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Structural Requirements for Recognition of the Human Immunodeficiency Virus Type 1 Core during Host Restriction in Owl Monkey Cells

Brett M. Forshey, Jiong Shi, and Christopher Aiken*

Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee

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Human immunodeficiency virus type 1 (HIV-1) infection of simian cells is restricted at an early postentry step by host factors whose mechanism of action is unclear. These factors target the viral capsid protein (CA) and attenuate reverse transcription, suggesting that they bind to the HIV-1 core and interfere with its uncoating. To identify the relevant binding determinants in the capsid, we tested the capacity of viruses containing Gag cleavage site mutations and amino acid substitutions in CA to inhibit restriction of a wild type HIV-1 reporter virus in owl monkey cells. The results demonstrated that a stable, polymeric capsid and a correctly folded amino-terminal CA subunit interface are essential for saturation of host restriction in target cells by HIV-1 cores. We conclude that the owl monkey cellular restriction machinery recognizes a polymeric array of CA molecules, most likely via direct engagement of the HIV-1 capsid in target cells prior to uncoating.

Following cell entry, retrovirus particles release their ribonucleoprotein cores containing the viral RNA payload into the host cytoplasm. The capsid plays a key role in early postentry steps, as alterations in its structure result in severe loss of infectivity (6, 30, 39, 43, 49). Biochemical analyses of subviral complexes recovered from human immunodeficiency virus type 1 (HIV-1)-infected cells suggest that the core undergoes "uncoating," which we define as specific disassembly of the viral capsid. Because reverse transcription complexes and preintegration complexes purified from cells acutely infected with HIV-1 lack significant quantities of viral capsid protein (CA) (8, 23), it has generally been assumed that HIV-1 uncoats immediately following entry into the cytoplasm. However, recent studies indicate that uncoating is a finely tuned process and that the viral capsid may persist in the cytoplasm prior to undergoing disassembly (10, 31, 36).

The HIV-1 core consists of a viral genome housed within a conical viral capsid that is generated during virion maturation. In HIV-1 particles, the capsid is composed of approximately 1,500 CA monomers. The 231-amino-acid CA of HIV-1 is comprised of two distinct, globular domains whose structures are known. The wedge-shaped, amino-terminal domain (amino acids 1 to 151) contains seven α-helices, a β-hairpin, a β-turn, and a solvent-exposed loop that binds the host cell protein cyclophilin A (CypA) (11, 14, 33). The smaller carboxyl-terminal domain consists of four α-helices and is essential for HIV-1 particle assembly (1, 12, 30, 33).

HIV-1 CA is translated as a segment of a viral structural protein precursor, the Gag polyprotein. During particle budding, the viral protease (PR) is activated and cleaves Gag, releasing matrix (MA), CA, p2, nucleocapsid (NC), and p6 proteins. Release of both ends of CA is required for formation of the mature viral capsid. Cleavage of the MA-CA junction induces refolding of the amino-terminal domain of CA into a β-hairpin structure that appears to promote the CA-CA intermolecular interactions necessary for proper capsid formation (48). Cleavage at the CA-p2 junction, the final step in Gag processing, disrupts a putative α-helix that spans the cleavage site. Release of p2 is required for complete condensation of the capsid shell around the ribonucleoprotein complex (15, 51). Reconstruction analysis of cryoelectron microscopic images of cylindrical polymers of CA assembled in vitro has led to a structural model for the HIV-1 capsid. In this model, CA is organized into helical arrays of hexameric rings, with the precise shape of the core dictated by the location and number of pentameric defects (13, 27, 33).

In addition to its roles in assembly and early postentry events in infection, CA is also the target of species-specific restriction factors (5, 25, 36, 47). Host restriction of retroviruses was first described in inbred mice that were resistant to infection by specific strains of murine leukemia virus (MLV) (16, 22, 28, 29). A murine restriction factor, Fv1, was identified by positional cloning and was shown to have sequence similarity to Gag proteins of endogenous retroviruses (3). Fv1 targets MLV CA and inhibits infection at a postentry step, following the accumulation of viral DNA but prior to proviral integration into the host genome (22, 26, 38). However, a direct interaction between Fv1 and CA has not been demonstrated, and the mechanism of restriction remains unknown.

Recently, the Fv1-like restriction of retroviruses has also been described in nonmurine cells (2, 5, 17–19, 41, 45, 46). Cells of many Old World monkey species restrict HIV-1 infection, while New World monkey cells restrict simian immunodeficiency virus SIVmac (2, 18, 36). Many nonmurine cell lines also restrict N-tropic MLV infection (44, 45). These restrictions are referred to as Lv1 and Ref1 in simian and human cells, respectively, and they act to prevent reverse transcrip-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Vanderbilt University School of Medicine, A-5301 Medical Center North, Nashville, TN 37232-2363. Phone: (615) 343-7037. Fax: (615) 343-7392. E-mail: chris.aiken@vanderbilt.edu.

tion. Restriction is saturated at high levels of input virions or virus-like particles. Although no Fv1 ortholog has been identified in primate cells, Lv1 and Ref1 restrictions also target the CA proteins of susceptible viruses, as elegantly demonstrated through the use of viruses containing chimeric Gag genes (25, 34, 36). Several cellular genes that mediate Lv1 and Ref1 restriction have recently been identified. A rhesus macaque protein, TRIM5α, inhibited infection by HIV-1 but not SIV or MLV when expressed in permissive human cells (42). RNA interference experiments further demonstrated that TRIM5α is necessary for Lv1 restriction in macaque cells. A human allele of TRIM5α exhibited only modest HIV-1 inhibitory activity, supporting the identification of TRIM5α as a species-specific host restriction factor. Human TRIM5α was then shown to be a key mediator of Ref1 restriction of N-tropic MLV (20, 24, 37, 53). Interestingly, African green monkey TRIM5α restricts both MLV and lentiviruses (20, 24, 53). In owl monkey cells, in contrast to rhesus cells, the ability of CA to bind host cell CypA is required for restriction of HIV-1. Disruption of the CA-CypA interaction, via mutations in CA or by the addition of cyclosporine A, allows HIV-1 to escape restriction in owl monkey cells (47). Recently, owl monkey Lv1 was identified as *TRIMCyp* (34a, 40). *TRIMCyp* encodes a fusion between TRIM5 and CypA, and accounts for the cyclosporine-sensitive restriction of HIV-1 observed in owl monkey cells. Collectively, these studies demonstrate a major role of TRIM5α in host restriction and suggest that divergent retroviruses may be restricted by a common mechanism.

The mechanism of retroviral host restriction is unknown. An attractive hypothesis is that host factors engage the incoming viral capsid and perturb uncoating in target cells. To probe the relationship between host restriction and HIV-1 uncoating, we examined the structural requirements for Lv1 recognition of the HIV-1 core in owl monkey cells. By quantifying the ability of mutant HIV-1 particles to inhibit restriction of a green fluorescent protein (GFP)-expressing HIV-1 reporter virus, we found that a stable viral capsid and properly folded CA amino terminus are required for engagement of the incoming HIV-1 core by Lv1. We conclude that the host restriction machinery recognizes a critical number of CA subunits in the context of the polymeric viral capsid.

MATERIALS AND METHODS

Cells and viruses. HeLa, 293T, and owl monkey kidney (OMK) cells were cultured in Dulbecco's modification of Eagle medium (Cellgro) supplemented with 10% fetal bovine serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml) at 37°C and 5% CO_2. HIV-1 proviral DNA constructs R9 and NL4-3, encoding full-length open reading frames for all HIV-1 structural and accessory genes, were used for these studies. Env-deficient HIV-1 proviruses were created by transferring the EcoRI-BamHI fragment of NL4-3ΔE to the parental provirus. NL.CA/NC mutants were created by transferring the BssHII-SpeI fragment from R9 CA mutant (E28A/E29A, P38A, M39D, D51A, and Q63A/Q67A) viruses into NL.MA/NCΔE (52). NL.CA/NC carboxy-terminal CA mutants (E128A/R132A, R143A, K203A, and Q219A) were created by segment overlap PCR. Amplified regions of the resulting mutant proviruses were verified by DNA sequencing. All of the viruses used in this study were pseudotyped by the vesicular stomatitis virus glycoprotein (VSV-G). Viruses were produced by calcium phosphate transfection of 293T cells (20 μg of plasmid DNA per 2×10^6 cells) as previously described (4). Proviruses were cotransfected with a plasmid expressing the VSV envelope glycoprotein (pHCMV-G) (54). One day after transfection, culture supernatants were harvested, clarified by passing through 0.45-μm-pore-size syringe filters, and frozen in aliquots at −80°C. The CA content of the virus stocks was quantified by p24-specific enzyme-linked immunosorbent assay (50) after stocks were boiled in 0.1% sodium dodecyl sulfate for 5 min to solubilize immature Gag proteins.

Analysis of HIV-1 infection. The reporter virus HIV-GFP (21) pseudotyped by VSV-G was used to assay infection of OMK cells. Cells (20,000 cells per well in 12-well plates) were infected with various concentrations of reporter virus in the presence of polybrene (5 μg/ml) in a total volume of 300 μl. One day later, complete medium (1 ml) was added. Two days after infection, cells were harvested by trypsinization and were fixed by the addition of an equal volume of phosphate-buffered saline containing 4% paraformaldehyde. GFP expression was quantified by flow cytometry by using a Becton Dickinson FACSCalibur, and the percentage of GFP-expressing cells was quantified with Cellquest software. A minimum of 5,000 cells was analyzed for each sample. Inhibition assays were performed by infection with a fixed quantity of HIV-GFP virions (2 ng of p24) in the presence of increasing concentrations of VSV-G-pseudotyped wild-type or mutant virions.

RESULTS

Use of HIV-1 particles to inhibit restriction in restrictive target cells. A key feature of retroviral postentry restriction is that the relevant host cell machinery can be saturated at high levels of input virus (5, 18, 47). As shown in Fig. 1a, inoculation of OMK cells with a fixed quantity of HIV-GFP reporter virions together with increasing amounts of wild type, non-GFP-encoding particles led to a significant enhancement of infection by the reporter virus (approximately 100-fold). Previous studies have also shown that noninfectious, virus-like particles can also function as decoys for restriction, thus indicating that productive infection by the decoy virus is not a requirement for the saturation of host cell restriction factors. This ability of noninfectious HIV-1 particles to inhibit restriction provides a convenient and quantitative assay for studying the molecular requirements for Lv1 restriction factor recognition of the viral core.

The inhibition of restriction requires active viral PR. Lv1 restriction targets the CA domain of Gag (25, 36, 47). Previous studies reported that HIV-1 virus-like particles containing only unprocessed Gag (due to deletion of *pol*) were incapable of inhibiting restriction (5, 34). By contrast, particles containing Gag and PR retained activity, indicating that the viral PR is required (5). To determine whether viral PR activity is essential for recognition of the HIV-1 core by simian restriction factors, we tested HIV-1 particles containing an inactivated viral PR for their ability to inhibit restriction in OMK cells. These immature virions were produced either by transfection of a mutant provirus encoding a PR active site mutation (PR^-) or through production of wild-type virus in the presence of an HIV-1 PR inhibitor (Crixivan). In both cases, the PR-inactivated virions were unable to enhance HIV-GFP infection of OMK cells (Fig. 1a). Similar results were observed with these viruses by using the HIV-1-restrictive rhesus macaque line FRhK-4 as target cells (data not shown). We conclude that an active viral PR is required for the inhibition of restriction by HIV-1 particles in rhesus macaque and owl monkey cell lines.

Restriction factor recognition requires processing of Gag at the MA-CA junction. The inability of PR-inactivated virus to inhibit infection suggested that proteolytic cleavage of Gag is necessary for recognition by the restriction factor. To test this hypothesis, we assayed an HIV-1 mutant containing substitutions that prevent processing of all six cleavage sites in Gag (Fig. 1b, MA/p6) (52). These particles contain only unprocessed Gag despite the presence of an active viral PR. We

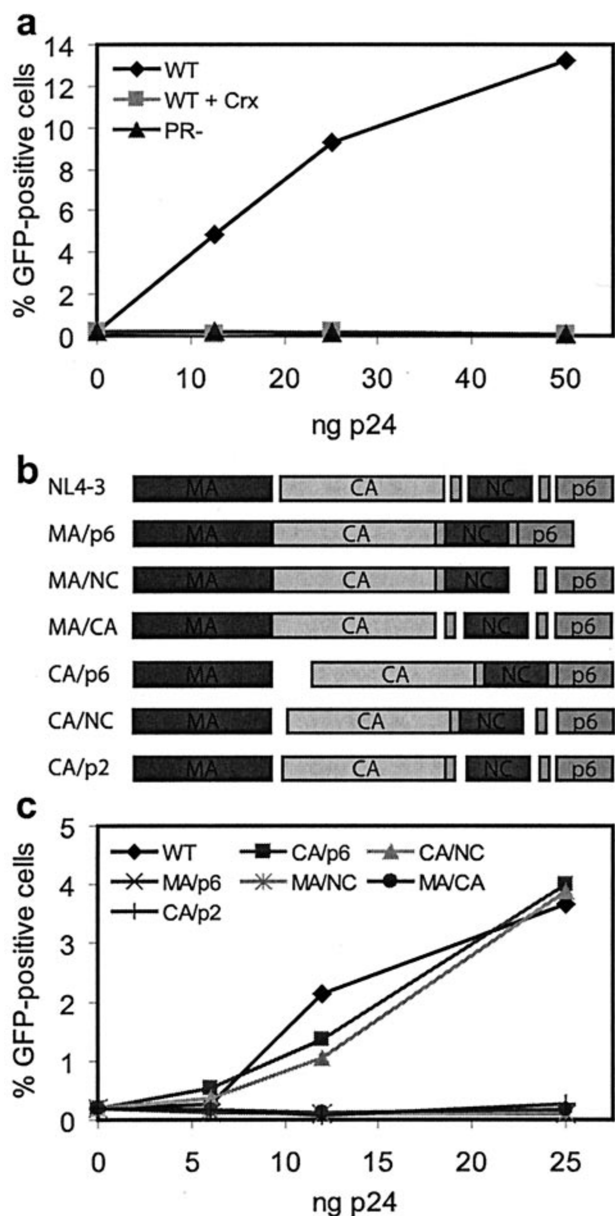

FIG. 1. Cleavage of Gag is required for the inhibition of intracellular host restriction by HIV-1. Cultures of OMK cells were inoculated with a fixed quantity of HIV-GFP particles and various doses of the indicated viruses. Cells were cultured for 2 days and analyzed for GFP expression by flow cytometry. (a) Analysis of immature HIV-1 particles containing inactivated PR. Immature particles were generated from a proviral construct containing mutations in the PR active site (PR^-) or by producing wild-type virions in the presence of the HIV-1 PR inhibitor Crixivan (Crx). (b) Schematic of Gag cleavage site mutants. Isoleucine substitutions were generated at the P1 position of the indicated junctions, thereby rendering these sites resistant to PR. (c) Assays of Gag cleavage site mutants for the inhibition of restriction. Values shown are the averages of duplicate determinations and are representative of three independent experiments.

previously demonstrated that MA/p6 particles, when pseudotyped with VSV-G, are capable of efficient fusion with target cells (52). MA/p6 was incapable of inhibiting restriction in OMK cells (Fig. 1c), demonstrating that the processing of Gag at one or more sites is necessary for restriction factor recognition.

To identify Gag cleavage events necessary for the inhibition of restriction, we employed a series of proviral clones containing mutations at various Gag cleavage sites, allowing selective processing of the viral polyprotein (Fig. 1b) (51, 52). As observed for the MA/p6 mutant, MA/CA and MA/NC particles were unable to enhance infection by the HIV-GFP reporter virus (Fig. 1c). These results demonstrate that cleavage of the MA-CA junction of Gag is essential for recognition of incoming cores by the host restriction machinery. We also analyzed two previously characterized mutants, CA/p2 and CA/NC, which prevent release of p2 and p2-NC, respectively (51). CA/p2 particles lack conical cores and contain partially condensed capsids that are unstable in vitro. By contrast, CA/NC cores are stable, consisting of unprocessed CA-p2-NC protein. We also included a virus containing additional mutations preventing cleavage between NC and p6 (CA/p6). Coinfection with HIV-GFP revealed that CA/p2 particles were markedly reduced in their ability to inhibit restriction. By contrast, the CA/NC and CA/p6 particles inhibited restriction with potencies comparable to wild-type HIV-1 (Fig. 1c). Thus, unlike the requirement for cleavage at the MA-CA junction, the inhibition of restriction on owl monkey cells does not require the processing of Gag between CA and NC. These results, together with the reported instability of CA/p2 cores, suggested that saturation of restriction is functionally linked to the stability of the viral capsid.

Restriction factors recognize a stable HIV-1 capsid. To further test the putative link between restriction factor recognition and HIV-1 capsid stability, we employed a series of HIV-1 variants that contain point mutations in CA which alter the capsid stability (10). We have previously shown that, within the limits of detection, these mutations reduce HIV-1 infectivity without affecting virus assembly, budding, cone formation, or reverse transcriptase activity. Importantly, the mutations did not alter the condensation of the conical core as revealed by electron microscopy of sectioned virions (49). Thus, the impaired infectivity of these mutants is likely due to an uncoating defect in target cells. Mutants were identified that contain capsids that are more stable than those of the wild type (E45A and E128A/R132A) (1), about half as stable those of the as wild type (Q63A/Q67A and R143A) (2), and too unstable to be isolated (P38A, K203A, and Q219A) (3). To test the capacity of these viruses to inhibit restriction by Lv1, we titrated the mutant virions onto OMK cells with a fixed quantity of the HIV-GFP reporter virus. Unlike wild-type HIV-1, CA mutants with unstable capsids were markedly reduced in their ability to enhance infection by HIV-GFP (Fig. 2). Among these, K203A particles were the most severely impaired, exhibiting no detectable activity. Other mutants with unstable capsids (P38A, Q63A/Q67A, R143A, and Q219A) were also significantly less active in the assay than wild-type virions. By contrast, the E45A and E128A/R132A mutant particles, which contain hyperstable capsids, enhanced infection as efficiently as wild-type HIV-1 (Fig. 2b). These results establish a strong genetic correlation between capsid stability and the ability of HIV-1 to saturate Lv1 restriction.

Mutations that prevent cleavage of the CA-NC junction restore activity to HIV-1 particles containing unstable cores. An

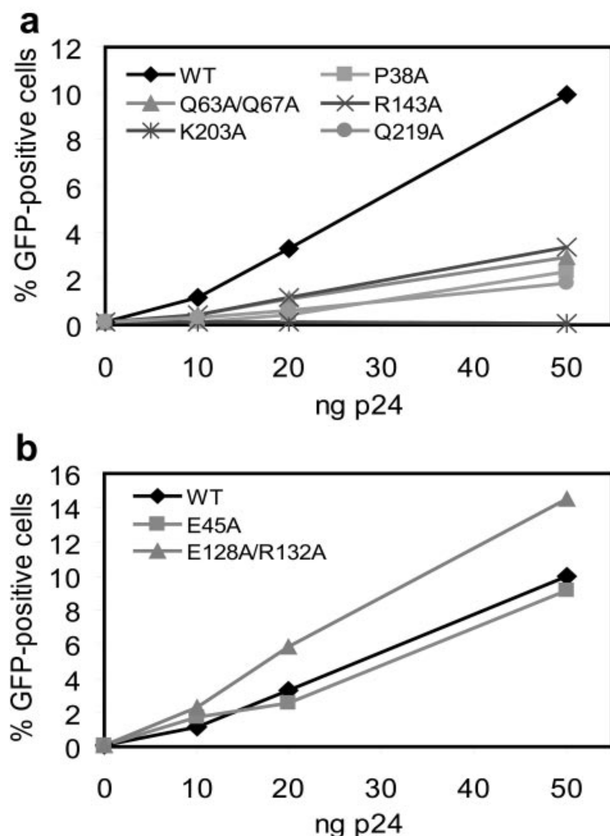

FIG. 2. Mutations decreasing HIV-1 capsid stability also result in an impaired ability to inhibit Lv1 restriction. Viruses with CA point mutations were tested for their ability to enhance HIV-GFP infection of OMK cells. Results with HIV-1 mutants containing unstable and hyperstable capsids are shown in panels a and b, respectively. Results shown are the averages of duplicate determinations and are representative of three independent experiments.

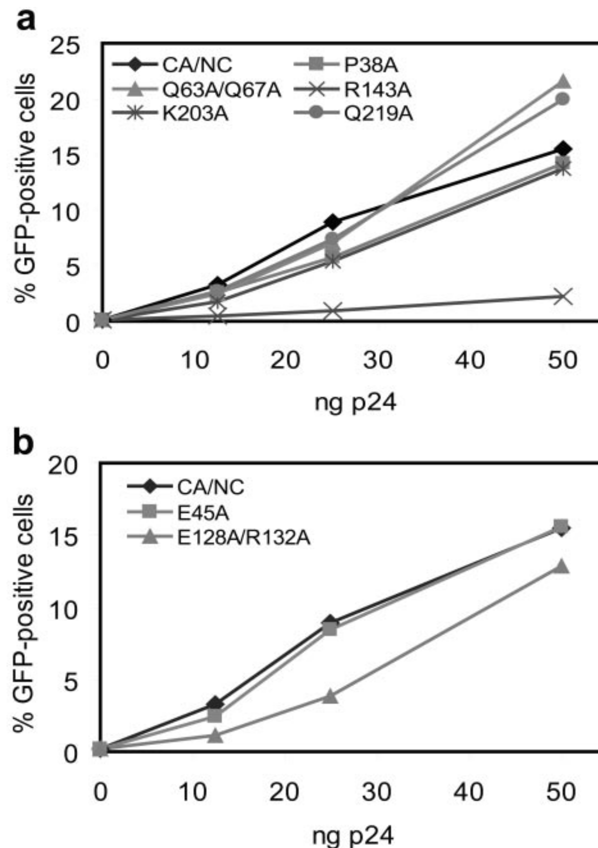

FIG. 3. CA/NC cleavage site mutants restore the ability of CA point mutants to inhibit Lv1 restriction. Viruses were generated from proviral clones containing CA/NC cleavage site substitutions and the indicated substitutions in CA. OMK cells were infected with the indicated numbers of mutant particles and HIV-GFP particles. CA point mutations which normally result in unstable and hyperstable capsids are shown in panels a and b, respectively. Results shown are the averages of duplicate determinations and are representative of two independent experiments.

alternative explanation for the inability of the CA point mutants to inhibition Lv1 restriction is that the mutations may inhibit recognition by the relevant host factors. Therefore, to determine whether a reduction in core stability specifically accounts for the loss in inhibition activity, we took advantage of the ability of the CA/NC cleavage site mutant to inhibition restriction. CA/NC cores are more stable than fully mature viral cores (51), presumably due to linkage of CA to the viral RNA via NC. Therefore, the aforementioned CA point mutations were transferred into the CA/NC cleavage mutant background in order to stabilize the cores. All such mutants exhibited a corresponding increase in core stability (data not shown). Furthermore, in the context of the CA/NC mutations, all but one of the CA point mutants potently enhanced infection by the reporter virus (Fig. 3). Thus, the impaired ability of the CA point mutants containing unstable cores to inhibit restriction was rescued by Gag cleavage site mutations that restore capsid stability to the mutant cores. The sole exception was the R143A mutant. This substitution resulted in impaired inhibition even when present in the CA/NC background, suggestive of a reduced ability of the restriction factor to bind to this mutant form of CA. As a negative control, the G89V substitution, which prevents CypA binding, was also tested in the CA/NC virus background (Fig. 4). OMK cells are permissive for infection by the G89V mutant virus, indicating that CypA binding is required for restriction in OMK cells. The CA/NC substitutions failed to rescue the inability of this mutant to inhibit restriction, indicating a disruption of restriction factor recognition for this mutant. Collectively, these results demonstrate that saturation of Lv1 by incoming HIV-1 depends on the stability of the viral capsid.

Lv1 recognition in OMK cells requires a properly folded CA amino-terminal domain. The finding that a stable capsid shell is important for the inhibition of restriction suggests that Lv1 recognizes a multimeric CA interface and not merely individual CA molecules. During maturation of HIV-1, cleavage of the MA-CA junction induces the folding of the CA amino terminus, resulting in the formation of a surface conducive to CA dimerization. Our finding that cleavage of the MA-CA junction is necessary for the inhibition of restriction led us to hypothesize that Lv1 recognizes a specific structure induced by the proper folding of the amino-terminal domain of CA. To test this hypothesis, we assayed the ability of mutant particles containing substitutions in this region that map to helices 1 and

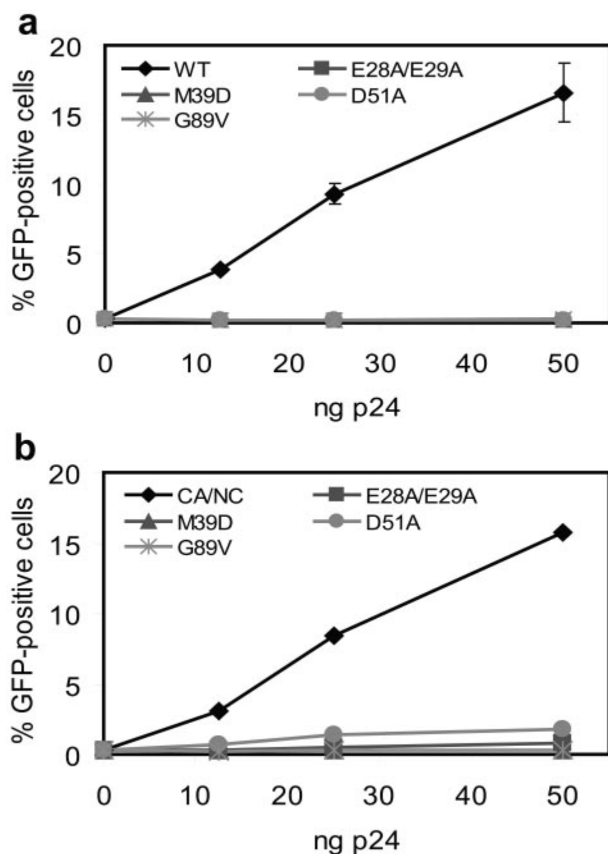

FIG. 4. N-terminal CA assembly mutants fail to inhibit Lv1 restriction of HIV-1 infection. OMK cells were coinfected with HIV-GFP particles and viruses containing N-terminal CA substitutions generated in proviral constructs lacking (a) or containing (b) the CA/NC cleavage site mutations. Values shown are the averages of duplicate determinations and are representative of two independent experiments.

2 (E28A/E29A, M39D, and D51A) and specifically disrupt the proper folding of the amino terminal β-hairpin. These mutations inhibit the intermolecular packing of amino-terminal helical bundles, thereby dramatically inhibiting CA assembly in vitro (48). HIV-1 particles containing these mutations were incapable of inhibiting restriction (Fig. 4a). Furthermore, despite their core-stabilizing effects, the CA/NC cleavage site mutations failed to restore full activity to amino-terminal region point mutants, demonstrating that the mutations prevent restriction factor recognition through an effect distinct from a loss of capsid stability (Fig. 4b). We conclude that Lv1 recognition of the incoming viral capsid requires the proper intermolecular packing of adjacent CA molecules. These results further indicate that the cellular restriction machinery recognizes a polymer of CA subunits present within the intact capsid shell.

DISCUSSION

HIV-1 infection of simian cells is inhibited by intracellular factors that act at an early postentry step, possibly at the level of viral uncoating. Restriction can be saturated by HIV-1 virus-like particles, suggesting that the relevant factors directly bind the incoming viral core. Our study revealed three specific viral structural requirements for the engagement of incoming HIV-1 capsids by a simian restriction factor. First, the viral PR must cleave the MA-CA junction, thereby allowing the refolding of the amino terminus of CA. Cleavage between CA and NC is not an absolute requirement, as demonstrated by the ability of the CA/NC cleavage mutant to inhibit restriction. Secondly, proper intermolecular packing of the amino-terminal helices of adjacent CA molecules is necessary, because mutations that disrupt these interactions compromised the ability of HIV-1 particles to inhibit restriction. Third, an intact capsid is important for restriction factor engagement, as mutant viruses containing unstable cores exhibited poor inhibition activity. Taken together, these findings indicate that a host restriction factor recognizes an intermolecular epitope in the capsid shell created by amino-terminal interactions between CA subunits. These results also strengthen our previous conclusion that some CA substitutions specifically perturb HIV-1 uncoating in target cells (10), as the inhibition phenotype observed in target cells correlated well with measurements of core stability in vitro. We conclude that our previously described biochemical assays of HIV-1 core stability provide a biologically relevant approach for studying viral uncoating in target cells.

Our results indicate that the CA subunit interface formed during HIV-1 maturation is essential for restriction factor recognition. Mutants that are defective for processing the MA-CA junction were unable to inhibit restriction. Release of MA from CA, as in wild-type, CA/NC, or CA/p6 particles, allowed engagement of the restriction factor. During maturation, cleavage of the MA-CA junction induces formation of an intermolecular CA subunit interface. The free amino terminus of CA refolds into a β-hairpin, promoting movement of CA helices 1 and 2 in the amino terminal domain of CA (48). These helices form intermolecular contacts in adjacent CA molecules that stabilize the hexameric rings of CA, which are organized into higher-ordered helical arrays to form the viral capsid (14, 27, 48). It is also possible that MA sterically interferes with restriction factor access when present in the core as an MA/CA fusion protein. However, point mutations in CA residues, important for folding of the amino-terminal β-hairpin, also destroyed the ability of fully mature HIV-1 particles to saturate Lv1 restriction, even when the mutant capsids were stabilized by substitutions blocking cleavage between CA and NC. Collectively, these results indicate that the restriction factor in OMK cells recognizes a specific structure formed by the N-terminal domain of CA in the context of an appropriate array of hexameric CA rings. If the core engages a polymeric complex of restriction factors, the requirement for an intact capsid for the inhibition of restriction could also result from high-avidity interactions with the restriction machinery.

Although cleavage of MA/CA was necessary for saturation of restriction, our data also demonstrated a distinct requirement for a stable viral capsid, as shown by two major lines of evidence. First, CA/p2 virions were markedly impaired for inhibition of restriction. CA/p2 capsids are incompletely condensed and are unstable (51) due to the appended p2 domain. By contrast, CA/NC virions efficiently inhibited restriction. These particles contain capsids that are stabilized, likely through cross-linking of the attached NC domains by viral

RNA. Second, point mutations in CA that destabilize the viral capsid also impaired the ability of HIV-1 particles to saturate restriction. Mutants in this class (P38A, Q63A/Q67A, R143A, K203A, and Q219A) are poorly infectious, yet they display no detectable assembly or maturation defects and are competent for cell entry (10, 49). Four of these mutants regained inhibition activity when the capsids were stabilized by additional mutations preventing the release of NC. These results demonstrated that the inability of the majority of the viruses with CA point mutations to inhibit restriction stems from a reduction in capsid stability rather than from a direct recognition defect. By contrast, mutant viruses with stable cores (E45A and E128A/R132A) saturated restriction as well as, or better than, wild-type virions, and the inhibition potency of these virions was not increased by the addition of CA/NC cleavage site substitutions. These findings demonstrate that a partially or fully intact CA shell is recognized and bound by Lv1 and that premature dissolution of the viral capsid following entry into the cytoplasm prevents association with the restriction factor.

Hatziioannou and coworkers have also analyzed several of the CA mutants used in the present work for their ability to infect human and simian cell lines (19). They reported that the E128A/R132A mutant, which was markedly reduced in its ability to infect human cells, was four- to sixfold more infectious than wild-type HIV-1 in rhesus macaque and African green monkey cell lines, and infection was comparable to wild-type levels in OMK cells. The preferential loss of E128A/R132A infectivity in human cells suggests that the mutant cores may be partially resistant to restriction (19). In our study, this mutant efficiently inhibited restriction in OMK cells, indicating that the mutant cores retain the ability to bind the restriction factor. This paradox may be resolved by postulating that binding of the restriction factor is not sufficient to mediate restriction, as has previously been suggested (35). Interestingly, the cores of E128A/R132A virions are approximately twice as stable as the core of the wild type (10), suggesting that the apparent resistance of this mutant to Lv1 restriction may result from the hyperstability of its core. It is tempting to speculate that if restriction triggers premature capsid uncoating, the enhanced stability of the E128A/R132A core renders this virus resistant to the inhibitory effects of restriction while preserving its ability to associate with the restriction factor.

Recently, Stremlau and coworkers identified TRIM5α from rhesus macaque cells as an HIV-1-specific restriction factor (42). The viral determinants of restriction factor binding in rhesus macaque cells clearly differ from those of owl monkey cells (the target cells analyzed in the present study). Virions containing the G89V substitution in CA failed to inhibit restriction in OMK cells (47), yet they potently enhanced HIV-GFP infection of restrictive rhesus macaque cells (our unpublished observations). Remarkably, in owl monkey cells, Lv1 restriction is mediated by a TRIM5-CypA fusion protein (34a, 40). Thus, the differential recognition of HIV-1 CA by Lv1 in rhesus and owl monkey cells is readily explained by the identities of the restriction factors in these cells. It remains to be determined whether the structural requirements for Lv1 recognition in owl monkey cells, as revealed in the present study, will extend to Lv1 restriction in other simian cell lines.

Previous studies have suggested that the HIV-1 core disassembles soon after penetration into the target cell, mainly due to the paucity of CA present in HIV-1 preintegration and reverse transcription complexes recovered from infected cells (7, 8, 32). Our results suggest that the viral capsid persists in the cytoplasm for a period of time prior to undergoing uncoating. In permissive human cells, mutant HIV-1 particles with unstable cores are poorly infectious and fail to complete reverse transcription (9, 10). Lv1 restricts infection at the level of reverse transcription by targeting CA (19, 25, 34, 46). We show here that HIV-1 particles do not efficiently inhibit restriction if their cores are unstable, indicating that the relevant host restriction factor (TRIMCyp) recognizes an intact polymeric viral capsid in the target cytoplasm. Collectively, these findings indicate that the uncoating of the viral core is a key step in HIV-1 infection that is highly sensitive to changes in capsid stability. Uncoating thus represents a novel and attractive target for HIV-1 therapy.

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REFERENCES

1. **Accola, M. A., B. Strack, and H. G. Gottlinger.** 2000. Efficient particle production by minimal Gag constructs which retain the carboxy-terminal domain of human immunodeficiency virus type 1 capsid-p2 and a late assembly domain. J. Virol. **74:**5395–5402.
2. **Besnier, C., Y. Takeuchi, and G. Towers.** 2002. Restriction of lentivirus in monkeys. Proc. Natl. Acad. Sci. USA **99:**11920–11925.
3. **Best, S., P. Le Tissier, G. Towers, and J. P. Stoye.** 1996. Positional cloning of the mouse retrovirus restriction gene Fv1. Nature **382:**826–829.
4. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
5. **Cowan, S., T. Hatziioannou, T. Cunningham, M. A. Muesing, H. G. Gottlinger, and P. D. Bieniasz.** 2002. Cellular inhibitors with Fv1-like activity restrict human and simian immunodeficiency virus tropism. Proc. Natl. Acad. Sci. USA **99:**11914–11919.
6. **Dorfman, T., A. Bukovsky, A. Ohagen, S. Hoglund, and H. G. Gottlinger.** 1994. Functional domains of the capsid protein of human immunodeficiency virus type1. J. Virol. **68:**8180–8187.
7. **Farnet, C. M., and W. A. Haseltine.** 1991. Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. J. Virol. **65:**1910–1915.
8. **Fassati, A., and S. P. Goff.** 2001. Characterization of intracellular reverse transcription complexes of human immunodeficiency virus type 1. J. Virol. **75:**3626–3635.
9. **Fitzon, T., B. Leschonsky, K. Bieler, C. Paulus, J. Schroder, H. Wolf, and R. Wagner.** 2000. Proline residues in the HIV-1 NH2-terminal capsid domain: structure determinants for proper core assembly and subsequent steps of early replication. Virology **268:**294–307.
10. **Forshey, B. M., U. von Schwedler, W. I. Sundquist, and C. Aiken.** 2002. Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication. J. Virol. **76:**5667–5677.
11. **Gamble, T. R., F. F. Vajdos, S. Yoo, D. K. Worthylake, M. Houseweart, W. I. Sundquist, and C. P. Hill.** 1996. Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. Cell **87:**1285–1294.
12. **Gamble, T. R., S. Yoo, F. F. Vajdos, U. K. von Schwedler, D. K. Worthylake, H. Wang, J. P. McCutcheon, W. I. Sundquist, and C. P. Hill.** 1997. Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein. Science **278:**849–853.
13. **Ganser, B. K., S. Li, and W. I. Sundquist.** 1999. Assembly and analysis of conical models for the HIV-1 core. Science **283:**80–83.
14. **Gitti, R. K., B. M. Lee, J. Walker, M. F. Summers, S. Yoo, and W. I. Sundquist.** 1996. Structure of the amino-terminal core domain of the HIV-1 capsid protein. Science **273:**231–235.
15. **Gross, I., H. Hohenberg, T. Wilk, K. Wiegers, M. Grattinger, B. Muller, S. Fuller, and H.-G. Krausslich.** 2000. A conformational switch controlling HIV-1 morphogenesis. EMBO J. **19:**103–113.
16. **Hartley, J. W., W. P. Rowe, and R. J. Huebner.** 1970. Host-range restrictions of murine leukemia viruses in mouse embryo cell lines. J. Virol. **5:**221–225.
17. **Hatziioannou, T., S. Cowan, and P. D. Bieniasz.** 2004. Capsid-dependent and -independent postentry restriction of primate lentivirus tropism in rodent cells. J. Virol. **78:**1006–1011.

18. **Hatziioannou, T., S. Cowan, S. P. Goff, P. D. Bieniasz, and G. J. Towers.** 2003. Restriction of multiple divergent retroviruses by Lv1 and Ref1. EMBO J. **22:**385–394.
19. **Hatziioannou, T., S. Cowan, U. K. Von Schwedler, W. I. Sundquist, and P. D. Bieniasz.** 2004. Species-specific tropism determinants in the human immunodeficiency virus type 1 capsid. J. Virol. **78:**6005–6012.
20. **Hatziioannou, T., D. Perez-Caballero, A. Yang, S. Cowan, and P. D. Bieniasz.** 2004. Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5α. Proc. Natl. Acad. Sci. USA **101:**10774–10779.
21. **He, J., Y. Chen, M. Farzan, H. Choe, A. Ohagen, S. Gartner, J. Busciglio, X. Yang, W. Hofmann, W. Newman, C. R. Mackay, J. Sodroski, and D. Gabuzda.** 1997. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature **385:**645–649.
22. **Jolicoeur, P.** 1979. The Fv-1 gene of the mouse and its control of murine leukemia virus replication. Curr. Top. Microbiol. Immunol. **86:**67–122.
23. **Karageorgos, L., P. Li, and C. Burrell.** 1993. Characterization of HIV replication complexes early after cell-to-cell infection. AIDS Res. Hum. Retrovir. **9:**817–823.
24. **Keckesova, Z., L. M. Ylinen, and G. J. Towers.** 2004. The human and African green monkey TRIM5alpha genes encode Ref1 and Lv1 retroviral restriction factor activities. Proc. Natl. Acad. Sci. USA **101:**10780–10785.
25. **Kootstra, N. A., C. Munk, N. Tonnu, N. R. Landau, and I. M. Verma.** 2003. Abrogation of postentry restriction of HIV-1-based lentiviral vector transduction in simian cells. Proc. Natl. Acad. Sci. USA **100:**1298–1303.
26. **Kozak, C. A., and A. Chakraborti.** 1996. Single amino acid changes in the murine leukemia virus capsid protein gene define the target of Fv1 resistance. Virology **225:**300–305.
27. **Li, S., C. P. Hill, W. I. Sundquist, and J. T. Finch.** 2000. Image reconstructions of helical assemblies of the HIV-1 CA protein. Nature **407:**409–413.
28. **Lilly, F.** 1970. Fv-2: identification and location of a second gene governing the spleen focus response to Friend leukemia virus in mice. J. Natl. Cancer Inst. **45:**163–169.
29. **Lilly, F., and T. Pincus.** 1973. Genetic control of murine viral leukemogenesis. Adv. Cancer Res. **17:**231–277.
30. **Mammano, F., A. Ohagen, S. Hoglund, and H. G. Gottlinger.** 1994. Role of the major homology region of human immunodeficiency virus type 1 in virion morphogenesis. J. Virol. **68:**4927–4936.
31. **McDonald, D., M. A. Vodicka, G. Lucero, T. M. Svitkina, G. G. Borisy, M. Emerman, and T. J. Hope.** 2002. Visualization of the intracellular behavior of HIV in living cells. J. Cell Biol. **159:**441–452.
32. **Miller, M. D., C. M. Farnet, and F. D. Bushman.** 1997. Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. J. Virol. **71:**5382–5390.
33. **Momany, C., L. C. Kovari, A. J. Prongay, W. Keller, R. K. Gitti, B. M. Lee, A. E. Gorbalenya, L. Tong, J. McClure, L. S. Ehrlich, M. F. Summers, C. Carter, and M. G. Rossmann.** 1996. Crystal structure of dimeric HIV-1 capsid protein. Nat. Struct. Biol. **3:**763–770.
34. **Munk, C., S. M. Brandt, G. Lucero, and N. R. Landau.** 2002. A dominant block to HIV-1 replication at reverse transcription in simian cells. Proc. Natl. Acad. Sci. USA **99:**13843–13848.
34a. **Nisole, S., C. Lynch, J. P. Stoye, and M. W. Yap.** 2004. A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. Proc. Natl. Acad. Sci. USA **101:**13324–13328.
35. **Owens, C. M., B. Song, M. J. Perron, P. C. Yang, M. Stremlau, and J. Sodroski.** 2004. Binding and susceptibility to postentry restriction factors in monkey cells are specified by distinct regions of the human immunodeficiency virus type 1 capsid. J. Virol. **78:**5423–5437.
36. **Owens, C. M., P. C. Yang, H. Gottlinger, and J. Sodroski.** 2003. Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. J. Virol. **77:**726–731.
37. **Perron, M. J., M. Stremlau, B. Song, W. Ulm, R. C. Mulligan, and J. Sodroski.** 2004. TRIM5α mediates the postentry block to N-tropic murine leukemia viruses in human cells. Proc. Natl. Acad. Sci. USA **101:**11827–11832.
38. **Pryciak, P. M., and H. E. Varmus.** 1992. Fv-1 restriction and its effects on murine leukemia virus integration in vivo and in vitro. J. Virol. **66:**5959–5966.
39. **Reicin, A. S., A. Ohagen, L. Yin, S. Hoglund, and S. P. Goff.** 1996. The role of Gag in human immunodeficiency virus type 1 virion morphogenesis and early steps of the viral life cycle. J. Virol. **70:**8645–8652.
40. **Sayah, D. M., E. Sokolskaja, L. Berthoux, and J. Luban.** 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature **430:**569–573.
41. **Schmitz, C., D. Marchant, S. J. Neil, K. Aubin, S. Reuter, M. T. Dittmar, and A. McKnight.** 2004. Lv2, a novel postentry restriction, is mediated by both capsid and envelope. J. Virol. **78:**2006–2016.
42. **Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski.** 2004. The cytoplasmic body component TRIM5α restricts HIV-1 infection in Old World monkeys. Nature **427:**848–853.
43. **Tang, S., T. Murakami, B. E. Agresta, S. Campbell, E. O. Freed, and J. G. Levin.** 2001. Human immunodeficiency virus type 1 N-terminal capsid mutants that exhibit aberrant core morphology and are blocked in initiation of reverse transcription in infected cells. J. Virol. **75:**9357–9366.
44. **Towers, G., M. Bock, S. Martin, Y. Takeuchi, J. P. Stoye, and O. Danos.** 2000. A conserved mechanism of retrovirus restriction in mammals. Proc. Natl. Acad. Sci. USA **97:**12295–12299.
45. **Towers, G., M. Collins, and Y. Takeuchi.** 2002. Abrogation of Ref1 retrovirus restriction in human cells. J. Virol. **76:**2548–2550.
46. **Towers, G. J., and S. P. Goff.** 2003. Post-entry restriction of retroviral infections. AIDS Rev. **5:**156–164.
47. **Towers, G. J., T. Hatziioannou, S. Cowan, S. P. Goff, J. Luban, and P. D. Bieniasz.** 2003. Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. Nat. Med. **9:**1138–1143.
48. **von Schwedler, U. K., T. L. Stemmler, and W. I. Sundquist.** 1998. Proteolytic refolding of the HIV-1 capsid protein amino-terminus facilitates viral core assembly. EMBO J. **17:**1555–1568.
49. **von Schwedler, U. K., K. M. Stray, J. E. Garrus, and W. I. Sundquist.** 2003. Functional surfaces of the human immunodeficiency virus type 1 capsid protein. J. Virol. **77:**5439–5450.
50. **Wehrly, K., and B. Chesebro.** 1997. p24 antigen capture assay for quantification of human immunodeficiency virus using readily available inexpensive reagents. Methods **12:**288–293.
51. **Wiegers, K., G. Rutter, H. Kottler, U. Tessmer, H. Hohenberg, and H.-G. Krausslich.** 1998. Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites. J. Virol. **72:**2846–2854.
52. **Wyma, D. J., J. Jiang, J. Shi, J. Zhou, J. E. Lineberger, M. D. Miller, and C. Aiken.** 2004. Coupling of human immunodeficiency virus type 1 fusion to virion maturation: a novel role of the gp41 cytoplasmic tail. J. Virol. **78:**3429–3435.
53. **Yap, M. W., S. Nisole, C. Lynch, and J. P. Stoye.** 2004. Trim5αprotein restricts both HIV-1 and murine leukemia virus. Proc. Natl. Acad. Sci. USA **101:**10786–10791.
54. **Yee, J. K., T. Friedmann, and J. C. Burns.** 1994. Generation of high-titer pseudotyped retroviral with very broad host range. Methods Cell Biol. **43:**99–112.