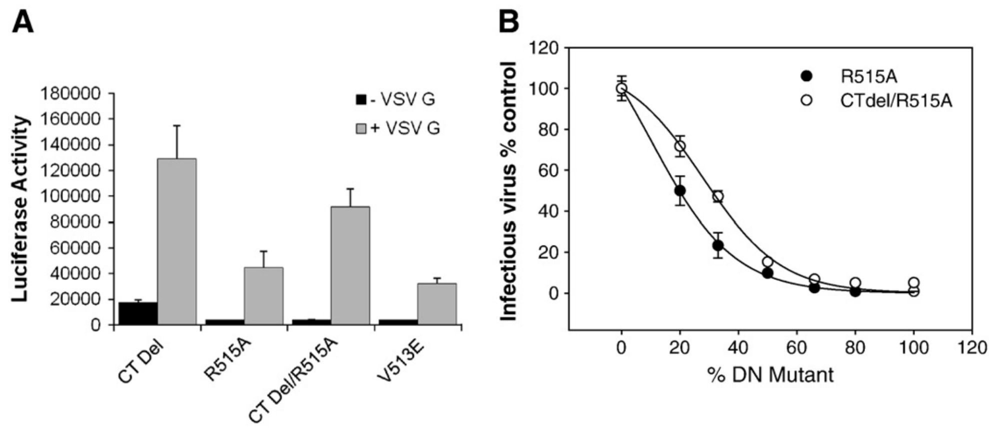

Fig. 6. The cytoplasmic tail partially contributes to the inhibitory effect of R515A Env. (A) HeLa cells were transfected with the indicated Env mutant DNA in the presence (+) or absence (−) of VSV envelope. Virus supernatants were collected 48 h post-transfection and used to infect TZM cells. Data from TZM cells was analyzed 24 h post-infection by reading the plate on a luciferase reader. Data represent mean ± SD from triplicate observations. One representative from 2 independent experiments is shown. (B) HeLa cells were transfected with varying ratios of pNL4-3 CTDel plasmid along with CTDel/R515A. Virus supernatants were used to infect TZM cells and assayed as in (A). Curves were fitted using the SigmaPlot software. Data are mean ± SD from triplicate observations. One representative from 2 independent experiments is depicted.

major homology region (MHR) of HIV Gag. However, whether these mutations could be used for anti-HIV gene therapy and the mechanism by which they show DN effects remained undetermined. Also, it is unclear whether Gag and Env mutants in combination would show better inhibition or other potential benefits when compared to single mutants. Our data show that CA mutants are more potent inhibitors of WT virus than MA mutants as evident from the enhanced inhibitory activity of Q155N and Y164A over 1GA. Characterization of the mechanism of action of the Gag mutants revealed some interesting findings. For one, the Y164A mutant is not only restricted in budding by itself but also inhibits release of WT HIV Gag, suggesting a DN effect at this step. Moreover, fluorescence microscopy analysis of DN Gag mutants alone or of WT Gag in the presence of 1:1 ratio of DN Gag mutants suggested defects in Gag membrane localization. However, when equal RT cpm of virus derived from co-transfection assays was used for second-round infections, the progeny virus particles were once again severely defective in infectivity. These data suggest that Y164A inhibits not only release but also infectivity of WT virions. The defects induced by the DN Gag when used to inhibit WT Gag were evident from our electron microscopy analysis where we saw aberrant virion morphology at a 1:1 ratio of WT to DN DNA. That these virions were defective in a post-entry event was further suggested by the VSV-G rescue experiments, in which WT + Y164A virions could not be rescued for infection even after pseudotyping with VSV-G. Finally, real-time PCR analysis using primers to detect early stages of reverse transcription confirmed these findings. This suggests that these aberrant virions most likely are defective at both entry and post-entry steps.

The Env mutants that have been studied for HIV inhibition include R515A and V513E (41.2). R515A is defective in the cleavage site between gp120 and gp41 and hence produces an uncleaved gp160 precursor protein which is obviously defective in fusion (Iwatani et al., 2001). V513E on the other hand has a substitution in the gp41 fusion peptide resulting in loss of fusion activity (Freed et al., 1992). While the mechanism by which these proteins are defective is different, interestingly they both act as DN inhibitors of WT HIV. We chose to study R515A in greater detail because of the lack of processing of gp160 to gp120 and gp41 would minimize soluble gp120 shedding from transduced/infected cells and limit any toxic side effects mediated by circulating gp120 (Gougeon, 2003; Perfettini et al., 2005). Interestingly, we also found that R515A was not only defective by itself but also inhibited WT virion infection at 1:1 ratio, indicating a DN phenomenon. Characterization of this DN effect using VSV-G rescue experiments suggested that the inhibition was not restricted to viral entry but also potentially at a post-entry step as VSV-G failed to rescue virion infectivity to WT levels. These data however need to be interpreted with caution as it is not known whether the DN Env mutants have a deleterious effect on VSV-G incorporation or entry. Based on the VSV-G rescue experiments we hypothesized that the post-entry defects seen with R515A might be mediated by interaction of the tail of uncleaved Env with Gag as the interaction of the HIV gp41 cytoplasmic tail with Gag has been implicated in post-entry defects in certain Gag and Env mutants (Freed and Martin, 1995a,b, 1996; Murakami and Freed, 2000a, b; Wyma et al., 2000). This was supported by our observation that deletion of the gp41 cytoplasmic tail in context of the HIV R515A mutant (CTDel/R515A) resulted in a better recovery of virus infectivity with VSV-G pseudotyping as well as reduced dominant negative effects.

The ultimate goal of anti-HIV gene therapy is to be able to transduce CD34+ stem cells ex vivo and then repopulate in HIV-infected patients to regenerate an HIV- resistant immune system. To this end, we tested our vectors for transduction of CD34+ stem cells ex vivo followed by differentiation into macrophages and challenge with HIV. Although our transduction efficiencies were low (20–40% in 2 different experiments), the fact that there was inhibition of HIV replication in stem cell-derived MDMs even under limited transduction efficiency is quite encouraging. Although it is possible that p24 staining underestimates the percentage of transduced cells the proof of principle of the potency of this dual Gag and Env lentiviral construct is demonstrated here. We believe that sorting of lentiviral transduced cells for analyses will further enhance the potency of inhibition. It is tempting to speculate further that the transduced cells could interfere with cell–cell transmission and/or cell–cell fusion between untransduced cells, thereby limiting HIV replication. Studies are under way to test this hypothesis as it would provide an added benefit if/when these vectors are used for gene therapy purpose.

Although our results suggest that transduced cells maintain long-term gene expression, the expression of DN forms of HIV proteins in differentiated T cells may be detrimental due to cellular toxicity or immune recognition and CTL mediated lysis. Recently a number of groups have proposed and developed conditional vectors that express the DN HIV gene only after infection with WT HIV. This has been achieved by making the DN gene ORF dependent on HIV rev and tat proteins (Ding et al., 2002; Unwalla et al., 2004a). This strategy would produce the DN inhibitor of HIV only in the cells that get infected. This suggests that the DN inhibitors should be able to inhibit late steps in HIV life cycle like assembly and budding and subsequent infectivity

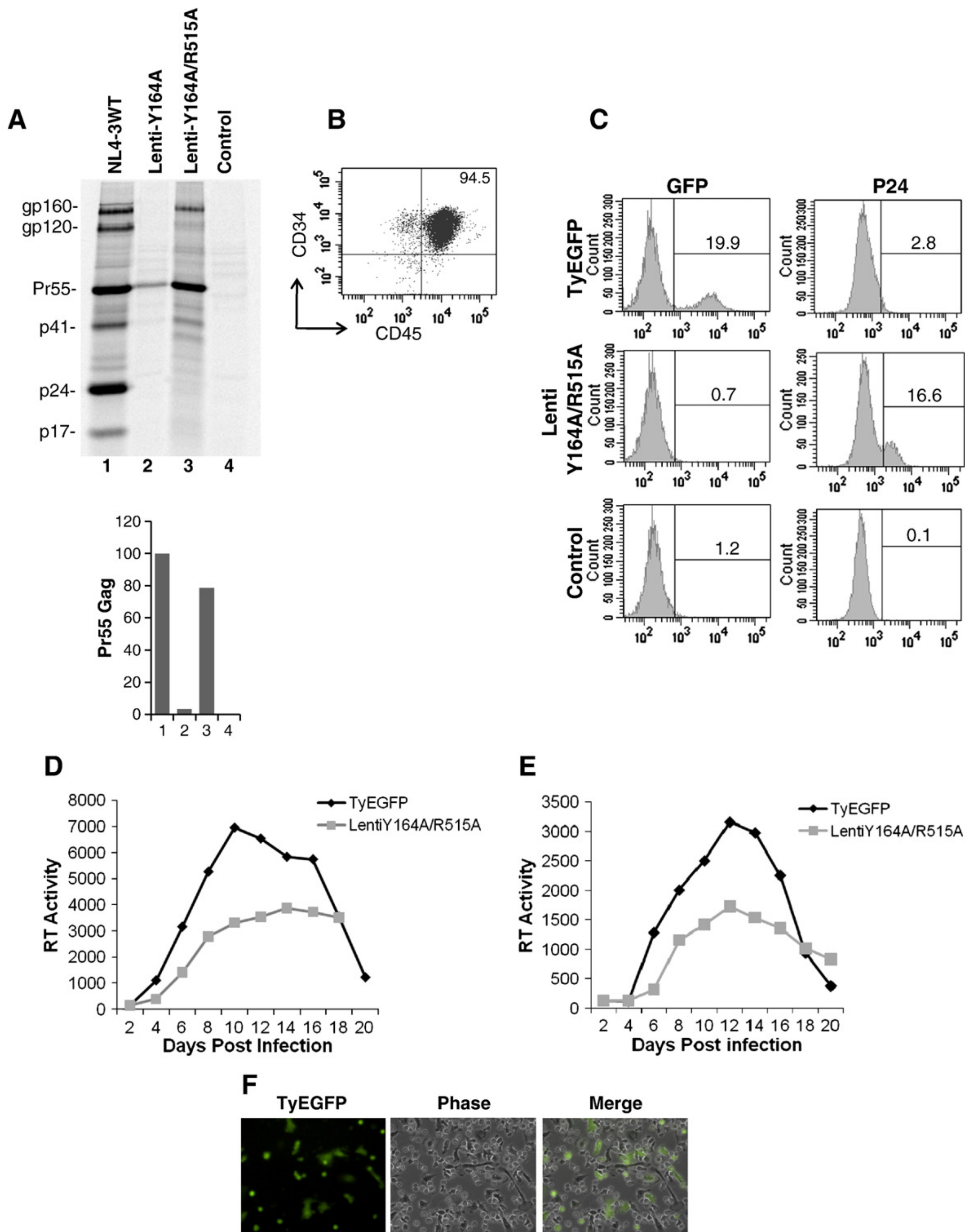

Fig. 7. The lentiviral vector expressing Y164A/R515A DN mutants inhibits HIV replication in hematopoietic stem cell-derived MDMs. (A) HeLa cells were transfected with WT HIV-1 or the lentiviral vector expressing Gag-Y164A, Gag Y164A and Env R515A or empty lentiviral vector. Cells were labeled with [^{35}S]Met/Cys and analyzed as described in Fig. 3. (B) CD34+ cells were isolated from human cord blood and the purity of the isolated population was determined by staining with human CD45 and CD34 antibodies. (C) CD34+ stem cells were transduced with GFP expressing lentiviral vector TyEFeGFP or Y164A/R515A lentiviral vector. Cells were assayed for GFP or p24 expression 72 h post-transduction by flow cytometry. (D) Stem cells transduced with the above lentiviral vectors were treated with GM-CSF for 7–10 days for differentiation into MDMs. Cells were then infected with pNLAD8 virus. Culture supernatants were harvested every other day for determination of RT activity. (E) Cord blood derived stem cells were expanded for 4 days in the presence of Stem Span media and CC110 cocktail. The cells were then treated with GM-CSF for 7–10 days for differentiation into MDMs and then transduced with the above lentiviral vectors. Forty-eight hours post-transduction, cells were infected with pNLAD8 virus. Culture supernatants were harvested every other day for determination of RT activity. (F) Stem cells transduced with TyEGFP lentivirus in (D) were analyzed for GFP expression at day 22.

of progeny viruses. We believe that our Gag and Env DN vectors represent good candidates for adaptation into conditional vectors. Further studies are under way to delineate the minimal Gag and Env domains required for DN HIV inhibition for improvement/development of this vector for in vivo anti-HIV gene therapy. Moreover, studies are required for validation of this vector for HIV inhibition in vivo which is now possible with the newer humanized mouse models for HIV.

Materials and methods

Cells, transfection, infection and reagents

HeLa and 293T cells were maintained in DMEM supplemented with 5% or 10% fetal bovine serum (FBS), respectively. Transfection of HeLa and 293T cells was performed using the ExGen 500 reagent (Fermentas). All virus stocks were prepared in HeLa cells unless otherwise indicated. pNL(AD8) virus stocks for infection of monocyte derived macrophages (MDMs) were prepared in 293T cells. TZM-bl cells were obtained from J. Kappes through the NIH AIDS Research and Reference Reagent Program and cultured in DMEM supplemented with 10% FBS. TZM-bl infections were performed in the presence of 20 μg/ml DEAE dextran (Sigma). HIV immunoglobulin (HIV-Ig) was provided by the NIH AIDS Research and Reference Reagent Program. SupT1 and Jurkat T cells were maintained in RPMI medium supplemented with 10% FBS. Human cord blood was obtained from NDRI (Philadelphia, PA) and stem cells were isolated using the Easy Sep human CD34+ selection kit (Stem Cell Technologies). CD34+ cells were cultured in Stem Span SFEM media supplemented with the CC110 cocktail (Stem Cell Technologies). Differentiation of CD34+ cells into macrophages was induced by recombinant human M-CSF (100 ng/ml) from R&D Systems, Minneapolis, MN.

Virus replication, virus cell fusion, cell-to-cell fusion and PCR assays

For virus replication, Jurkat T cells were infected with 20,000 RT cpm of different virus stocks. Culture supernatants were harvested and cells were split every other day or every 3 days for measurement of RT activity. Virus cell fusion assays were conducted as described before. TZM cells were infected with equal volumes or equal RT cpm of virus stocks in the presence of 20 μg/ml DEAE dextran. Cells were lysed approximately 24 h post-infection using the britelite plus reagent (PerkinElmer) and plates were read on a luciferase reader. Curve fits and IC_{50} calculations were performed using the SigmaPlot software. Cell to cell fusion/apoptosis assays were conducted as described previously (Garg et al., 2009). Briefly, HeLa cells were transfected with the various HIV Env expression constructs. Twenty-four hours post-transfection, an equal number of TZM (for fusion assays) or 5 times more SupT1 (for apoptosis detection) cells were added to the wells. Fusion was assayed 5–6 h post-addition of TZM cells using the britelite plus reagent and reading plates on a luciferase plate reader. Apoptosis was measured after overnight coculture of SupT1 cells using annexin V (BD Biosciences) staining and analysis using the BD Facs Canto flow cytometer. For detection of early and late reverse transcription products by real-time PCR, DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen). Real-time PCR was conducted using the SYBR Green PCR master mix (Applied Biosystems). Primers for detection of early envelope based PCR products have been described by Lee et al.(2009). CCR5 gene was used as the internal control for calculation of delta delta CT. Results were expressed as fold decrease from WT infected cells.

DNA constructs

The full length HIV proviral clone pNL4-3 (Adachi et al., 1986), its myristoylation-deficient mutant pNL4-3/1GA (Freed et al., 1994) and HIV molecular clone Lai and Lai/CTDel have been described previously (Garg et al., 2007). The CCR5-tropic clone pNL(AD8) has been previously described (Freed et al., 1995) . The HIV Gag mutants were constructed in pNL4-3 derived Gag using specific primers and the site directed mutagenesis kit (Stratagene). The Env mutants were constructed in the Lai Env backbone as above. Double Gag and Env mutants were constructed by replacing the EcoR1/Xho1 fragment containing the respective mutations into the pNL4-3 backbone. Molecular clone encoding Gag-monomeric red fluorescent protein (mRFP1) (pNL4-3/Gag-mRFP) has been described previously (Chukkapalli et al., 2008) and was kindly provided by V. Bokyo and Wei-Shau Hu. For cloning the Gag-Y164A and Env-R515A mutants into a lentiviral vector, we first amplified the Y164A Gag region from our NL4-3 based vectors and used TOPO cloning to insert the Gag region into the ViraPower Lentiviral expression systems (Invitrogen) to generate pLenti-Y164A. Subsequently the Env region from R515A Env vector was cut with EcoR1 Xho1 and subcloned downstream of the Gag region to generate pLenti-Y164A/R515A. Lentiviruses were prepared by transfecting 293T cells with the lentiviral vector and helper constructs in combination with VSV-G plasmid for pseudotyping as per the manufacturer's protocol (Invitrogen). GFP expressing control lentiviral vector (pTY-EFeGFP) was kindly provided by the AIDS reference reagent program and packaged the same as above.

Radioimmunoprecipitation

One day post-transfection, cells were metabolically labeled with [^{35}S]Met/Cys (PerkinElmer) in RPMI-1640 medium supplemented with 10% FBS but devoid of Met and Cys. For Env incorporation assays, cells were labeled with [^{35}S]Cys (PerkinElmer) in RPMI-1640 medium supplemented with 10% FBS but devoid of Cys. Procedures for preparation of cell lysates, ultracentrifugation of virions, and immunoprecipitation of cell and virion-associated material with HIV-Ig have been described elsewhere (Freed et al., 1994). Virus release efficiency was calculated as the amount of virion p24 divided by total Gag (cell $Pr55^{Gag}$ + cell p24 + virion p24).

Immunofluorescence and EM analysis

Immunostaining of cells was performed as described (Joshi et al., 2006), with minor modifications. Cells plated on Nunc chamber slides were rinsed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in 100 mM sodium phosphate buffer (pH 7.2) for 20 min. Following fixation, cells were thoroughly rinsed with PBS, permeabilized using 0.1% Triton X-100/PBS for 2 min, and incubated for 10 min with 0.1 M glycine/PBS to quench the remaining aldehyde residues. Cells were then blocked with 3% BSA/PBS for 30 min, followed by incubation for 1 h with primary antibody appropriately diluted in 3% BSA/PBS. After 3 washes in PBS, cells were incubated for 30 min with secondary antibody diluted in 3% BSA/PBS. Cells were then washed and mounted using Aqua Poly/Mount (Polysciences Inc). Images were acquired using the NikonTi microscope and analyzed using the NIS Elements AR software. For EM analysis, transfected cells were fixed using buffer containing 2% glutaraldehyde and 100 mM sodium cacodylate and stored at 4 °C. Samples were then sectioned and analyzed by transmission EM (Freed et al., 1994).

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.03.028.

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