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Identification of Amino Acid Residues in the Human Immunodeficiency Virus Type-1 Reverse Transcriptase Tryptophan-repeat Motif that are Required for Subunit Interaction Using Infectious Virions

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The human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) functions as a heterodimer (p51/p66), which makes disruption of subunit interactions a possible target for antiviral drug design. Our understanding of subunit interface interactions has been limited by the lack of virus-based approaches for studying the heterodimer. Therefore, we developed a novel subunit-specific mutagenesis approach that enables precise molecular analysis of the heterodimer in the context of infectious HIV-1 particles. Here, we analyzed the contributions of amino acid residues comprising the Trp-motif to RT subunit interaction and function. Our results reveal important inter- and intra-subunit interactions of residues in the Trp-motif. A tryptophan cluster in p51 (W398, W402, W406, W414), proximal to the interface, was found to be important for p51/p66 interaction and stability. At the dimer interface, residues W401, Y405 and N363 in p51 and W410 in p66 mediate inter-subunit interactions. The W401 residue is critical for RT dimerization, exerting distinct effects in p51 and p66. Our analysis of the RT heterodimerization enhancing non-nucleoside RT inhibitor (NNRTI), efavirenz, indicates that the effects of drugs on RT dimer stability can be examined in human cells. Thus, we provide the first description of subunit-specific molecular interactions that affect RT heterodimer function and virus infection *in vivo*. Moreover, with heightened interest in novel RT inhibitors that affect dimerization, we demonstrate the ability to assess the effects of RT inhibitors on subunit interactions in a physiologically relevant context.

Keywords: reverse transcriptase; subunit-specific; Trp-motif; dimerization; NNRTI

Introduction

The reverse transcriptase (RT) of human immunodeficiency virus type-1 (HIV-1) is a heterodimeric enzyme comprised of 51 kDa and 66 kDa subunits.[1,2] The RT heterodimer (p51/p66) is generated *via* proteolytic processing of the Gag-Pol polyprotein precursor ($Pr160^{Gag\text{-}Pol}$) by the viral protease (PR) during assembly/maturation of the virus particle.[3,4] The p66 subunit is divided into two domains, polymerase and RNase H. The p51 subunit is produced by PR cleavage of the p66 subunit and removal of the RNase H domain.[5,6] Crystallographic analysis subdivides the polymerase domain into the fingers, palm, thumb and connection subdomains.[7] Since the subunits are derived from the same coding region, and the relative arrangement of RT subdomains differs markedly between p51 and p66, a mutation in one subunit is structurally and functionally non-equivalent to the same mutation in the other subunit. For this reason, and because mutations in the RT domain of $Pr160^{Gag\text{-}Pol}$ may be pleiotropic,

Abbreviations used: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; PR, protease; NNRTI, non-nucleoside RT inhibitor; EFV, efavirenz; mAb, monoclonal antibody; pdb, Protein Data Bank.

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impairing virus assembly or maturation,[8–10] subunit-specific structure/function studies of RT have not been possible with infectious HIV-1.

HIV-1 RT dimerization has been investigated using a number of complementary non-physiologic, *in vitro* approaches. Formation of the heterodimer has been defined as a two-step process.[11,12] The first step involves a concentration-dependent association of the two subunits. This is followed by a slow isomerization/maturation step that involves interactions of the fingers and thumb of p51 with the palm and RNase H of p66, respectively. Specifically, residues 52–55 and 135–140 of the p51 fingers appear to interact with residues 85–96 of the p66 palm,[13–15] while residues 280–295 of the p51 thumb subdomain appear to contact residues 536–545 of RNase H.[16–19] The first step in RT dimerization apparently involves interactions between hydrophobic residues in the connection subdomains of p51 and p66. This is believed to include residues W401–W410 of p66 and residues P392–W401 of p51.[15,20,21] The connection subdomain is distinctive in having six tryptophan residues and a tyrosine between amino acid residues 398–414. This motif is well conserved among the primate lentiviruses, and has been appropriately dubbed the tryptophan-repeat motif (Trp-motif). In a recent study, using a yeast two-hybrid approach to analyze Trp-motif mutations, residues $p66^{W401}$ and $p66^{W414}$ were shown to be involved in RT dimerization.[21] Mutagenesis of other aromatic amino acids that lie between these two residues did not affect subunit interaction. Since $p66^{W401}$ and $p66^{W414}$ are not located at the dimer interface, the authors suggested that repositioning of structural elements between these residues accounted for their results. A comprehensive investigation of the Trp-motif has not been possible due to an inability to express most recombinant p51 Trp-motif mutants in yeast and bacteria.[21] Others have also reported that mutation of certain hydrophobic residues impairs recombinant p51 protein expression in bacteria, while the same mutation in p66 is expressed normally.[10]

The HIV-1 RT functions as a heterodimer and several studies have suggested that the dimerization process of RT could be targeted for HIV antiretroviral therapy.[22,23] Synthetic peptides corresponding to the connection subdomain (Trp-motif) have been reported to disrupt dimerization.[20,23,24] Perhaps most notably, a short peptide matching RT residues 395–404 was shown to inhibit heterodimerization *in vitro* and virus replication in cell culture.[20] Recent studies of non-nucleoside reverse transcriptase inhibitors (NNRTI) have heightened interest in compounds that interfere with RT conformational flexibility as a novel drug design concept.[25,26] NNRTI are a group of small hydrophobic compounds with diverse structures that inhibit HIV-1 RT (see Balzarini for a review).[27] NNRTIs interact with HIV-1 RT by binding to a site on the p66 subunit of the heterodimer. This results in both short-range and long-range distortions of the RT structure. NNRTIs have been shown to interfere directly with the global hinge-bending mechanism that controls the cooperative motions of the p66 fingers and thumb subdomains required for RT function.[28,29] In yeast, several NNRTIs were recently shown to enhance p51/p66 subunit association as a result of a specific interaction of drug with p66.[30] Clearly, understanding the biophysical parameters defining subunit interactions will be integral to designing effective inhibitors of RT.

We recently reported a novel approach that enables subunit-specific analysis of p51 and p66 in infectious virions.[31] We demonstrated that a Vpr-p51 fusion protein is incorporated into HIV-1 virions through an interaction between Vpr and the Gag precursor polyprotein, and when co-expressed p66 is specifically and selectively packaged as a Vpr-p51/p66 complex. Processing by the viral PR liberates Vpr, generating functional heterodimeric RT (p51/p66) and infectious viral particles. Thus, quantitative amounts of the p51 subunit incorporated into virions are relatively constant, with the amount of p66 present in the virion being a function of dimerization. By exploiting this approach we have now determined the contribution of amino acid residues comprising the Trp-motif to RT subunit interaction and virus infection. We demonstrate that inter-subunit interactions between the connection subdomains include W401, Y405 and N363 in p51 and W410 in p66, and that mutation of these residues impairs RT function and virus infectivity. The W401 residue of the Trp-motif was found to be of central importance. Mutation of this amino acid simultaneously in both subunits is deleterious to RT dimerization and virus infection. The RT heterodimerization enhancing drug, efavirenz (EFV), rescued this dimerization defect in a dose-dependent manner. Additionally, we demonstrate that intra-subunit interactions between tryptophan residues comprising a hydrophobic cluster (W398, W402, W406, W414) proximal to the connection subdomain interface are important for p51/p66 subunit interaction and stability. Our findings reveal important new insights into the biophysical nature of the Trp-motif and its role at the connection subdomain dimer interface.

Results

Structural analysis of the putative RT dimerization domain (tryptophan motif)

The Trp-motif of HIV-1 is comprised of aromatic amino acids in the connection subdomain (between amino acid positions 398 and 414). Alignment analysis shows that the six tryptophan residues (W398, W401, W402, W406, W410 and W414) and tyrosine (Y405) are conserved within the connection subdomain of most primate lentiviruses (Figure 1(a)). The most conserved residue amongst all the lentiviruses is W398 (data not shown). To understand the interactions at the dimerization interface between the two connection subdomains of HIV-1

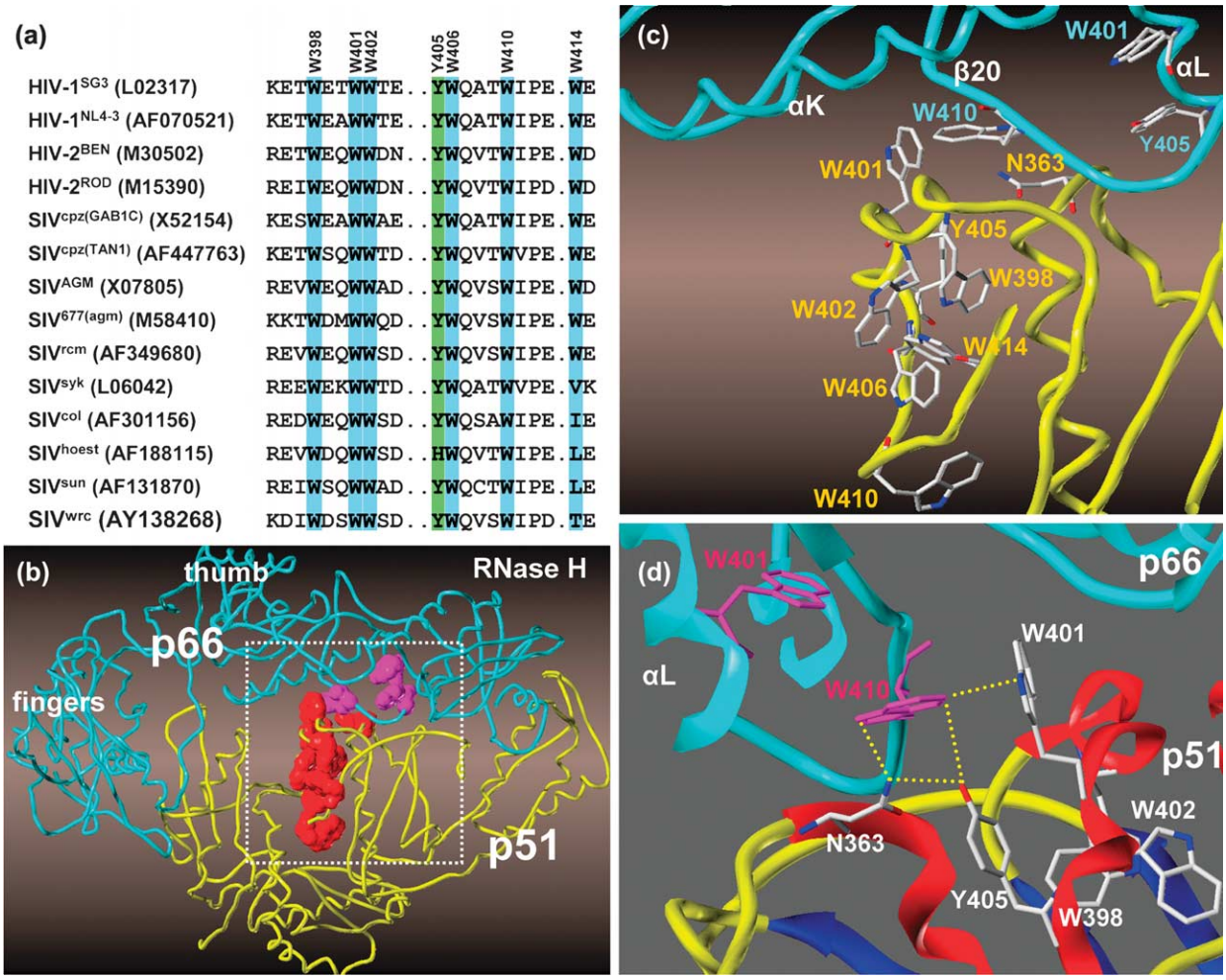

Figure 1. Structural analysis of RT connection subdomain. (a) Alignment of Trp-motifs of primate lentiviruses. The Pol amino acid sequences of representative strains of primate lentiviruses were aligned using MegAlign (DNASTAR, Inc.). HIV-1 RT sequence (amino acid residues 395–415) is shown along with corresponding alignments for other indicated primate lentiviruses. (b) Ribbon representation of the p66 (cyan) and p51 (yellow) subunits in the crystal structure of the complex of HIV-1 RT with double-stranded DNA and incoming tenofovir-diphosphate (pdb file 1T05).[46] For clarity, only the protein is shown. The tryptophan-rich motif and other p51 residues at the interface of the two subunits are shown in Van der Waals volumes (red). Residues W401 and W410 of the p66 subunit are shown at or near the interface also in Van der Waals volumes (magenta). (c) Magnification of the area in the box shown in (b). Shown are the side-chains of residues of the tryptophan motif and of the interface that were mutated in this study. (d) Ribbon representation of the interface between p66 and p51. W410 of the p66 subunit is shown to have extensive interactions with residues of the p51 subunit (p51-N363, p51-W401, and p51-Y405).

RT, we compared several crystal structures of HIV-1 RT, including unliganded (1DLO), in complex with substrates (pdb codes 1TO5, 1RTD, 1HYS, 1N6Q) or NNRTIs (1HNI, 1SV5, 1S6P, 1S9E, 1DTT, 1BMQ, 1FK9). The overall structure of the dimerization interface appears to be conserved among the various RT complexes. Stabilization of the heterodimer appears to involve direct, as well as indirect interactions between residues from the two subunits. Specifically, a key direct interaction appears to involve three p51 residues from the β18-αK (N363) loop, the αL helix (W401), and the αL-β20 loop (Y405), that are within interacting distance of residue W410 located in the αL-β20 loop of p66 (Figure 1(b)–(d)). In addition to these interactions, the W401 in p51 is also within interacting distance with p66 residue P412 at the base of the β20-sheet in p66. Indirect interactions may also play a role and involve residues that are proximal to the p66 or the p51 part of the interface. In the p51 subunit, a cluster of four Trp residues (W398, W402, W406 and W414) is proximal to the p51 interface residues (Y405, N363, and W401) (Figure 1(b)–(d)).

Expression and virion incorporation of hetromeric RT containing p51 Trp-motif mutations

The p51 Trp-motif residues were independently mutated to alanine in the pLR2P-vpr-p51-IRES-p66 (vpr-p51/p66) expression plasmid. Wild-type and each of the mutant DNAs were cotransfected into 293T cells with the RT-IN defective M7 proviral DNA, and progeny virions were examined by

immunoblot analysis. The control RT-IN-minus M7 particles (Figure 2(a), lane 2) did not contain RT. A similar level of both RT subunits (p51 and p66) was detected in particles derived by cotransfection of M7 and the wild-type vpr-p51/p66 expression plasmid (lane 3). The pLR2P-vpr-Δp51-IRES-p66 (vpr-Δp51/p66) control plasmid does not express p51, and the absence of detectable p66 (lane 4) confirmed that its incorporation was dependent on the expression of p51 (Vpr-p51). Analysis of $p51^{W398A}$/p66 (lane 5) showed p51 and p66 in the virion, however, an additional band was also detected migrating just below p66. This band was confirmed to be a product of the p66 subunit by probing with monoclonal antibody (mAb) 7E5 specific to the RNase H domain (data not shown). To a lesser extent, a similar p66 product was also seen in some of the other p51 mutants. Notably, the aberrant p66 product seemed to associate with mutants of residues (W398A, W402A, W406A and W414A) that cluster together proximal to the heterodimer interface (Figure 1(c)). Wild-type HIV-1 SG3 virions were analyzed as an additional control (lane 1). Immunoblot analysis using mAb to CA confirmed that approximately the same amount of each virus was analyzed (Figure 2(b)). Examination of transfected cells by immunoblotting with polyclonal anti-Vpr serum (Figure 2(c)) demonstrated that all of the mutants expressed Vpr-p51 (lanes 5–11) at levels similar to that of wild-type Vpr-p51 (lane 3). A replica blot probed with 7E5 mAb confirmed that p66 was expressed at a similar level among the transfected cells (Figure 2(d)). The amount of cellular protein analyzed was similar, as demonstrated by probing for the α-tubulin protein (Figure 2(e)).

To examine if the aberrant p66 was due to misprocessing by the viral protease (PR), 293T cells were cotransfected with a PR-defective proviral DNA (PR catalytic mutant: D25A) and vpr-$p51^{W398A}$/p66. Detection of the aberrant p66 product in these virions (data not shown), suggested that it was generated independently of the HIV-1 PR.

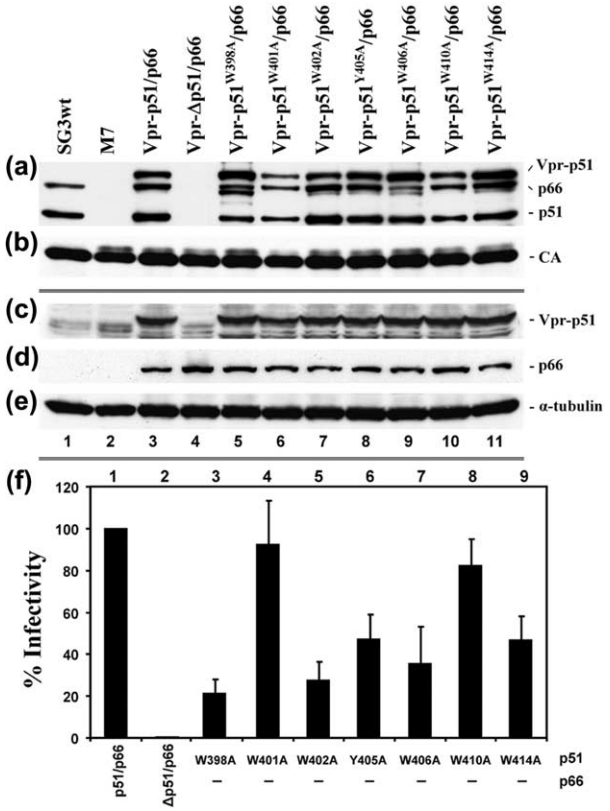

Figure 2. Analysis of p51 Trp-motif mutants. M7 proviral DNA was transfected into 293T cells alone or together with wild-type or mutant vpr-p51/p66 and vpr-IN expression plasmid DNAs. Transfection-derived virions were analyzed by immunoblotting for (a) RT (p51/p66) and (b) CA (p24). Expression of Vpr-p51 (c), p66 (d) and α-tubulin (e) in the transfected 293T was examined by immunoblotting. (f) Infectivity of p51 Trp-motif mutants. The infectivity of virions containing alanine substitutions in the p51 Trp-motif was analyzed using the TZM-bl reporter cell line as described in Materials and Methods. Infectivity is expressed as a percentage of the wild-type *trans*-RT heterodimer (Vpr-p51/p66) complemented virions.

Functional analysis of p51 Trp-motif RT mutants

The functionality of the p51 Trp-motif mutants was analyzed in a single-round infectivity assay, using the TZM-bl reporter cell line.[32] Virions were generated by cotransfecting 293T cells with M7, wild-type or mutant vpr-p51/p66 and vpr-IN. vpr-IN was included since M7 lacks IN, which is required for efficient initiation of reverse transcription and for integration of the nascent viral cDNA.[31,33] The infectivity of virions containing the wild-type *trans*-RT (Vpr-p51/p66) was normalized to 100% (Figure 2(f), lane 1). The infectivity of M7 derived by cotransfection with vpr-Δp51/p66 was less than 0.2% compared to vpr-p51/p66 (lane 2). Mutations in the tryptophan cluster (W398A, W402A, W406A and W414A) decreased infectivity to less than 50% (lanes 3, 5, 7 and 9, respectively), with the W398A mutant being the most defective. The infectivity of the $p51^{W401A}$/p66 mutant (lane 4) was similar to that of wild-type, while the $p51^{Y405A}$/p66 mutant (lane 6) was reduced to about 50%. The $p51^{W410A}$/p66 mutant (lane 8) had little effect on infectivity, consistent with our previous report on this mutant.[31]

Subunit-specific mutagenesis of Trp-motif residues at the heterodimer interface

Our analysis of inter-subunit interactions was focused initially on mutagenesis of individual residues to either alanine or leucine. The infectivity of the wild-type RT *trans*-heterodimer was normalized to 100% (Figure 3(a), lane 1). The vpr-Δp51/p66 was less than 0.2% infectious (lane 2), as reported previously. Replacement of $p51^{W401}$ with either alanine or leucine did not affect viral infectivity (lanes 3 and 4). Both the $p51^{Y405A}$/p66

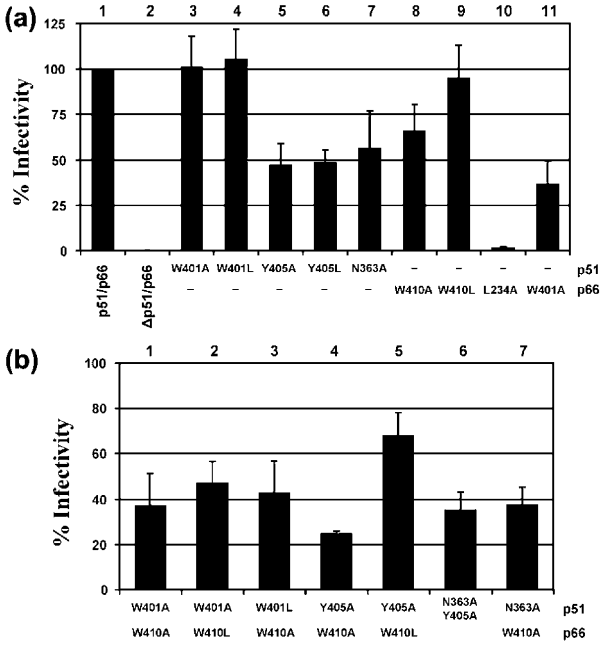

Figure 3. Analysis of Trp-motif residues located at the RT heterodimer interface. Trp-motif residues that lie within interacting distance at the dimer interface were mutated. The infectivity of virions containing single (a) or dual (b) mutations was analyzed by the TZM-bl reporter cell assay. Infectivity is expressed as a percentage of the wild-type *trans*-heterodimer control.

and p51^{Y405L}/p66 mutant RTs reduced infectivity to approximately half of that of the wild-type *trans*-heterodimer (lanes 5 and 6, respectively). Mutation of N363 in p51 to alanine also reduced infectivity, albeit to a lesser extent than the 405 mutations (lane 7). The replacement of p66^{W410} with alanine caused a slight reduction in infectivity (lane 8), while the leucine substitution had no effect (lane 9). The p51/p66^{L234A} and p51/p66^{W401A} mutants, reported previously as mutations that affect dimer formation, were included as controls in our experiments.[21,34] The p51/p66^{L234A} mutant reduced infectivity to less than 5% (lane 10), while the p51/p66^{W401A} mutant was approximately 40% infectious (lane 11).

To further delineate these Trp-motif interactions, residues that lie within interacting distance of each other were mutated in pairs. Mutations were made in conjunction at residues W401 and W410 of p51 and p66, respectively. Infectivity analysis of p51^{W401A}/p66^{W410A}, p51^{W401A}/p66^{W410L} and p51^{W401L}/p66^{W410A} suggested that mutagenesis of both residues together reduced viral infectivity (approximately 40%) to a significantly greater extent compared to that of the single mutations (Figure 3(b), lanes 1–3). Analysis of RT containing simultaneous mutations at p51^{Y405} and p66^{W410} indicated that substitution of both residues with alanine decreased infectivity to about 25% of the wild-type heterodimer (lane 4). In contrast, the p51^{Y405A}/p66^{W410L} double mutant (lane 5) did not show an additive negative effect and did not reduce virus infectivity when compared to the p51^{Y405A}/p66 single mutant, suggesting that mutagenesis of p66^{W410} to leucine does not affect its interaction with Y405 of p51. Our model predicts that the residue N363 in p51 interacts with both p51^{Y405} and p66^{W410}. The p51$^{N363A;Y405A}$/p66 (lane 6) and p51^{N363A}/p66^{W410A} (lane 7) virions had similar infectivity, which was reduced to approximately 35% of wild-type and substantially lower than the respective single mutants. Immunoblot analysis detected only a slight reduction in Vpr-p51-mediated p66 incorporation in some of the double mutants (data not shown).

Analysis of the inter-subunit interface in provirus

Careful consideration of our results indicated an interaction at the dimer interface between residues p51^{W401} and p66^{W410} that is important for subunit interaction. Additional analysis of the W401A and W410A mutations was conducted in the context of the complete HIV-1 NL4-3 proviral clone. The wild-type or mutant proviral DNAs were transfected into 293T cells and progeny virions were analyzed for infectivity. The infectivity of virus containing the W401A mutation was less than 5% of that of wild-type (Figure 4(a), lanes 1 and 3). In contrast, W410A caused an increase in virus infectivity (lane 4). The non-infectious RT-minus M7 clone was included as negative control (lane 2). Notably, immunoblot analysis showed a significantly reduced amount of the W401 mutant RT in virions, compared to either the wild-type or W410A mutant (Figure 4(b)). Probing a replica blot with mAb to CA confirmed that approximately the same amount of each virus was analyzed (Figure 4(c)).

Analysis of W401A mutation in the RT *trans*-heterodimer

To determine the effect of the W401A proviral DNA mutation on RT, the mutation was analyzed by subunit-specific *trans*-complementation, where the mutation was present in p51, p66 or both p51 and p66. The infectivity of virions complemented with the wild-type *trans*-heterodimeric RT was normalized to 100% (Figure 5(a), lane 1). Subunit-specific mutagenesis of p51 (p51^{W401A}/p66) did not significantly affect viral infectivity, as described above (lane 3), while mutagenesis of the p66 subunit (p51/p66^{W401A}) reduced infectivity to about 40% (lane 4). The effect of this mutation in both subunits (p51^{W401A}/p66^{W401A}) was quite dramatic (lane 5), reducing infectivity to levels similar to that observed for the W401A mutant provirus.

Analysis of virions produced by co-expressing vpr-p51^{W401A}/p66 demonstrated, as expected, wild-type levels of both subunits (Figure 5(b), lanes 1 and 3). However, when the W401A mutation was present in the p66 subunit (vpr-p51/p66^{W401A}),

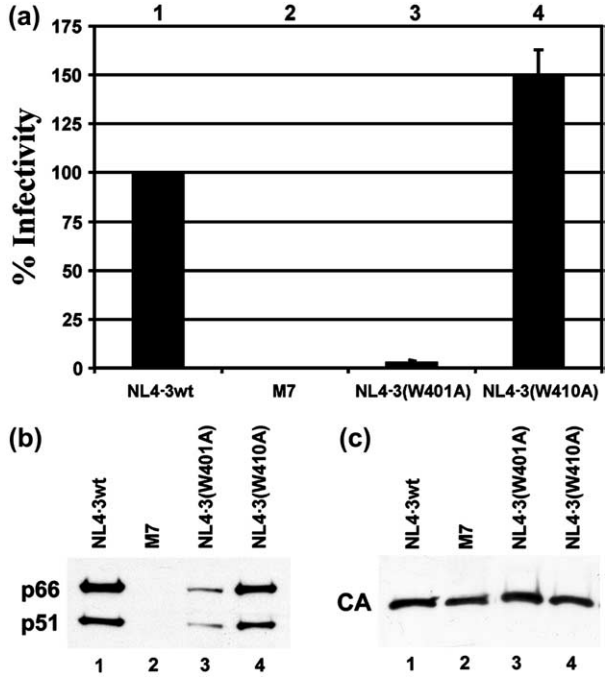

Figure 4. Analysis of W401 and W410 mutations in HIV-1 provirus. (a) The importance of RT Trp-motif residues W401 and W410 for viral infectivity was analyzed using the HIV-1 NL4-3 molecular clone. Infectivity was determined using TZM-bl reporter cells and the results are expressed as a percentage of wild-type NL4-3. Virions derived by transfection of the wild-type and mutant proviral DNAs were also analyzed by immunoblotting using mAbs to RT (b) and CA (c).

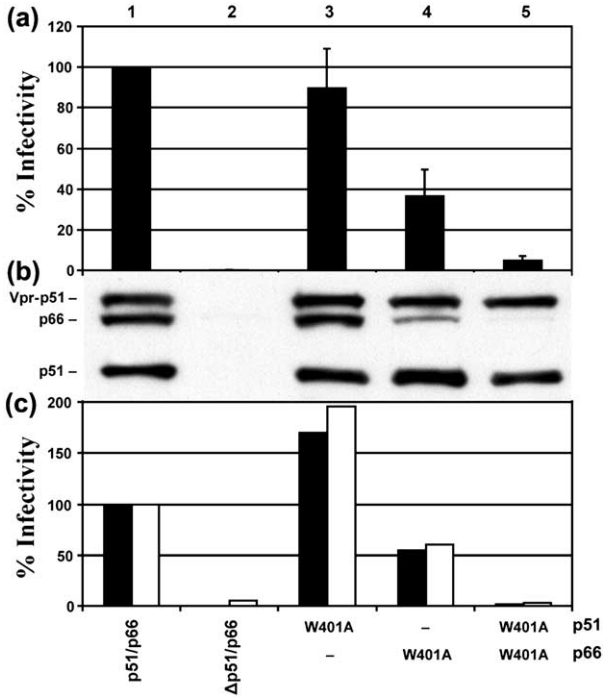

Figure 5. Subunit-specific analysis of the W401A mutant. The W401 residue was mutated in p51, p66 or p51 and p66. Transfection-derived virions containing the respective mutant *trans* RTs, were analyzed for (a) infectivity on TZM-bl cells and (b) virion incorporation of p51 and p66 by immunoblotting. (c) Virion infectivity was determined using the TZM-bl reporter cells (black bars) and the JLTRG-R5 reporter T cell line (white bars). Infectivity is expressed as a percentage of the wild-type *trans*-RT control.

the incorporation of p66 into M7 virions was reduced (lane 4). Interestingly, the presence of W401A in both p51 and p66 further reduced the amount of p66 detected in virions (lane 5). In all cases, reduced virion p66 was also seen using a polyclonal RT antiserum and the amount of p66 expressed in the cells was normal (data not shown). The decrease in virion p66 observed with the p51/$p66^{W401A}$ and $p51^{W401A}$/$p66^{W401A}$ mutants was identical to that observed when virions were produced using a PR-defective proviral DNA (PR catalytic mutant: D25A) in place of M7 (data not shown). This result confirmed that less p66 was detected in the M7 virions because the W401A mutation(s) impaired p66 virion incorporation. Importantly, this result ruled out the possibility that less p66 was detected due to overprocessing and conversion of p66 to p51 by the viral protease subsequent to virion assembly.

To analyze the infectivity of *trans*-RT complemented virions in a target cell that is more physiologically relevant, the JLTRG-R5 reporter cell line was used. These cells are derived from JLTRG cells and are of Jurkat T cell lineage (unpublished results).[35] The JLTRG-R5 cells have stably integrated EGFP reporter under control of the HIV-1 long terminal repeat (LTR), and thus EGFP expression is induced by virus infection. Infectivity for the wild-type *trans*-RT heterodimer was normalized to 100% (Figure 5(c), lane 1). The Δp51/p66 exhibited infectivity below 5% (lane 2). The W401A mutation in p51 did not reduce viral infectivity (lane 3), while W401A in p66 reduced infectivity to about 50% of that of the wild-type *trans*-RT (lane 4). The presence of W401A simultaneously in p51 and p66 significantly decreased infectivity (lane 5). The data shown in Figure 5(c) are from a single experiment in which infectivity in the JLTRG-R5 and TZM-bl cell lines was compared head-to-head. Among the different mutants that were analyzed, the result for the $p51^{W401A}$/p66 mutant was an outlier compared with the mean of all the previous experiments using the TZM-bl cell line for analysis. Importantly, the infectivity results from the experiment shown in Figure 5(c) for the JLTRG-R5 cell line paralleled those of the TZM-bl cell line. These data support the conclusion that the results of RT subunit-specific function in TZM-bl indicator cell are consistent with those generated using T cells, a more physiologically relevant target, and emphasize the biological relevance of our approach. The results indicate that the *trans*-RT heterodimer complemented virions can be analyzed

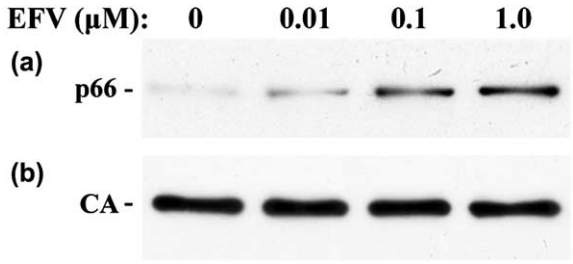

Figure 6. Effect of NNRTIs on RT subunit interactions. Virions were generated by cotransfection of 293T cells with M7 and *trans*-RT dimerization-defective mutant plasmid vpr-p51^{W401A}/p66^{W401A}. The dimerization enhancing NNRTI EFV was added to the culture medium 12 hours after DNA transfection at concentrations ranging from 0.01 μM to 1.0 μM. The transfection-derived virions were collected 48 hours later and analyzed by immunoblot using mAbs to (a) RNase H and (b) CA.

in multiple human-derived reporter cell lines including more physiologically relevant T cell lines.

Efavirenz enhances subunit interaction in the *trans*-RT W401A double mutant

To examine the effect of dimerization enhancing drugs on the dimerization-defective W401A mutant *trans*-heterodimeric RT, EFV was added to the producer cells (transfected 293T cells) at concentrations ranging from 0.01 μM to 1.0 μM. Examination of virion-associated p66^{W401A} incorporation, which is dependent upon interaction with p51^{W401A} (Vpr-p51^{W401A}), showed that EFV rescued subunit dimerization in a dose-dependent manner (Figure 6(a)). Equal virion protein loading was confirmed by probing a replica blot with anti-CA mAb (Figure 6(b)). The amount of virion-associated p51 and Vpr-p51 was equal in both the absence and presence of drug (data not shown). Similar results were also observed for other second generation NNRTIs that show promise in early clinical trials (data not shown).

Discussion

RT subunit interactions have been extensively analyzed using biochemical and other non-physiological methods. The lack of a virus-based approach for analyzing subunit interactions has hampered our understanding of the dimerization process, and possibly the development of RT antagonists that affect subunit interactions. Two significant problems have hindered structure/function studies of RT using infectious virus. First, RT is encoded and assembled into virions as part of the Pr160$^{Gag\text{-}Pol}$ polyprotein, and consequently, mutations in RT/Pr160$^{Gag\text{-}Pol}$ may be pleiotropic, affecting multiple steps of the viral life cycle such as assembly, maturation, etc.[8–10,36] Analogous with our results for the W401A proviral mutant, Yu *et al*. have reported that mutations in the polymerase primer grip decrease virion-associated RT due in part to premature Gag-Pol processing.[8] The second problem is due to the heterodimeric nature of the RT. The asymmetry of the p51 and p66 subdomains entails that a mutation in one subunit is structurally and functionally non-equivalent to the same mutation in the other subunit. Thus, we developed a novel *trans*-complementation approach for analyzing the RT heterodimer in precise molecular detail in the context of infectious virions. By exploiting this approach, we have addressed several relevant questions concerning HIV-1 RT biology that were previously experimentally inaccessible. Primarily, these include (i) the role of hydrophobic amino acid residues comprising the Trp-motif for subunit interaction and RT function, (ii) the contribution of amino acid residues at the p51/p66 connection subdomain interface to RT dimerization and virus infection, and (iii) the availability of a biologic approach capable of assessing the effects of both dimerization enhancing and disrupting drugs.

Several studies have suggested that the hydrophobic residues in the Trp-motif are important for RT dimerization.[15,37] A recent study, utilizing a yeast two-hybrid approach demonstrated that residues p66^{W401} and p66^{W414} are important for subunit interaction.[21] Because neither of these residues is located at the dimer interface, the authors inferred that repositioning of structural elements in the p66 αL-β20 loop, which lie between these residues, affected p51–p66 interaction and accounted for their observation. Since heterodimer formation was not affected by mutagenesis of the other aromatic amino acids in the p66 αL-β20 loop, it was suggested that backbone carbonyl groups in the loop and not specific amino acid side-chains provide the important interactions at this interface. However, the inability to express most of the p51 Trp-motif mutants precluded a comprehensive examination of this p51/p66 interface. Structural analysis of interactions in the Trp-motif with residues at the subunit interface in several complexes of RT with substrate or inhibitors shows that the side-chain of W410 in the p66 αL-β20 loop is consistently within interacting distance of p51 residues W401, Y405, and N363 (Figure 1(d)). *Trans*-complementation analysis of these putative inter-subunit interactions showed that mutation of individual residues at this interface, with the exception of p51^{W401}, caused a measurable decrease in virus infectivity. Simultaneous mutagenesis of two inter-subunit residues within interacting distance of each other further impaired viral infectivity, suggesting that this effect was most likely due to effects on subunit interactions. The data from immunoblots (not shown) indicate similar to wild-type (Vpr-p51/p66) levels and processing of the mutant *trans*-RT of the two subunits. The decreased infectivity could be explained as follows: (i) The destabilization of the heterodimer is not sufficient to cause disassociation. Thermodynamic evaluations

of p51/p66 have estimated a relatively high Gibbs free energy of dimer stabilization (approximately 10–12 kcal/mol), corresponding to a dissociation constant of ~10.0 nM.[14,38] This has necessitated that *in vitro* dimer dissociation studies be carried out in the presence of denaturants like urea or acetonitrile.[14,23,39] Thus, a precedent for dimer destabilization without disassociation in the absence of extrinsic factors exists. However, destabilization, albeit not sufficient to cause disassociation, could impair RT function and consequently virus infectivity. (ii) It is possible that mutations may have global effects on the folding of the subunits.

The most severe effect on heterodimerization was observed for the $p51^{W401A}/p66^{W401A}$ mutant. The presence of W401A in both subunits markedly impaired p51–p66 interaction, directly evidenced by a significant decrease in Vpr-$p51^{W401A}$-mediated $p66^{W401A}$ packaging. Based on our data, the structural analysis of several RT crystal structures and previous reports on the Trp-motif, we believe that repositioning the αL-β20 loop by mutating $p66^{W401}$ and disruption of interactions involving $p51^{W401}$, $p51^{W405}$, $p51^{N363}$ and $p66^{W410}$ account for our findings. Our subunit-specific mutational analysis of the W401 RT mutants demonstrates that W401 of the p66 and p51 subunits has distinct structural roles in the stabilization of the RT heterodimer. In p51 the W401A mutation appears to affect the interactions at the interface, through disruption of the π–π interactions with $p66^{W410}$. However, the p66-W401A mutation appears to affect the folding of the p66 subunit because it is at the interface of two helices (αL and αK) (Figure 1(c)). Hence, when both subunits are mutated the different effects appear additive.

Our structural analysis of the dimer interface in several RT crystal structures highlighted the potential importance of a cluster of four tryptophan residues in p51 (W398, W402, W406 and W414) proximal to the dimer interface. While these four p51 tryptophan residues do not directly interact with p66 residues, they are clustered together through hydrophobic interactions and seem poised to indirectly affect the dimer interface by their proximity to residues Y405, W401, and N363 of p51 that are at the p51–p66 interface (Figure 1(b) and (c)). Our subunit-specific mutagenesis of these residues suggests that the Trp cluster affects the interaction between p51 and p66 (Figure 2). Interestingly, alanine substitution resulted in a misprocessed form of p66 that was detected in virions. *Trans*-complementation analysis using PR-defective virus indicates that a cellular protease is responsible for the aberrant processing of p66. We suggest two possible explanations for this observation. First, the p51 Trp mutants may interact with and incorporate into virions a smaller processed form of p66 generated in the cell. Our failure to detect the aberrant p66 in cell lysates, suggests that it is present at a significantly lower level than wild-type p66. Alternatively, these p51 Trp mutants might form unstable heterodimers in which p66 is misfolded and thus, susceptible to proteolytic processing by a cellular protease. If this were true, it is interesting to note that dimer instability causes p66 misprocessing instead of normal processing to generate p51, which could associate with disassociated p66 to give functional RT heterodimer. The defect in infectivity seen with the mutants containing misprocessed p66 further supports this interpretation. Although p66 misprocessing seems to occur as a consequence of the atypical manner in which RT is expressed *via* the *trans*-complementation approach, our results suggest that residues W398, W402, W406 and W414 are important for proper RT subunit interactions.

NNRTIs are a chemically diverse group of largely hydrophobic compounds that inhibit HIV-1 RT by binding in a hydrophobic pocket near the polymerase active site in the p66 subunit. NNRTIs have been described that can either stabilize or destabilize the RT heterodimer. Various NNRTIs have also been found to induce increased β-gal activity in the yeast two-hybrid system, due to enhanced RT subunit association.[30] In particular, EFV binding to the NNRTI hydrophobic pocket enhanced RT heterodimerization, including RT with p51/p66 W401 mutations. Additionally, both the 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-beta-D-ribofuranosyl 3′-spiro-5″-(4″-amino-1″,2″-oxathiole 2″,2″-dioxide) (TSAO) thymine derivatives and the *N*-acyl hydrazones are classes of compounds that show inhibition characteristics similar to NNRTIs. Although these drugs may not bind to the well-defined NNRTI binding pocket of HIV-1 RT, they bind to a region of RT close to and partially overlapping this site. Furthermore, in the presence of a denaturant like urea these compounds have been shown to destabilize HIV-1 RT heterodimerization.[14,39] Our results show a dose-dependent enhancement of dimerization of the $p51^{W401A}/p66^{W401A}$ RT mutant in the presence of EFV. Since our approach for analyzing subunit-specific structure/function is conducted in a context that is physiologically relevant to HIV-1 replication, it should provide valuable insight into the ability of small molecule inhibitors to affect RT subunit interactions. Since RT is incorporated into virions as part of the $Pr160^{Gag\text{-}Pol}$ precursor polyprotein, it may be argued that initial dimerization events may occur at the level of the polyprotein, making inhibitors that act at the level of p51–p66 interactions inconsequential at the level of $Pr160^{Gag\text{-}Pol}$ dimerization. A recent study, albeit carried out in bacteria, suggests that EFV enhances proteolytic processing of a segment of $Pr160^{Gag\text{-}Pol}$.[40] Despite the need to carefully consider these factors, our system represents the first viable approach for analyzing the effects of RT dimerization inhibitors in the context of virus infectivity. With heightened interest in compounds that inhibit RT conformational flexibility as a novel drug design concept,[26] especially for drugs that will be effective against mutating RT targets, our results indicate the utility

of this novel *trans*-complementation approach for assessing the effects of RT inhibitors on subunit interactions using infectious virions in a context that is more physiologically relevant.

Materials and Methods

Cells, antibodies and antiviral drugs

The 293T, JC53,[41] and TZM-bl cell lines[32] were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). The JLTRG-R5 cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 15% FBS and gentamycin (0.1 mg/ml). Antibodies used included polyclonal anti-RT and anti-Vpr sera[42] and mAbs to human α-tubulin (Sigma), HIV-1 CA (183-H12-5C, contributed by Bruce Chesebro and Hardy Chen) and HIV-1 RT and RNase H (8C4 and 7E5, respectively, contributed by Dag E. Helland), obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The NNRTI EFV was obtained from the NIH AIDS Research and Reference Reagent Program.

Plasmid constructs

The HIV-1 pSG3 (SG3) proviral clone (Genbank accession no. L02317)[43] was used to produce wild-type virus, and to construct the RT-deficient proviral clone and all recombinant RT and IN expression plasmids (for abbreviations of plasmids see Table 1). The RT-IN-minus $pSG3^{M7}$ (M7) proviral construct described previously[31] was used for *trans*-complementation analysis with all the pLR2P-based RT and IN expression plasmids. For expressing the RT subunits *in trans*, the pLR2P-vpr-p51-IRES-p66 (vpr-p51/p66) plasmid[31] was modified by including 135 bp of PR sequence 5′ of RT. This increased the molecular mass of the Vpr-RT fusion protein and enabled visual separation from p66 by immunoblot analysis. Briefly, p51-encoding sequence was PCR amplified from SG3 using primers containing BglII and MluI restriction sites, respectively. The internal ribosome entry site (IRES) was PCR amplified from the encephalomyocarditis virus (EMCV) (Genbank accession no. NC_001479) with primers containing MluI and XmaI sites, respectively. The p51 and IRES DNA fragments were digested with corresponding endonucleases and ligated simultaneously into the BglII-XmaI cut pLR2P-vprRT.[44] The vpr-p51/p66 was modified in that the N-terminal protease cleavage (PC) site of RT was maintained by including 135 bp of PR-encoding sequence 5′ of RT compared to 33 bp of PR sequence in the original construct. The vpr and p51 coding sequences were placed in-frame, with a translational stop codon (TAA) to terminate RT expression at amino acid residue 440, which is the full-length p51 subunit. The vpr-p51 reading frame was followed by the IRES and then the p66-encoding DNA sequence. Mutant derivatives of vpr-p51/p66 (Table 1) were constructed using PCR-based site-directed mutagenesis and cloning into the BglII-MluI or XmaI-XhoI sites for p51 and p66-containing DNA fragments, respectively. The pLR2P-vpr-Δp51-IRES-p66 (vpr-Δp51/p66) control expression plasmid was constructed to contain a translational stop codon at the first amino acid position of p51.[31] This plasmid controls for non-specific incorporation of p66 into viral particles. All mutant clones were confirmed by nucleotide sequence analysis. The pLR2P-vprIN (vpr-IN) expression vector has been described.[44]

Table 1. Abbreviations for plasmids

Plasmid	Abbreviation
$pSG3^{wt}$	SG3
$pSG3^{M7}$	M7
pLR2P-vpr-p51-IRES-p66	vpr-p51/p66
pLR2P-vpr-Δp51-IRES-p66	vpr-Δp51/p66
pLR2P-vpr-p51-IRES-$p66^{MUTANT}$	vpr-p51/$p66^{MUTANT}$
pLR2P-vpr-$p51^{MUTANT}$-IRES-p66	vpr-$p51^{MUTANT}$/p66
pLR2P-vpr-IN	vpr-IN

The abbreviations for plasmids used in this study are listed above. All mutants were abbreviated in a similar style as shown for ^{MUTANT}.

Transfection and analysis of virus infectivity

DNA transfections were performed on monolayer cultures of 293T cells grown in six-well plates using the calcium phosphate DNA precipitation method. Unless otherwise noted, each cell monolayer (well) was transfected with 6 μg of proviral DNA, 3 μg of the vpr-p51/p66 constructs and 1 μg of the vpr-IN construct. Culture supernatants from the 293T cells were collected 60 hours post-transfection, clarified by low-speed centrifugation (1000*g*, ten minutes), and filtered through 0.45 μm pore-size sterile filters. The clarified supernatants were analyzed for HIV-1 p24 concentration by ELISA (Beckman-Coulter Inc.).

Virus infectivity was assessed using the TZM-bl reporter cell line as described.[33] Briefly, virus-containing supernatants were normalized for p24 antigen concentration, serially diluted (fivefold dilutions) and used to infect monolayer cultures of TZM-bl cells. At 48 hours post-infection, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) reagent as described.[45] Following X-gal staining, the blue-stained cells were counted using a light microscope. Wells containing between 30 and 300 blue cells were used to calculate the infectious units of virus per ng of p24 antigen (IU/p24-ng).

The ability of *trans*-RT-containing virions to infect T cells was tested by quantitatively analyzing infection of the JLTRG-R5 reporter T cell line. 12-well flat-bottomed culture plates containing 1.0×10^5 JLTRG-R5 cells were infected at a multiplicity of infection (MOI) of 5.0 (as determined by the TZM-bl assay) for the wild-type Vpr-p51/p66 complemented virions. The other *trans*-RT-containing virion preparations were normalized for p24 antigen concentration equivalent to that of the wild-type *trans*-RT. The total volume was adjusted to 1 ml and the infection was carried out at 37 °C for 24 hours. At 24 hours post-infection, 1 ml of fresh RPMI 1640 was added to each well and culture was continued at 37 °C for an additional 48 hours. Then the cells were washed (2×) in phosphate buffered saline (PBS). The cell pellet was resuspended in 50 μl PBS and then fixed in 1% paraformaldehyde (in PBS). The expression of EGFP was measured using a FACStar Plus flow cytometer with CellQuest software (Becton Dickinson).

Effects of NNRTIs on *trans*-RT subunit interaction

DNA transfections were performed on monolayer

cultures of 293T cells grown in six-well plates using the FuGENE 6 Transfection Reagent (Roche), as recommended by the manufacturer. The M7 and vpr-p51/p66-based plasmids were used at a ratio of 2:1. At 24 hours post-transfection, the specified concentrations of drug were added. Culture supernatants from the 293T cells were collected 60 hours post-transfection, clarified by low-speed centrifugation (1000*g*, ten minutes), and filtered through 0.45 μm pore-size sterile filters. The clarified supernatants were processed for and analyzed by immunoblot as described below.

Immunoblot analysis

Transfection-derived virions were concentrated by ultracentrifugation through 20% (w/v) sucrose cushion (125,000*g*, two hours, 4 °C) using a SW41 rotor (Beckman Inc.). Pellets were solubilized in Laemmli loading buffer (62.5 mM Tris–HCl (pH 6.8), 0.2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol), boiled, and proteins were separated on 12.0% (w/v) polyacrylamide gels containing SDS. Following electrophoresis, proteins were transferred to nitrocellulose (0.2 μm pore size) by electroblotting and incubated for one hour at room temperature in blocking buffer (5% (w/v) non-fat dry milk in PBS). The blocked blots were exposed to an appropriate primary antibody for one hour in blocking buffer with constant mixing. After extensive washing, bound antibodies were detected by chemiluminescence using horseradish peroxidase-conjugated species-specific secondary antibodies (Southern Biotechnology Associates, Inc.).

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